University of Southampton

Doctoral Thesis

Synthesis, biodistribution and efficacy of monomeric and multimeric antisense oligonucleotides in visceral tissue and the central nervous system

Author:
Michael Moazami

Supervisor:
Dr. Jonathan Watts

Watts group
Chemistry

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"It is not the critic who counts; not the man who points out how the strong man stumbles, or where the doer of deeds could have done them better. The credit belongs to the man who is actually in the arena, whose face is marred by dust and sweat and blood; who strives valiantly; who errs, who comes short again and again, because there is no effort without error and shortcoming; but who does actually strive to do the deeds; who knows great enthusiasms, the great devotions; who spends himself in a worthy cause; who at the best knows in the end the triumph of high achievement, and who at the worst, if he fails, at least fails while daring greatly, so that his place shall never be with those cold and timid souls who neither know victory nor defeat."

Theodore Rosevelt

UNIVERSITY OF SOUTHAMPTON

Abstract

Faculty of Natural and Environmental Sciences
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Doctor of Philosophy

Synthesis, biodistribution and efficacy of monomeric and multimeric antisense oligonucleotides in visceral tissue and the central nervous system

by Michael Moazami

Antisense oligonucleotides (ASOs) are a promising class of therapeutics for treating diseases of a genetic origin. ASOs are able to modulate the expression of disease-causing RNA, interact with the immune system and affect the endogenous regulation of genes. ASOs are, however, poorly taken up into some tissue types which limits their potential therapeutic benefit. This work aimed to improve the biodistribution and efficacy of antisense oligonucleotides by synthesising them as dendrimers – discrete structures comprising multiple oligonucleotides around a central core. Hence, the various synthetic approaches are described for attempts to access these molecules, their effects on *in vitro* and *in vivo* distribution and silencing of the long non-coding RNA MALAT1. It is demonstrated that larger dendrimers are more rapidly taken up into cultured cells than monomeric oligomers and that the compounds distribute differently in the mouse. Nevertheless, silencing efficacy both *in vitro* and *in vivo* seems to peak with monomeric or dimeric structures. It is anticipated that the rapid uptake of these dendrimers may be able to be translated into improved silencing by modifying their chemistry in the future.

To adequately assay the biodistribution of these oligonucleotides, the available techniques discussed in the literature are compared. Dissatisfied with the current state of the art, the process of preparing a polyclonal antibody directed against the chemically modified backbone found in most therapeutic oligonucleotides is described. It is shown that the antibody generated is able to be used for immunohistochemistry and immunofluorescence, and that it shows promise for use as a quantitative assay for determining oligonucleotide concentration in tissue.

Finally, efforts in improving oligonucleotide chemistry and structure for silencing disease-causing RNA in the CNS are descrived. Using *C9ORF72* repeat-expanded Amyotrophic Lateral Sclerosis as a target disease, it is show that some oligonucleotides are intrinsically and surprisingly toxic on central administration and that this toxicity can be ameliorated by reduction of the phosphorothioate content of the backbone. The generation of a preclinical lead compound is described that will, building on this work, be screened for toxicity in non-human primates and advanced to a compassionate care trial in two patients, pending FDA approval.

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Abbreviations

^{1}H NMR	Hydrogen Nuclear Magnetic Resonance
^{13}C NMR	Carbon Nuclear Magnetic Resonance
Ac_2O	Acetic Anhydride
AcOH	Acetic acid
ALS	Amyotrophic Lateral Sclerosis
AMA	Ammonia/ Methylamine
AuNP	Gold nanoparticle
BBB	Blood-brain Barrier
$BM(PEG)_2$	1,8-Bismaleimido-diethyleneglycol
BMD	Becker Muscular Dystrophy
BNA	Bridged Nucleic Acid
BTT	Benzylthiotetrazole
CBV	Circulating Blood Volume
$CDCl_3$	d-Chloroform
COSY	Correlation Spectroscopy
CPG	Controlled Pore Glass
CuAAC	Copper-catalysed Azide-Alkyne Cycloaddition
CyDye	Cyanine Dye
DCC	*N*,*N*′-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DDTT	3-[(Dimethylaminomethylene)amino]-3*H*-1,2,4-dithiazole-5-thione
DIPEA	N,N-Diisopropylethylamine

DMAP	Dimethylaminopyridine
DMD	Duchenne Muscular Dystrophy
DMT	Dimethoxytrityl
DNA	Deoxyribonucleic Acid
DOPE	1,2-Dioleoyl-glycero-3-phosphoethanolamine
DOTMA	*N*-[1-(2,3,-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride
DTME	Dithiobismaleimidoethane
EDITH	3-Ethoxy-1,2,4-dithiazoline-5-one
EIA	Enzyme Immunoassay
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GalNAc	N-acetylgalactosamine
HEG	Hexaethylene glycol
HLA	Human Leukocyte Antigen
HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	1-Hydroxybenzotriazole hydrate
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence
hsiRNA	Hydrophobic small-interfering nucleic acid
IACUC	Institutional Animal Care and Use Committee
IPA	Propan-2-ol
lcaa CPG	Long-chain alkyl amino Controlled Pore Glass
lncRNA	Long non-coding RNA
LRMS	Low Resolution Mass Spectrometry
MALAT1	Metastasis-Associated Lung Adenocarcinoma transcript 1
MCA	Maleimidecaproic acid
MCAPFP	Maleimidecaproic acid pentafluorophenol ester
MOE	Methoxyethyl
MRI	Magnetic Resonance Imaging

mRNA	Messenger Ribose Nucleic Acid
miRNA	Micro Ribose Nucleic Acid
NBF	Neutral Buffered Formalin
ncRNA	Non-coding RNA
NMR	Nuclear Magnetic Resonance
ODN	Oligodeoxynucleotide
OMe	Methoxy
***p*-TsOH**	*p*-Toluenesulfonic acid dihydrate
PAGE	Polyacrylamide Gel Electrophoresis
PAMAM	Polyaminoamidine
PBS	Phosphate Buffered Saline (pH 7.4)
PET	Positron Emission Tomography
PMO	Phosphorodiamidite Morpholino
PNA	Peptide Nucleic Acid
PS	Phosphorothioate
QC	Quality Control
RES	Reticuloendothelial System
RNA	Ribonucleic Acid
RNAP	RNA Polymerase
siRNA	Small-interfering RNA
SNA	Spherical Nucleic Acid
SPAAC	Strain-promoted Azide-Alkyne Cycloaddition
SPECT	Single-Photon Emission Computerised Tomography
t-RNA	Transfer RNA
TBAB	Tetrabutylammonium bromide
TBAF	Tetrabutylamminium fluoride
TBAI	Tetrabutylammonium iodide
TBDPS	tert-Butyldiphenylsilyl
TCAA	Trichloroacetic acid

tcDNA	Tricyclo DNA
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TIPS	Triisopropylsilyl
TOCSY	Total Correlation Spectroscopy
TLC	Thin-layer Chromatography
TLR	Toll-like receptor
UHPLC-ESI	Ultra High Performance Liquid Chromatography Electrospray Ionisation
UTC	Untreated Control
UV-Vis	Ultraviolet-visible

Chapter 1

Introduction

1.1 The Flow of Genetic Information

The central dogma of molecular biology is that DNA is transcribed to make RNA which in turn is translated to give a functional protein.[1] This has proven to be a gross oversimplification of the diverse biology undertaken by the macromolecules of life.[2] While at its core the central dogma remains true, it notably omits all non-coding RNAs as well as some significant processing steps that occur to RNA between DNA and protein formation.

1.1.1 Messenger RNA and Protein from DNA

Within the nucleus of each somatic cell in a eukaryotic organism are a number of pairs of chromosomes comprised of DNA, each containing a subset of the genes required for the organism. Genes are transcribed as they are required into precursor messenger RNA (pre-mRNA) using an RNA Polymerase (RNAP) along with several other proteins such as transcription factors. RNAP is a multi-functional protein that is able to break the dsDNA hydrogen bonding to form a 'transcription bubble'. It then assembles RNA

nucleotides that are complementary to the template strand of the unwound DNA. Precursor mRNA is not all functional in coding for a given protein. In order to provide fully functional mRNA, pre mRNA is processed (attaching a 5′-cap, necessary for ribosomal recognition and nuclease resistance and a 3′-polyA tail, necessary for nuclease protection) and spliced, attaching the coding exons and removing the non-coding introns, thus forming a continuous strand of mature, coding mRNA. The 3′ and 5′ untranslated regions (UTRs) as well as the 5′ cap and 3′ polyA tail do not get translated. The 3′ UTR serves to regulate gene expression, notably by acting as a binding site for microRNAs (miRNAs) (see section 1.1.2.2). The 5′-UTR acts similarly to the 3′-UTR in that it regulates expression, however it is not generally an accessible region for miRNA binding.[3–5]

Once processed and spliced, the pre-mRNA is termed either mature mRNA or simply mRNA. Mature mRNA is composed of sets of 3 nucleotides called codons; each codon codes for one of the 20 natural amino acids in accordance with the organism's genetic code. The ribosome translates the mature mRNA to form the final protein sequence, using tRNA to assemble the polypeptide which is then further modified by folding and processing.[6]

1.1.2 Noncoding RNAs

Non-coding RNA (ncRNA) is estimated to account for the majority of RNA by weight in a given cell, while mRNA is estimated to comprise only 3-7%.[7] Indeed only 1.5% of the produced RNA actually codes for protein.[8] With such a small amount of total RNA that actually codes for protein, it is important to consider the roles of non-coding RNAs.

Much of non-coding RNA was considered 'junk', that is, without observable function. This view has been challenged in recent years as some ncRNAs have been strongly implicated in diseases such as cancer.[9,10] ncRNAs come in many forms, some of which

interact with proteins and exert gene-regulatory effects[11] as a result while others exert their effects without protein assistance.[11]

Non-coding RNAs also include functional molecules such as ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNAs (snoRNAs) and many others that serve important roles in maintaining the cell. These will not be discussed here as they are not directly relevant to the work later discussed.

1.1.2.1 Long non-coding RNA

Long non-coding RNAs (lncRNA) form a defined group of ncRNAs with length >200 bases.[12] Approximately 300 lncRNAs have had their functions annotated[13], while ca. 100 000 human lncRNAs have been described.[14,15]

lncRNAs have been found to be important in cancers.[16] One important example is metastasis-associated lung adenocarcinoma transcript 1 (MALAT1).[16] MALAT1 overexpression is associated with tumours of the liver, breast, colon and many other tissues.[17]

1.1.2.2 MicroRNA

miRNA begins as a non-coding RNA in the nucleus called pri-miRNA, a hairpin RNA with a 5′-cap and 3′-polyadenylated tail. This is processed by Drosha (an endoribonuclease) to give pre-miRNA, a stem-loop structure. pre-miRNA is exported from the nucleus and processed by Dicer, which interacts with both the 3′ and 5′ end of the hairpin, cleaving it to give a double stranded miRNA.[18] Once the miRNA is generated, the guide strand becomes incorporated into RISC while the passenger strand is lost. miRNAs play a natural regulatory role in gene expression, and up or down regulation of miRNAs is implicated in several diseases, notably in several cancers.[18–20] miRNAs cause repression of gene expression by a complex mechanism that doesn't always involve cleavage of the target mRNA, including repression of translation and

acceleration of deadenylation and degradation of the target. miRNAs can cause upregulation of the target gene by binding to an inhibitory region of the 3′ UTR.[21] miRNAs are also able to bind naturally occuring antisense transcripts (NATs) resulting in gene upregulation. Crucially important to both the function and specificity of a miRNA is its seed sequence, a short (6-nucleobase from nucleotide 2-7) region at the 5′ end of the bound strand that must be perfectly complementary to the target.[22]

1.1.3 The Genetic Code and Genetic Disease

First base (5'-end)	Middle base	Third base (3'-end)			
		U	C	A	G
U	U	Phe	Phe	Leu	Leu
	C	Ser	Ser	Ser	Ser
	A	Tyr	Tyr	Stop	Stop
	G	Cys	Cys	Stop	Trp
C	U	Leu	Leu	Leu	Leu
	C	Pro	Pro	Pro	Pro
	A	His	His	Gln	Gln
	G	Arg	Arg	Arg	Arg
A	U	Ile	Ile	Ile	Met
	C	Thr	Thr	Thr	Thr
	A	Asn	Asn	Lys	Lys
	G	Ser	Ser	Arg	Arg
G	U	Val	Val	Val	Val
	C	Ala	Ala	Ala	Ala
	A	Asp	Asp	Glu	Glu
	G	Gly	Gly	Gly	Gly

Figure 1.1: The Standard Genetic Code

An organism's genetic code describes which 3-base codons correspond to which amino acids. Figure 1.1 shows the 'standard genetic code' (reproduced from[23]), that is, the genetic code most prevalent throughout the animal kingdom. The code is degenerate, that is, different codons code for the same amino acid. However, what is also evident is that the alteration of a single base in this code can produce an entirely different amino acid. As we have discussed, this code is dependent on the absolute DNA sequence and hence any alterations in the DNA sequence may manifest as alterations in the final protein. For example, switching the middle base from A to T in the sequence GAG, giving GTG, would yield a valine residue as opposed to a glutamic acid residue after transcription to RNA and translation to protein. This is known as a missense mutation; when it occurs at exon 1 of the β-globin gene this particular mutation is responsible for causing sickle-cell anemia.[24,25] Abrupt

termination of a protein strand by premature presence of a stop codon is referred to as a point nonsense mutation, while silent mutations occur when a change in the mRNA does not yield a different amino acid (UUU to UUC, for example, both yield phenylalanine). It is difficult to absolutely determine the propensity of a mutation to cause disease. If the mutation occurs at a critical point in the sequence, damaging the active site of a particular enzyme it may be catastrophic, however if it occurs in a non-coding region or at non-critical location in a protein, the mutation may go unnoticed. Genetic mutations may contribute to common ailments, for example familial hypercholesterolemia (1 in 500 on average), or may manifest as something considerably rarer, such as Huntington's Disease (about 1 in 15,000).[26,27]

1.1.4 Therapeutics

The earliest available remedies for the types of conditions caused by genetic mutations were those designed to treat the symptoms of such. Truly curative measures are rare, though not unheard of. In the example of sickle-cell disease, common supportive therapies include a lifelong commitment to infection prophylaxis, pain management and regular blood transfusions.[28] The only curative measure yet identified is transplantation of haematopoietic stem cells from HLA-identical siblings, a measure obviously unavailable to any sufferer without a sibling.[29]

The current outlook for sufferers of genetic disease depends on the disease in question. As noted earlier, therapeutic options for sickle-cell anaemia do exist. Sufferers of Huntington's however, are restricted to palliative medicines.[27] It is clear that there is a true need for development of specific drugs targeting genetic conditions.

1.2 Oligonucleotide Interactions

Oligonucleotides are small (typically 10-40 nt, can range from 5-300+ nt) strands of DNA or RNA. One of their many applications in the laboratory and in the clinic is modulating gene expression and silencing non-coding RNAs. Oligonucleotides bind to their targets primarily using Watson-Crick base pairing (Fig. 1.2) allowing them to target specific genes.

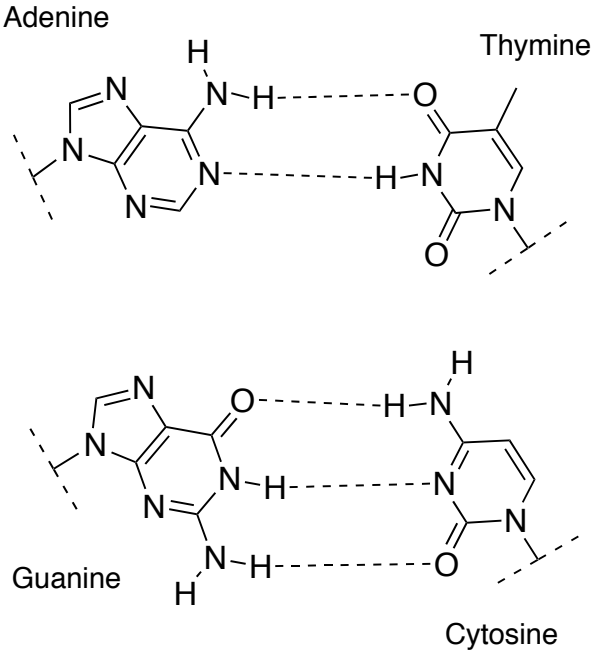


Figure 1.2: Watson-Crick Base Pairing

The diversity of potential RNA targets for oligonucleotides is one of the most exciting aspects of the field of therapeutic oligonucleotides. With the plethora of potential RNA targets detailed in section 1.1, oligonucleotide therapeutics are able to exert a perturbative effect at almost every level of the transfer of information from gene to phenotype. It is convenient to categorise the mechanisms of these perturbations by whether they result in degradation of target RNA or not.

Whether or not a certain oligonucleotide will degrade its target RNA or exert a non-destructive influence depends wholly on the chemical modifications incorporated. These modifications and their effects are discussed in section 1.5.

1.2.1 RNA-Cleavage Mediated Gene Modulation

A subset of oligonucleotide therapeutics exert their effect by causing direct degradation of target RNA. This requires the assistance of a nuclease enzyme, that is an enzyme capable of cleaving nucleic acids. There are two such mechanisms that are commonly utilised by oligonucleotide therapeutics.[30]

1.2.1.1 RNase H

RNase H is an enzyme with the ability to cleave the RNA strand of RNA•DNA hybrids.[31] It is essential in all higher eukaryotes for the cleavage of RNA•DNA hybrids produced during DNA replication and repair. The enzyme occurs in both the nucleus and the cytoplasm of cells[32,33] and is responsible for the RNA cleavage observed when cells or organisms are treated with classical antisense oligonucleotides. Indeed, the antisense effect was first described in 1978 by Paul C. Zamecnik and Mary L. Stephenson, who showed that addition of a 13-mer oligodeoxynucleotide (ODN) complementary to the terminal sequence of Rous sarcoma virus inhibited viral production in infected chick embryo fibroblast cultures.[34]

RNase H cleaves the target RNA, inducing gene silencing and liberating the ASO which may subsequently find another copy of its target RNA to bind.[30] This catalytic process can occur in the nucleus or in the cytoplasm, before or after splicing.[35] The prevalence of RNase H throughout the cell allows for antisense oligonucleotides to be effective silencing tools at every step of the RNA lifecycle.

1.2.1.2 RISC

The RNA-induced silencing complex (RISC) has been described briefly (section 1.1.2.2). RISC is the catalytic enzyme machinery that associates with both endogenous miRNAs and exogenous siRNA molecules. In the endogenous state, a transcribed dsRNA or hairpin RNA is processed by the nuclease Dicer to give an RNA duplex that subsequently loads into RISC. Argonaute (AGO), a nuclease found in the RISC, then cleaves the passenger strand leaving the guide strand loaded into the RISC, and able to catalytically cleave its perfect complement found elsewhere in the cell. Therapeutic siRNA molecules are administered as duplexes which can similarly be loaded into RISC, and silence their targets. RNAi factors are found both in the cytoplasm and the nucleus, hence much as for ASOs, it is possible for siRNA molecules to exert silencing effects throughout the cell.[36] Nevertheless, nuclear silencing with siRNA is a controversial topic. While silencing of some lncRNAs has been incontrovertibly demonstrated using siRNAs in the nucleus,[36,37] this is inconsistently described, suggesting that RISC-mediated nuclease activity in the nucleus may be the product of specific, presently not understood, circumstances.[38]

The differences between siRNA and miRNA can be summarised as follows (modified from[39]):

	siRNA	**miRNA**
Structure	21-23 nucleotide RNA duplex with 2-nucleotide 3′overhang	19-25 nucleotide RNA duplex with 2 nucleotide 3′overhang
Target Complementarity	Fully complementary to mRNA	Partially complementary to mRNA, typically targeting the 3′ untranslated region of mRNA
Target Nature	One intended target	Multiple targets
Nature of Regulation	Endonucleolytic cleavage of mRNA	Translational repression Degradation of mRNA (rarely some endonucleolytic cleavage)

Table 1.1: Differences between siRNA and miRNA

1.2.2 RNA-Blocking Gene Modulation

Some antisense oligonucleotides are able to modulate gene expression by binding at specific regions during RNA processing, by repressing translation and by repressing endogenous regulatory factors all without cleaving the target. Oligonucleotides that work by any of these mechanisms are not generally considered to be catalytic because one copy of oligonucleotide is needed per site.

1.2.2.1 Splice Switching

Perhaps the most prevalent use of non-cleaving oligonucleotides is in the field of splice correction. ASOs are able to bind pre-mRNA and block splice sites, redirecting splicing away from deleterious splice-variant proteins and towards the generation of desirable splice variants.[40] By binding to pre-mRNA in a steric-block fashion, these oligonucleotides are able to induce upregulation of a healthy splice variant and decrease the production of a

disease-causing splice variant.[41] Of interest in this regard is the condition β-thalassemia, caused by a mutation in intron 2 of the β-globin gene (IVS2-654). This induces aberrant splicing which has been shown to be repaired using a 2′-OMe phosphorothiate-modified oligonucleotide.[42] Splice-switching oligonucleotides are also proving useful in treating Duchenne Muscular Dystrophy (DMD). DMD is usually caused by a frame-shift mutation, leading to a premature stop codon and subsequent loss of one or more exons.[43] This loss of exons results in a frame-shift mutation, producing complete loss of dystrophin and a severe DMD phenotype.[44] The deletion of specific in-frame exons leads to the expression of a truncated, yet partly functional dystrophin protein, which exhibits a far milder phenotype called Becker Muscular Dystrophy (BMD).[44] Using splice-switching oligonucleotides to induce exon skipping in DMD can restore the original reading frame, producing a partly functional BMD-like dystrophin protein.[44] This has been shown to ameliorate the muscle damage associated with DMD and improve muscle function in affected mice.[44,45] Indeed one drug, eteplirsen, a PMO oligomer (section 1.5.2.4) has been approved for use in human patients with DMD.[46–48]

1.2.2.2 Anti-miRs

Anti-miRs are oligonucleotides that are designed to be complementary to miRNAs (section 1.1.2.2). Oligonucleotides that bind miRNAs prevent them from engaging with their targets and hence prevent the repression of the target, thus targeting a miRNA allows for upregulation of all of the genes under the control of the miRNA.[49]

Anti-miRs have been shown in model animals to effectively inhibit miR-122 reducing serum cholesterol,[49] miR-199b inhibiting cardiac hypertrophy,[50] miR-21 preventing autoimmune splenomegaly[51] and many others.[52] miR-10b inhibition was shown to induce upregulation of downstream genes with commensurate improvement in survival of glioblastoma-bearing mice.[9]

1.2.2.3 Translation Inhibition

Steric blocking oligonucleotides can be used to prevent translation of a disease mRNA by binding to the translation start site and preventing attachment of the ribosome. This mechanism of action has been used to inhibit replication of HIV[53] as well as N-myc in a neuroepithelioma cell line.[54]

1.3 Challenges with Oligonucleotides as Therapeutics

Because of their ability to manipulate expression of genes, oligonucleotides hold remarkable therapeutic potential for diseases of genetic origin. In order to fully realise this potential however, oligonucleotide drugs have significant hurdles to overcome to be effective as therapeutic molecules.

1.3.1 Size and Polarity

The Lipinksi rule of 5[55] implies that a good small molecule drug candidate will have fewer than 5 H-bond donors, 10 H-bond acceptors, a molecular weight (MWT) below 500 Da and a logP (log of the octanol/water partition coefficient) below 5. Oligonucleotides espouse none of these properties; a standard 20-mer oligomer will have 40-60 H-bond donors, greater than 40-60 H-bond acceptors, a molecular weight of ca. 7 kDa and a calculated logP of >100.[56] Chemical modification (discussed in section 1.5) can help to ameliorate some of the difficulties associated with such large molecules acting as drugs.

1.3.2 Immunological Challenges

The innate immune system can recognise specific patterns in oligonucleotides. Toll-like recepter 9 (TLR9) for example is activated by unmethylated CpG (a CG dinucleotide) present in bacterial DNA, but will also become activated following injection of an oligonucleotide containing an unmethylated CpG motif.[57–59] siRNAs can be recognised by TLR3 (which typically serves to recognise viral dsRNA) as well as TLRs 7 and 8,[60] though the specific motifs recognised by TLRs 7 and 8 are controversial.[61] TLR activation is associated with an inflammatory immune response which can result in treated patients experiencing flu-like symptoms post-administration.[62] This interaction of oligonucleotides with the innate immune system has however been explored with the intention to use it clinically. TLR9 agonist ASOs have been trialled clinically against melanoma[63], non small-cell lung cancer[64] and squamous cell carcinoma of the head and neck,[65] among others.[66] TLR9 agonist ASOs have been administered by intracerebral injection in patients with recurrent glioblastoma with some improvement in survival noted in a subset of the patient population.[67]

1.4 Delivery of Therapeutic Oligonucleotides

Delivery of oligonucleotide drugs is one of the most pressing challenges to be overcome in the field.[41] For oligonucleotides to exert their effect, they must first cross the cell membrane in order to interact with the target RNA. This is a trivial matter in cultured cells (section 1.4.2), however improving systemic administration of oligonucleotides in living organisms remains an active area of reserach.

1.4.1 Mechanisms of Internalisation

Uptake is thought to predominantly occur though either absorptive endocytosis and/or fluid phase pinocytosis depending on the system.[68–71] It was presumed that ASOs find

their target without a protein chaperone, however recent studies have shown that ASOs are significantly associated with several proteins once inside the cell.[68,72] Some proteins, such as Annexin A2, have been shown to affect endocytic trafficking and in doing so, to affect the degree of silencing observed.[73] Figure 1.3[74] shows the major pathways associated with both productive and non-productive internalisation of oligonucleotides.

Clathrin-mediated endocytosis is a well-studied pathway and describes the mechanism by which cells internalise substances that bind to cell-surface receptors. A significant proportion of the productive mechanisms for internalisation of oligonucleotides are clathrin-mediated. This occurs because of the oligonucleotide binding a cell-surface receptor that when bound by its endogenous ligand stimulates invagination of the clathrin-coated pit for the explicit purpose of internalising the bound ligand.[75] Oligonucleotides are also able to gain entry into cells through macropinocytosis,[76] pre-loading in to exosomes,[77] and *via* Nucleolin-1 binding.[78]

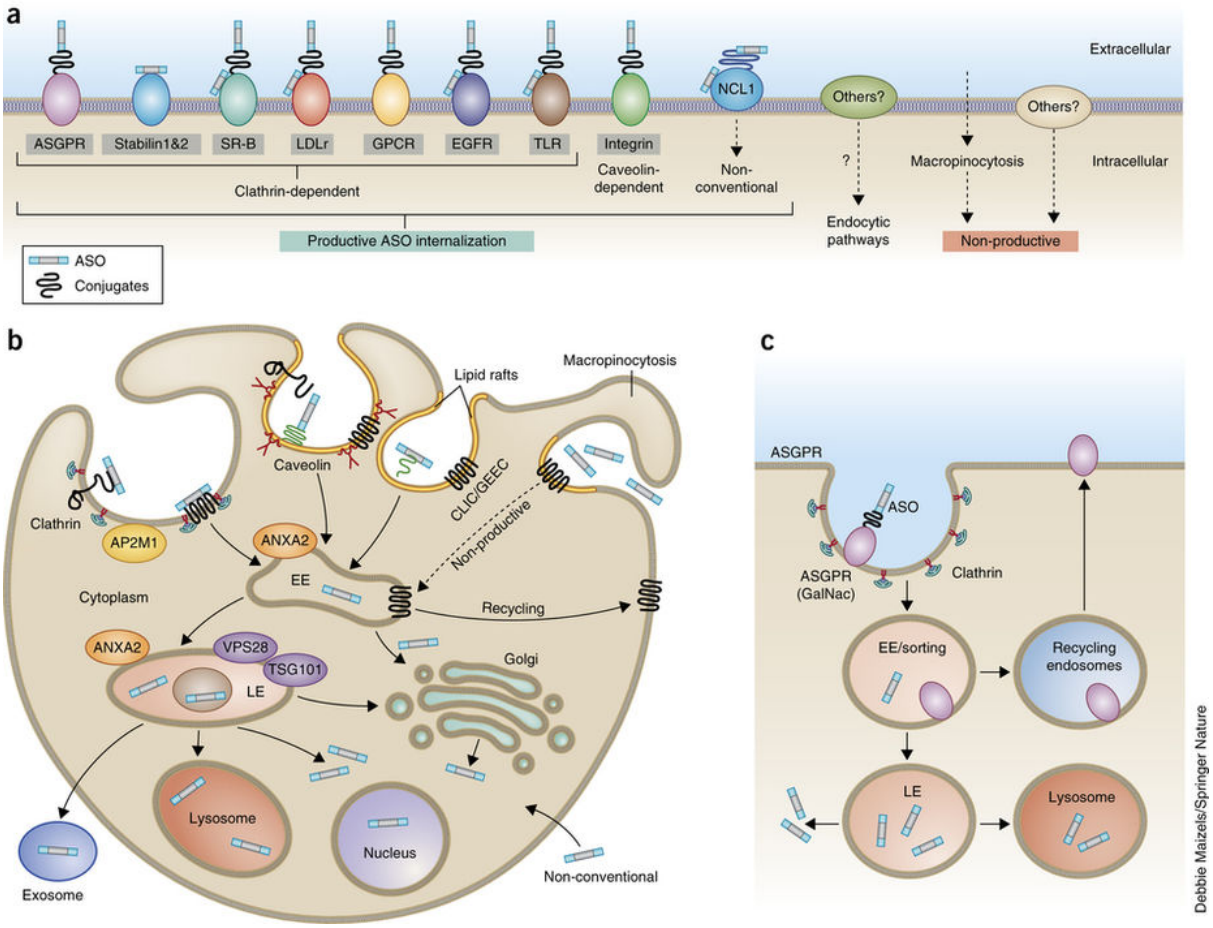


Figure 1.3: (a) Cell-surface ASO adsorption. Different cell surface proteins, including receptors, can interact with PS-ASOs either directly or via ligands conjugated to PS-ASOs. Cell-surface proteins can direct the internationalization of associated ASOs via clathrin- or caveolin-dependent endocytic pathways or via non-conventional endocytic pathways. Other proteins may also mediate ASO adsorption and internalization via different pathways. (b) PS-ASOs can enter cells via different endocytic pathways including macropinocytosis. Internalized ASOs can traffic from early endosomes (EE) to late endosomes (LE) and to lysosomes. ASOs must escape from endosomal organelles to reach the cytosol and nucleus to act on target RNAs. Some proteins (AP2M1 and ANXA2) may facilitate PS-ASO productive transport, whereas other proteins (VPS28, TSG101) may direct PS-ASO to non-productive pathways. The macropinocytosis pathway may represent a non-productive pathway for unformulated ASOs. PS-ASO internalization via non-conventional endocytic pathways is only conjecture. (c) ASOs conjugated with GalNAc enter cells via the interaction of the GalNAc moiety with the ASGPR receptor. PS-ASOs are internalized together with the ASGPR via clathrin-mediated endocytosis. The PS-ASO dissociates from ASGPR in early endosome, the receptor is recycled back to the plasma membrane, and the PS-ASO reaches the cytosol to act on target RNAs. Used with permission.[74]

1.4.2 Transfection into Cultured Cells

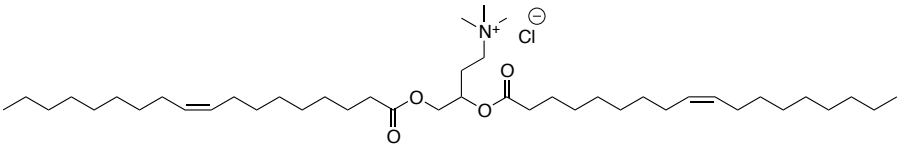


Figure 1.4: Lipid Transfection Agent DOTMA

In cultured cells, internalisation is often effected using a cationic lipid (e.g. N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) (Fig. 1.4)). This interacts with the anionic oligonucleotide, forming a complex that is able to permeate the cell and release the oligonucleotide when inside the cell.[30] Once internalised, the oligonucleotide must escape from endosomal vesicles in order to interact with the target RNA (and to avoid lysosomal degradation) – this has been effected using endosome membrane destabilising molecules (such as dioleylphosphatidylethanolamine (DOPE)).[69]

1.4.3 Delivery of Oligonucleotides *In Vivo*

Unfortunately, while cationic lipid-mediated techniques are useful for *in vitro* studies on cultured cells, they are impractical for *in vivo* systems owing to lipid-mediated toxicity.[79] Lipid nanoparticles are rapidly engulfed by macrophages of the the reticuloendothelial system (RES). Several strategies have been used to try and overcome this problematic immune engagement.[76] PEGylated lipid nanoparticles, for example, bind protein minimally and avoid phagocytosis by macrophages.[80] The most clinically advanced siRNA drug, patisiran (to treat transthyretin amyloidosis) is lipid encapsulated[81] and expected to be approved by the FDA in 2018.

Oligonucleotides have also been conjugated to small molecules to improve their delivery. Lipids (including anandamide),[82–84] folate,[85] as well as others have been used and shown to improve delivery.[86] Oligonucleotide conjugates are discussed in greater detail in section 1.5.4.

Conjugation of oligonucleotides to cell-penetrating peptides has also proven efficacious in *in vivo* studies, with several ASOs and siRNAs having been successfully delivered.[79,87] Significant progress has been made conjugating efficacious cell-penetrating peptides to non-charged oligonucleotides such as PMOs (1.5.2.4).[88]

1.5 Chemical Modifications

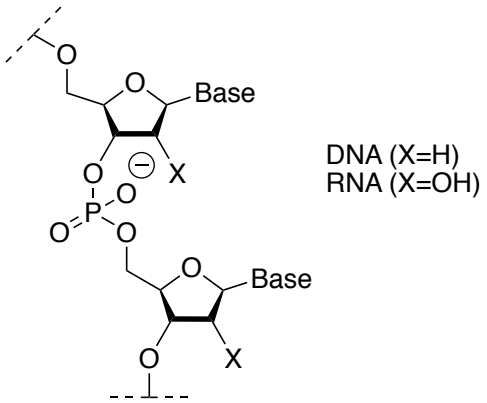


Figure 1.5: Unmodified backbone of native DNA or RNA.

Unmodified nucleic acids (Fig. 1.5) are very rapidly degraded by intracellular endonucleases and exonucleases.[70,89] Such degradation produces cytotoxic and antiproliferative nucleotide monophosphates as well as preventing the oligomer from interacting with its target genetic material.[90] Modifying oligonucleotides allows for their biological persistence and can improve potency allowing for more effective gene modification.

1.5.1 Backbone Modifications

1.5.1.1 Methylphosphonate Chemistry

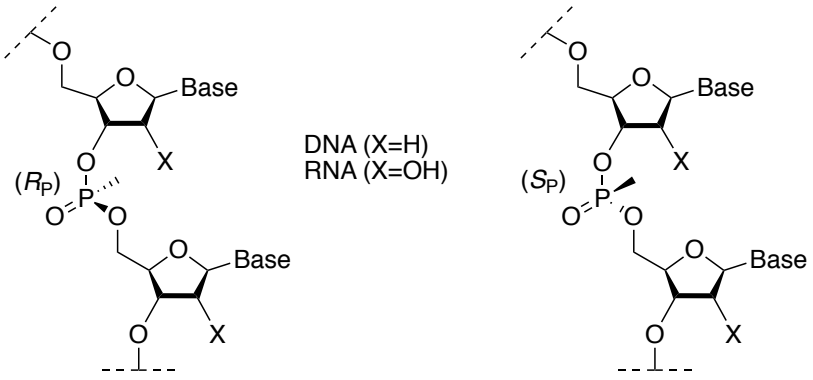


Figure 1.6: Methylphosphonate backbone

An early synthetic modification to oligonucleotides was the methylphosphonate analogue.[70] These non-charged oligomers, formed by replacement of a non-bridging oxygen with a methyl group (Fig. 1.6) have a high resistance to endogenous nucleases, have no charge along the structure, and are chiral at phosphorus.[91] Despite their enhanced stability, oligonucleotides consisting entirely of methylphosphonate nucleotides are unsuitable for use in a clinical setting; their non-ionic nature reduces their solubility but more importantly they are unable to recruit RNase H.[70,92]

1.5.1.2 Phosphorothioate

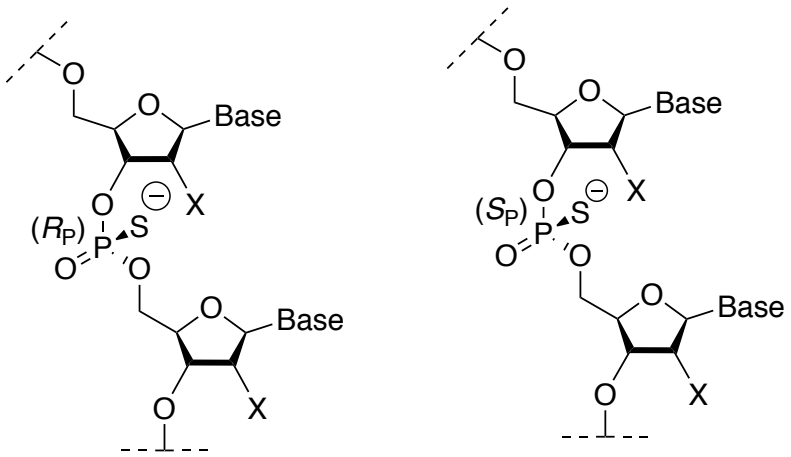


Figure 1.7: Phosphorothioate backbone

Phosphorothioate-modified oligodeoxynucleotides (PS-ODNs) were the first true breakthrough in the chemistry of oligonucleotides.[93,94] They contain a sulfur atom in place of a non-bridging oxygen atom (Fig. 1.7). PS-ODNs have an excellent resistance to endogenous nucleases and they retain their charge, facilitating good solubility. Phosphorothioate-modified oligonucleotides exhibit higher affinity binding to both plasma and cell-surface proteins which results in enhanced cellular uptake.[95] In addition, PS-ODNs exhibit interactions with several proteins, especially those with the ability to bind strongly to anionic molecules (such as heparin binding proteins), which can induce some toxicity.[41,96] This property is also responsible for some of the desirable pharmacokinetic properties observed, such as an increased serum half life.[41,97] PS-ODNs are able to induce cleavage of complementary RNA by recruiting RNase H.[30,70,96,98,99] The PS modification does however reduce affinity; the melting temperature of PS-ODN•RNA duplexes has been seen to decrease by up to 0.5 °C/nucleotide relative to the non modified complex.[41,68,96,99] In primate studies, rapid intravenous introduction of PS-ODNs has resulted in activation of the complement cascade.[100] The cause of this effect is thought to be as a result of the peak plasma concentration (>40-50 μg/mL) as slow intravenous infusion did not produce the

same effect.[100] These reservations aside, the PS-ODN modification is the common modification of choice in therapeutics. Four approved antisense therapeutics, mipomersen (familial hypercholesterolemia), fomivirsen (cytomegalovirus retinitis), alicaforsen (pouchitis) and nusinersen (spinal muscular atrophy) are fully phosphorothioate modified and 18-21 bases in length.[101–106]

1.5.1.3 Stereochemically Defined Phosphorothioate Linkages

The introduction of sulfur induces chirality at phosphorus, producing complex mixtures of diasterioisomers (2^{n-1}, where n is the length of the chain) that is used without isomeric separation. This mixture of diastereoisomers is completely inseparable post-synthetically, and early synthetic attempts to control stereochemistry at phosphorothioate linkages were tedious.[107] Initial attempts consisted of synthesising stereorandom dimers and purifying the single phosphorothioate-containing molecules using HPLC. The individual diastereoisomers could then be phosphitylated for use on an automated DNA synthesier. While this allowed for specific orientations of skip-stereoselective oligonucleotides, wholly stereoselective oligonucleotides remained inaccessible.[108] Clearly preparation of longer stretches of stereopure oligonucleotides required control at the synthetic level, as opposed to post-synthetic resolution of isomers. Interestingly, analysis of diastereoisomeric composition of various oligonucleotides synthesised in a stereorandom fashion indicated that the isomeric ratio is not 50:50, but that it varies significantly depending on the context of the sequence.[109] This is perhaps not surprising given the extremely chiral nature of the DNA molecule.

With significant research, chiral phosphoramidite chemistry was developed that was able to achieve a 98:2 diastereoisomeric ratio at each sulfurisation step. This allowed Ionis pharmaceuticals to investigate the *in vitro* and *in vivo* properties of stereochemically defined phosphorothioate linkages.[110] Dishearteningly, no significant improvements were noticed in the limited array of synthesised compounds, despite the marginal improvement

in melting temperature of the fully R_p oligonucleotide compared to the fully stereorandom molecule.

A significant body of work was completed more recently by Wave Biosciences,[111] wherein they disclosed that a specific diastereoisomeric pattern of phosphorothioate stereochemistry ($S_pS_pR_p$) significantly improved the *in vivo* efficacy, *in vitro* activity and *in vitro* metabolic stability of the approved stereorandom drug mipomersen.[111] Similar improvement have been shown with stereopure phosphorothioate oligonucleotides by Roche.[112] With this knowledge, it is reasonable to suggest that a significant body of future drug molecules will be diastereospecific.

1.5.2 Sugar Modifications

1.5.2.1 2′-O-Alkyl Ribose Modifications

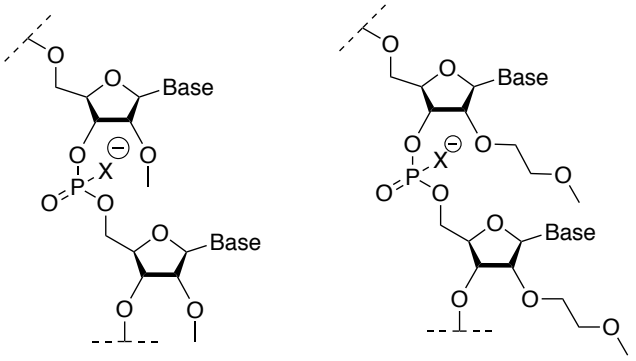


Figure 1.8: 2′-O-alkyl ribose modifications Left: 2′ O-Methyl RNA (OMe) Right: 2′ O-methoxyethyl RNA (MOE).
X= O or S.

Following PS-ODNs came some of the 2′-O-sugar modified oligonucleotides (Fig. 1.8).[68] These modifications increase the oligonucleotides' resistance to endogenous nucleases and increase binding affinity to target mRNA.[113] 2′-O-alkyl substituted oligonucleotides do not activate RNase H, and hence are unable to exert an antisense effect through the cleavage mechanism.[113,114] These oligonucleotides are, however, able

to induce gene silencing and splice-switching through the steric block mechanism at good therapeutic concentrations ($IC_{(50)}$= 2 nM) by targeting the 5′ end of the target mRNA, near the translation start site.[41,115,116]

1.5.2.2 LNA, cEt and tcDNA

The ribofuranose ring, unlike a 6-membered ring, does not have a strongly preferred conformation and is more flexible permitting several conformations (Fig. 1.9). Predominant biologically relevant minima are the 2′-endo, assumed by B-DNA, and 3′-endo, assumed by RNA and A-DNA.[117]

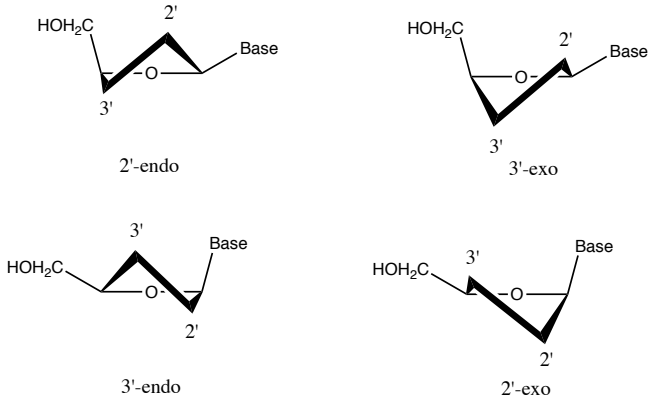


Figure 1.9: Ribose conformers. 2′ and 3′ positions shown unsubstituted for clarity.

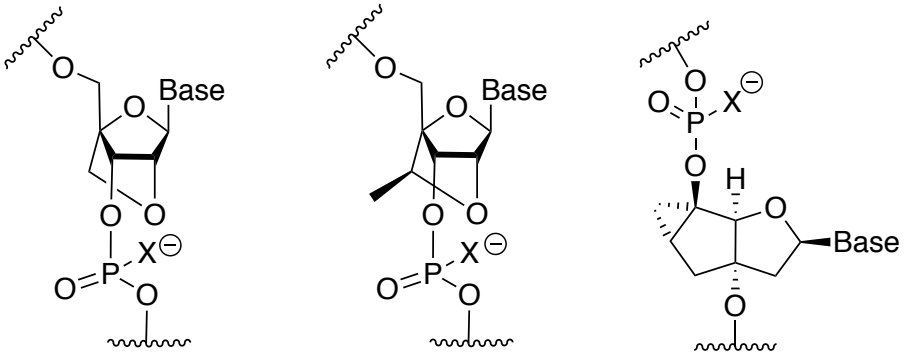


Figure 1.10: Modifications: Locked Nucleic Acid (LNA), S-constrained Ethyl (S-cEt) and TricycloDNA (tcDNA).
X= O or S.

LNA (Fig. 1.10) is a conformationally restricted derivative of RNA, forcing a 3′-endo configuration at the ribose sugar.[118] Inclusion of LNA nucleotides in either strand can increase the binding affinity of DNA and RNA homoduplexes as well as DNA•RNA hybrids.[119] Increases in the melting temperature of ca. 2-5°C per locked nucleotide when binding to DNA and ca. 4-8 °C when binding to RNA, providing excellent target binding.[120,121] While pure LNA is unable to recruit RNase H, DNA-LNA gapmers (see section 1.5.3) are able to do so, as long as the DNA gap is sufficient.[122,123]

c-Et modified nucleobases have many of the same properties as LNA, which one might expect given their similar structures. While there is a stereocenter present at the bridging carbon of the cEt nucleoside that is absent in LNA, only the (S) isomer is used therapeutically owing to marginally improved silencing effect and binding strength relative to the (R) isomer.[124,125]

Interestingly, LNA and cEt containing gapmers show significantly more hepatotoxicity than do corresponding MOE containing gapmers, and this has been shown to be a function of their ability to bind and cleave off-target RNAs.[126] Judicious sequence selection is necessary when using these high affinity modifications in gapmer oligonucleotides in order to prevent such toxicity.

tcDNA was first detailed in the literature in 1997.[127] Inclusion of tcDNA in an oligonucleotide provides an increase in melting temperature against RNA of ca. 1°C per inclusion.[128] While the improvement in binding is somewhat modest by comparison with the LNA and cEt modifications, it is typically the case that tcDNA comprises the full length of the oligomer as opposed to the sporadic inclusion of the other modifications.[128] tcDNA enjoys a somewhat privileged position in the current therapeutic zeitgeist as an excellent modification for induction of splice-correction, notably for DMD.[129,130] One of the major differences between tcDNA and other modifications is the reported improvement in functional biodistribution of tcDNA-containing oligonucleotides to both muscle and across the BBB at high systemic doses (200 mg/kg).[131]

1.5.2.3 Novel Bridged Nucleic Acids

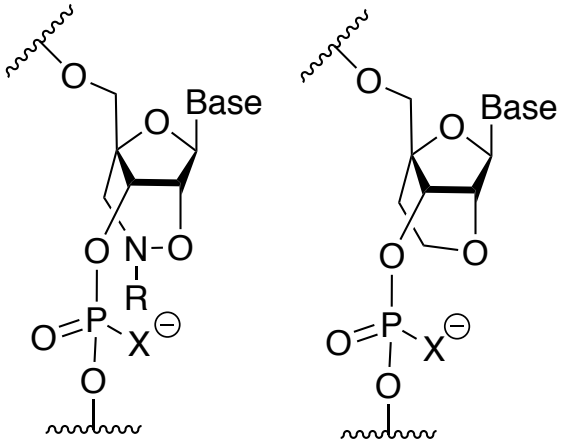


Figure 1.11: Modifications: 2′,4′-BNA^{NC} and ENA.
R= H, Me, Bn and others, X= O or S.

In addition to the previously discussed bridged nucleic acids, many other BNAs have also been synthesised. Two of the better-characterised modifications are shown in Figure 1.11.

2′,4′-BNA^{NC} was reported in the literature in 2007.[132] The N-Me version of the modification showed remarkable nuclease resistance (moreso than a phosphorothioate inclusion), and the NH and NBn versions also showed better nuclease resistance than native LNA. All members of this family of modifications had enhanced binding to target RNA to approximately the same extent as LNA.[132] Interestingly, when the N-Me modification was incorporated into oligonucleotides, it was shown to give more potent silencing than the equivalent LNA-containing analogue for shorter oligonucleotides.[127] *In vivo*, N-Me 2′,4′-BNA^{NC} containing oligonucleotides were of comparable or higher potency than their LNA analogues, with no significant differences in toxicity.[133]

ENA containing oligonucleotides were introduced in 2001, with the original work detailing a 5 °C increase in T_m per inclusion and 80 fold improvement in nuclease resistance compared with LNA.[134,135] Since this point, it has been shown that ENA-containing oligonucleotides are able to induce exon 19 skipping in dystrophin mRNA *in vitro*.[136] There is relatively little published information available on the *in*

vivo properties of ENA-containing oligonucleotides, with some data available that compares ENA chemistry favourably with MOE.[137] Despite this, there have been several clinical trials in humans using ENA containing oligonucleotides for dystrophin exon-skipping.[138,139]

1.5.2.4 Amide and Phosphorodiamidate Containing Sugar-free Oligonucleotides

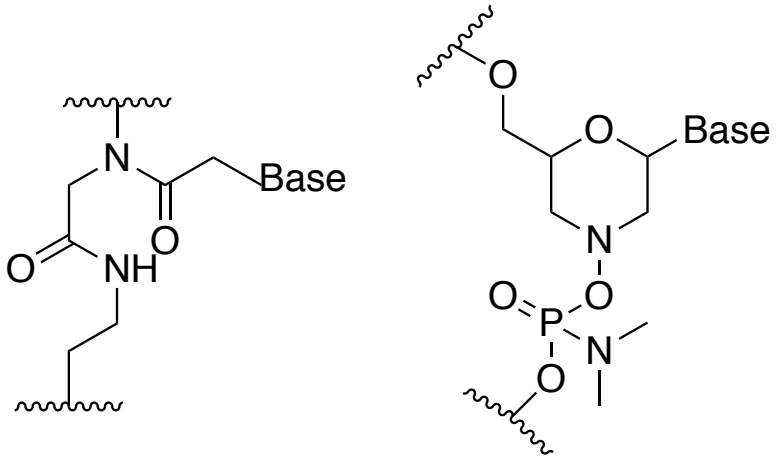


Figure 1.12: Sugar-free modifications
Left: PNA, Right: PMO

Further modifications attempted through modification of the ribose ring to improve nuclease resistance, target affinity, biostability and pharmacokinetics.[41,68] The diversity of this group of molecules is particularly rich featuring modifications at both the phosphate and sugar moieties of DNA.[41] The most studied of these analogues are peptide nucleic acid (PNA) and phosphoroamidate morpholino oligomer (PMO) (Fig. 1.12).[41] It should be noted that as all of the discussed modifications are on the ribose sugar, none of the following oligonucleotide derivatives are able to recruit RNase H, and instead produce their antisense effects by steric blockage.[118]

PNA is pseudopolypeptide DNA mimic based on N-(2-aminoethyl)glycine. PNA's neutral backbone renders it immune to nucleases and peptidases, also it prevents

nonspecific protein binding (i.e. from polyanion-recognising proteins), avoiding one source of unintended interaction.[68,140,141] PNA•DNA hybrids are not affected by electrostatic intrastrand repulsion as DNA•DNA duplexes are, owing to PNA's neutral backbone, this improves hybrid stability increasing both affinity and rate of association of PNA.[142,143] The neutral backbone however causes significant solubility issues, raising several concerns over cell permeability requiring further modifications for use even in cell studies.[118] Pharmacokinetic studies have revealed that PNAs are rapidly excreted from the body, presumably owing to their inability to adequtely bind proteins, decreasing their potential usability.[118]

1.5.3 Gapmers

To harness the beneficial stabilising effects of sugar modifications, but maintain the RNase H recruiting facility of PS-ODNs, chimeric 'Gapmers' (Fig. 1.13) were synthesised.[41,144] These consist of a stretch of phosphorothioate-modified DNA flanked on each side by extremely nuclease resistant 'wings'. Gapmers have the advantage of being resistant to nuclease degradation while also being able to recruit RNase H, as long as there are at least 6 consecutive PS-DNA residues present.[41,144–146]

Gapmers may also present a solution to RNase H's promiscuity.[146] RNase H is able to effect its activity on sequences of DNA•RNA hybrid that are only partly complementary.[147] This, coupled with its minimal requirement of 4-5 bases of complementarity, leads to the rather counterintuitive notion that longer sequences of RNase H competent oligonucleotide may be less specific.[146] A hypothetical 15-mer strand may be viewed as 8 overlapping 8-mers, this drastically decreases specificity within the genome from 1 in 10^9 for a 15-mer to 1 in 8192 for the sum of the 8-mers.[146,148] Gapmer oligonucleotides with an RNase H competent sequence of only 6-8 nt of DNA may reduce this undesirable, promiscuous cleavage.[41]

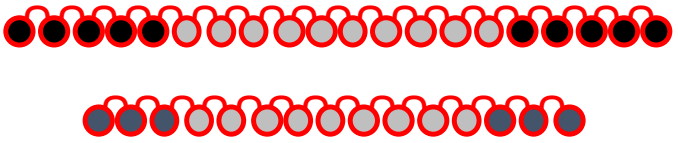


Figure 1.13: Schematic example of two potential gapmer designs, a 5-10-5 (top) and a 3-9-3 (bottom).

1.5.4 Oligonucleotide Conjugates

Small molecule conjugates are rapidly emerging as an additional 'modification' for therapeutic oligonucleotides. The main use of these small, typically hydrophobic conjugates is to improve the pharmacokinetic properties of siRNA molecules.[149] Cholesterol was the first of these, though the field has expanded to include fatty acids like palmitic acid[150] and cell-penetrating peptides[151]. The most promising of these conjugates have so far been various configurations of the N-acetyl-galactosamine (GalNAc) sugar, the most clinically advanced of which is shown in Figure 1.14.[152]

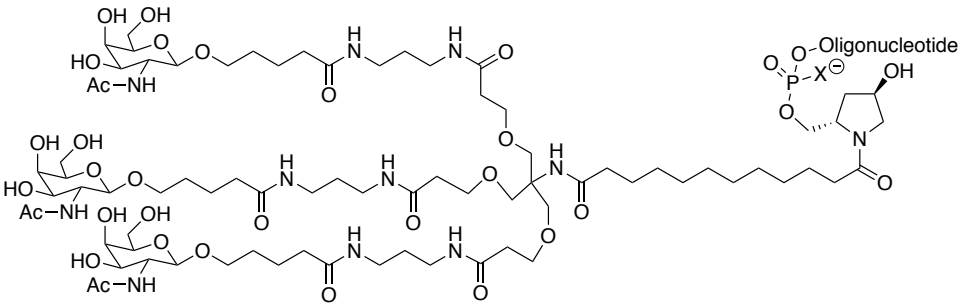


Figure 1.14: 'Triantennery' GalNAc conjugate developed by Alnylam Pharmaceuticals. X= O or S.

siRNAs conjugated to GalNAc show enhanced bioaccumulation in hepatocytes in the liver, owing to asialoglycoprotein receptor binding.[153] GalNAc conjugation has allowed

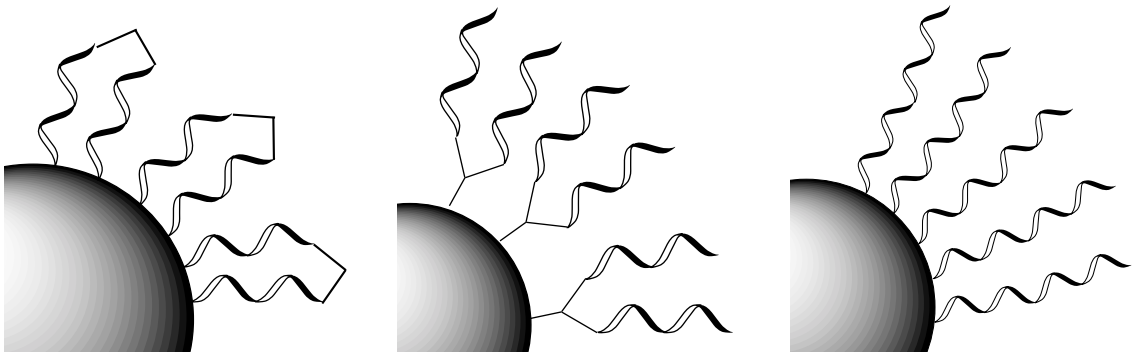
for rapid advancement of siRNA therapeutics targeting genes expressed in the liver in clinical trials. Antisense oligonucleotides have also been conjugated to various small-molecule moieties, including GalNAc, to similarly facilitate an improvement in pharmacokinetics and pharmacodynamics. Indeed, GalNAc conjugated ASOs demonstrate the same hepatocyte uptake as their siRNA counterparts.[154]Despite some initial drug candidate failures, there is hope that GalNAc conjugated oligonucleotide therapeutics will find their way into clinical use soon.[155]

1.6 Multimeric Oligonucleotides

Multimeric oligonucleotides have been reported in the literature in various iterations for years.[156–160] Work on the topic has accelerated in recent years subsequent to some quite remarkable discoveries about their unique properties. While sparse reports of multi (3+) armed dendrimers do indeed exist, there seems to be a dearth of work pressing past the more common dimeric structures. Studies comparing the biological properties of a homologous series of dendrimers are non-existent, and yet it was felt it necessary in understanding the biological properties of this unsatisfactorily categorised class of oligonucleotides.

1.6.1 Solid Phase Synthesis of Multimeric Oligonucleotides

Oligonucleotide dendrimers can be synthesised in several ways on solid support. Each of these methods has the benefit of being conducted entirely on an automated oligonucleotide synthesiser, using standard phosphoramidite chemistry. This allows for all reactions to be completed in automation as opposed to a separate post-synthetic step, in which some synthesised oligonucleotide may be lost. The principal disadvantage to this method is that purification can pose a challenge owing to the diminishing discrepancy between full length and n-x products resulting from the synthesis of larger oligonucleotides.



(a) Convergent approach **(b)** Divergent approach **(c)** Linear approach

Figure 1.15: Solid-phase synthetic routes to multimeric oligonucleotides, shown still bound to synthesis support (only dimers are shown).

1.6.1.1 Convergent Approach

Convergent synthesis of multimeric oligonucleotides (Fig. 1.15a) was pioneered by the Damha lab in 1993.[159] In this approach, oligonucleotides are grown on the solid support and then pinched off using a bisphosphoramidite reagent. In the original work, this was accomplished using the bisphosphoramidite derivative of rA (Fig. 1.16) to react simultaneously with two already grown strands. This can then be subjected to further oligonucleotide growth and pinching cycles to yield large oligonucleotide dendrimers.[159] This method has been used to synthesise multimeric oligonucleotides containing up to 14 arms (consisting solely of dT bases and the rA branching base). The full length product constitutes a small (but not quantitated) percentage of the overall product mixture.[159]

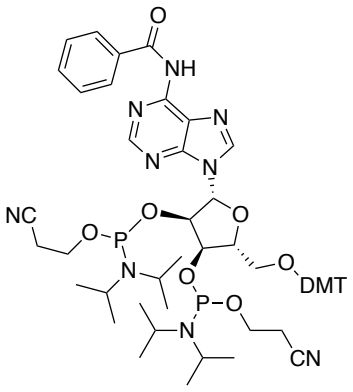


Figure 1.16: Bisphosphoramidite used in the original synthesis of Convergent Dendrimers

1.6.1.2 Divergent Approach

The divergent approach to multimeric oligonucleotides (Fig. 1.15b) was first introduced by the Southern lab in 1997.[156] Here, a 'branching' phosphoramidite is used to introduce multiple reactive sites from which an oligonucleotide can grow. Both 'doubler' and 'trebler' reagents were described (Fig. 1.17). Dendrimers containing up to 27 arms have been reported using this method with DNA phosphoramidites.[157]

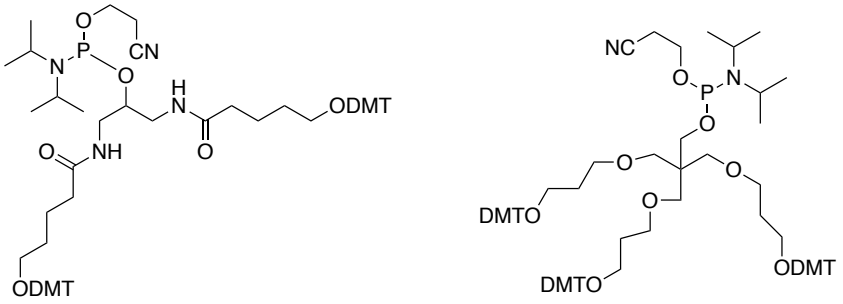


Figure 1.17: Doubler (left) and trebler (right) phosphoramidites.

1.6.1.3 Linear Approach



Figure 1.18: Schematic representation of a linear multimer consisting of 2 15-mers joined by 4 phosphate-containing bases.
Black borders and linkages = PO; red borders and linkages = PS

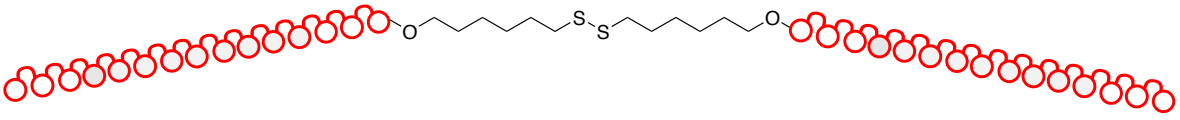


Figure 1.19: Schematic representation of a linear multimer consisting of 2 15-mers joined by a disulphide linker.

The linear approach towards making multimeric oligonucleotides (Fig. 1.15c) is the simplest, requiring no additional reagents beyond standard phosphoramidites. Oligonucleotides are grown in a stepwise fashion, typically with a biocleavable linker between strands. This often takes the form of a phosphodiester linked stretch of approximately four DNA nucleotides, a nuclease-labile stretch that allows for cleavage in the lysosome to give the individual active strands. The main benefit to in line synthesis is that, in effect, it is equivalent to synthesising one long oligomer, protocols for which have been established for years. In a very similar manner, two linear oligonucleotides have been linked by a disulphide-containing modifier (Fig. 1.19).[116]

In contrast to both the convergent and divergent approaches, this linear multimer exists as an oligonucleotide with both 3′ and 5′-ends presented. It also lacks a discernable 'shape'- the (> 3-armed) dendrimers produced by the other approaches are likely pseudo-spheroidal in nature as a result of steric and electrostatic repulsion, whereas linear multimers likely maintain their linearity (Fig. 1.18).

1.6.1.4 Polarity

Multimeric oligonucleotides can be synthesised in a certain polarity such that they present one end of their oligonucleotides as opposed to the other (Fig. 1.20). The nature of this polarity has significant effect on the immunogenicity of the resulting molecule (discussed in 1.6.3.3).

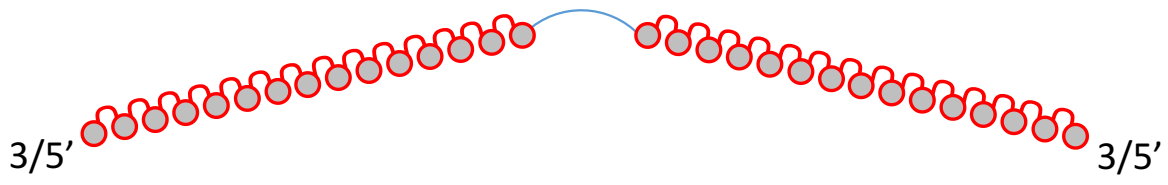


Figure 1.20: Schematic representation of a dimer consisting of 2 15-mers linked by some small molecule linker.
Black borders and linkages = PO; red borders and linkages = PS, blue curve = small molecule linker

As the usual direction of synthesis for an oligonucleotide on solid support is 3′ to 5′, one might expect that convergently synthesised dendrimers would yield structures with their 3′-end out, divergently dendrimers would yield structures with their 5′-end out and that linearly synthesised dendrimers would have one 3′ end and one 5′ end presenting. However, the convergent synthetic route is not typically used by groups looking to prepare 3′-end out dendrimers – more commonly, reversed phosphoramidites are employed (Fig. 1.21). Reversed phosphoramidites can also be used to prepare linear dendrimers targeting different genes, but maintaining the desired polarity. Oligonucleotide synthesis is described in section 1.7.

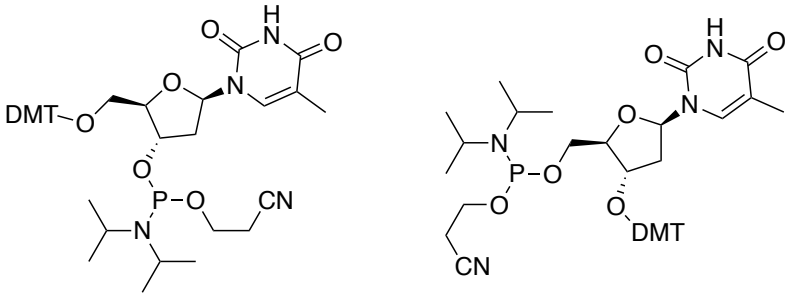


Figure 1.21: Left: standard 3′-phosphoramidite thymidine monomer, right: 'reversed' 5′-phosphoramidite thymidine monomer

1.6.2 Post-synthetic assembly of Multimeric Oligonucleotides

In contrast to the solid-phase approach, the post-synthetic route to dendrimeric oligonucleotides requires the synthesis and purification of the appropriately-modified oligonucleotide followed by chemical conjugation to a pre-defined core. The benefit to this approach is that oligonucleotides can be pre-purified, reducing the challenge of purifying n-x impurities[1] from the bulk reaction. The drawbacks are the necessity of chemical modification to allow for orthogonal conjugation, the necessity for the

[1] Purifying a 14-mer from an intended 15-mer requires separation of compounds that have 93% similarity in charge, separating a 59-mer from a 60-mer requires separation of compounds that are 98% similar, a significantly more challenging process.

synthesis of a small-molecule core and the difficulty of ensuring complete conversion to the fully-substituted dendrimer.

1.6.2.1 Click Chemistry

The use of click chemistry is a logical starting point in the assembly of dendrimers. Coined by Sharpless in 2001, the term 'click chemistry' describes a handful of extremely efficient chemical reactions that can be used to assemble larger, more complex molecules.[161] The exemplar reaction is the copper-catalysed azide-alkyne cycloaddition (CuAAC) reaction (Fig. 1.22).

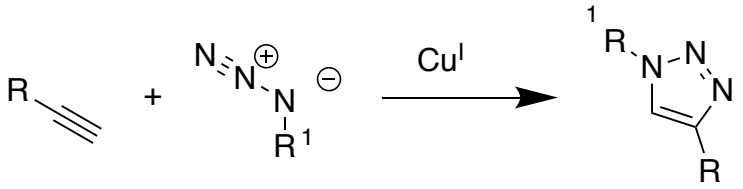


Figure 1.22: Copper-catalysed azide-alkyne cycloaddition reaction.

The CuAAC reaction has been used to assemble dendrimeric (2-4 single-stranded arms) oligonucleotides around an azide-containing pentaerythritol-based core[160] as well as a click-assembled core (3-9 double-stranded arms) (Fig. 1.23).[162]

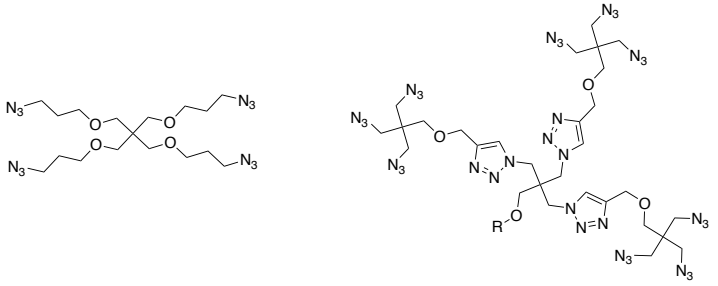


Figure 1.23: 4 and 9 arm azide-tagged cores for dendrimer assembly.

One of the major challenges with this approach is that of incomplete reaction leading to partially-formed dendrimers, which necessitates an additional purification step to isolate the desired material. It seems to be the case that the larger dendrimers are more challenging to make with this approach, which is intuitive considering the incumbent steric congestion as more oligonucleotides are added as well as the inherent electrostatic repulsion from the polyanionic backbones. The other major inherent challenge is the use of the toxic metal copper in the reaction. Failure to remove all traces of copper from the synthesised dendrimers can result in significant toxicity to treated cells and animals. Copper also has a tendency to induce DNA strand scission,[163] frequently resulting in degradation of oligonucleotides subjected to CuAAC reaction.

An alternative to the CuAAC is the strain-promoted azide-alkyne cycloaddition (SPAAC), a copper-free cycloaddition reaction that functions by virtue of a strained alkyne. Phosphoramidites containing this moiety are commercially available (Fig. 1.24) and have been used to prepare single-stranded oligonucleotide dimers.[164]

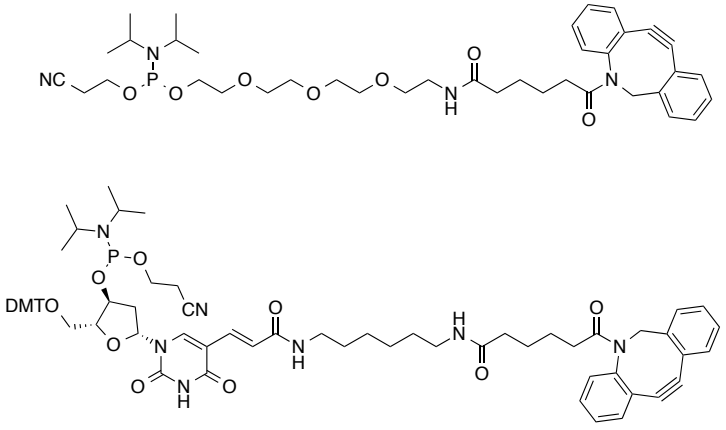


Figure 1.24: Some available strained alkyne-containing phosphoramidites.

1.6.2.2 Thiol Assembly

Another approach to multimerisation was published in 2010 by Mok *et al.*, relying on the use of dimeric maleimide-containing crosslinking molecules (Fig. 1.25) and thiol-modified siRNA.[165] The authors used disulphide-containing dithio-bismaleimido-ethane (DTME)

to serve as a biocleavable linker (relying on glutathione cleavage after endocytosis) as well as a non-cleavable bis-maleimido-polyethylene glycol ($BM(PEG)_2$) linker.

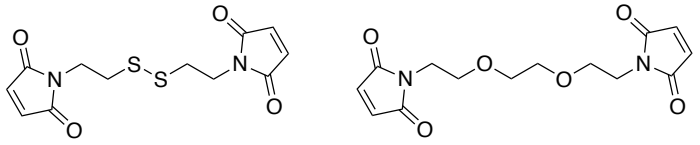


Figure 1.25: Cross linking bis-maleimide reagents DTME (top) and $BM(PEG)_2$ (bottom)

The sense and antisense strands were separately dimerised with both the biocleavable and the non-biocleavable linkers before being allowed to hybridise. The pre-dimerisation allowed for the formation of an equilibrium mixture of long-chain siRNAs.

This approach was extended in 2011 by the use of a trimeric maleimide-containing linker that allowed for the formation of 3-dimensional structures.[166] It is challenging to estimate the overall size of the dendrimers formed by this method, however because the assembly is mediated by base-pairing it is highly likely that the average length is sequence-dependent and proportional to the T_m of the duplex.[166]

1.6.2.3 Nanoparticles: Gold and Beyond

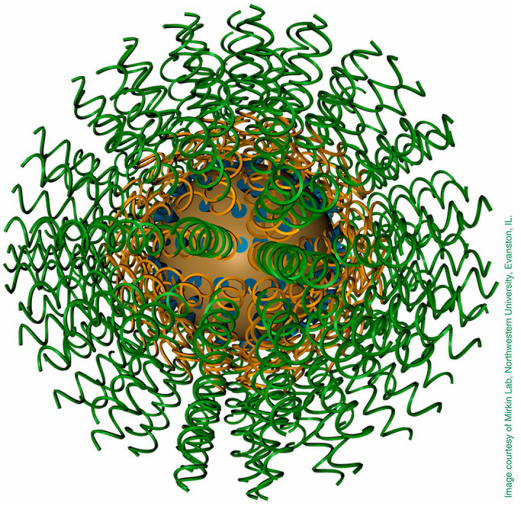


Figure 1.26: A typical ca. 13 nm gold nanoparticle functionalised with 80-100 thiol-modified oligonucleotides.

The first spherical nucleic acids (SNAs, Fig. 1.26) were introduced in 1996 by the Mirkin and Schultz groups.[167,168] In these introductory papers, thiolated DNA was attached to gold nanoparticles which were shown to assemble into colloids upon the addition of a linker DNA strand. Furthermore it was demonstrated that this was a thermally reversible process and that the DNA-AuNP conjugates were stable at high temperatures (80°C).[167]

Further work by the Mirkin group demonstrated that it was possible to alter the composition of the inorganic core using Ag[169], Fe_2O_3[170] and semiconductor[171] cores among others. Each core confers its own novel properties such as magnetism, luminescence, and others to the system.

Lipid nanoparticles, such as liposomes, are also able to interact with lipid-tagged oligonucleotides to form spherical nucleic acids.[172] When decorated with immunostimulatory oligonucleotides, these structures demonstrate more potent immune activation than the corresponding monomeric oligonucleotides.[173]

1.6.3 Functionality of Multimeric Oligonucleotides

1.6.3.1 Cellular Uptake

Multimeric oligonucleotides show generally improved uptake into cultured cells. A divergently assembled antisense dendrimer was synthesised and fluorescently tagged for uptake. The construct was fou times better taken up by cells relative to the monomeric oligonucleotide of the same sequence.[174] An oligonucleotide dimer linked by a biologically cleavable linker was shown to be taken up more effectively than a single oligonucleotide[175,176] and the same was found in neurones with an anandamide-tagged siRNA nonamer.[162] Larger SNA constructs demonstrate unexpected *in vivo* biodistribution, even crossing in to the brain in mice with established glioblastoma-like tumours.[177] SNAs are also taken up into almost all human cell types with the exception of red blood cells.[178,179] Interestingly, it was also shown that coreless SNAs[179] retained their uptake properties implying that the enhanced endocytosis is not as a result of the inorganic core.[180] Further experiments have shown that absorption of these SNAs occurs *via* scavenger receptors in contrast to the typically observed absorptive endocytosis and/or fluid phase pinocytosis for traditional oligonucleotides.[180,181]

It has also been shown that ASOs that self-assemble into nanoparticles are better taken up than ASOs that do not.[182]

1.6.3.2 Gene Silencing

Multimeric structures have shown improved silencing both *in vitro* and *in vivo*. A linear dimer comprising two LNA-containing antimiRs, joined by a short stretch of phosphodiester DNA exerted a more potent effect than did either antimiR administered alone. It was also shown that the use of two separate antimiRs (oligonucleotides complementary to miRNAs), targeted to different miRNAs could be made into a linear dimer with greater effect than either of the two administered individually. This

propensity for an enhancement in efficacy of dimeric antimiRs held true for RNase H recruiting gapmers. A heterodimeric oligonucleotide containing a biocleavable linkage between a gapmer against ApoC3 and a gapmer against ApoB showed enhanced silencing of both target genes compared to co-administration of the same gapmers.[175,176,183]

Bhagat *et al.* demonstrated that dimeric oligonucleotides with their 3′-ends out silence ca. 40% more effectively than the monomeric equivalent.[184] Similarly, a phosphorothioate DNA nonamer oligonucleotide dendrimer targeted against EGFR was shown to be ca. 60% more effective than the corresponding monomer.[174] Disulfide-linked dimeric oligonucleotides have been shown in mouse models of DMD to be at least as effective as the co-administration of the two separate oligonucleotides.[116]

Post-synthetically assembled trimer and nonamer siRNA directed against the rabies virus were shown to be more efficacious than the parent monomeric siRNA.[162]

SNA structures have also been shown to effectively silence target genes *in vitro* and *in vivo*.[177] For example, an SNA decorated with antisense oligonucleotides targeted against Her2, an oncogene, was able to induce similar silencing to the transfected monomeric oligonucleotide in cultured cells.[185]

1.6.3.3 Immunogenicity

Some oligonucleotides have intrinsic immunogenicity (as discussed in 1.3.2). Dendrimerised oligonucleotides have the ability to either enhance or obviate immunogenic responses. 5′-end out dimeric oligonucleotides (so-called 'Immunomers' (Fig. 1.20)) have been shown to robustly agonise TLR7 and TLR9 receptors *in vitro* and *in vivo* resulting in a significant increase in relevant cytokines.[186,187] Immunomers have demonstrated the ability to reduce the size of primary, as well as distant metastatic melanomas in humans refractory to anti PD-1 therapy.[188,189]

Oligonucleotides can also act to abrogate the immune response. Kandimalla *et al* demonstrated that monomeric and dimeric oligonucleotides containing CpG motifs with 2′-O-methyl C, 5′-methylC or 2′-O-methylG modifications abrogate the TLR7, 8 and 9-mediated immune response.[190,191] An immune antagonist oligonucleotide has completed phase 2 clinical trials for treatment of psoriasis and dermatomyositis.[192]

Dimeric oligonucleotides with their 3′-ends out (Fig. 1.20) do not seem to activate the immune system and are used as effective silencing tools (see section 1.6.3.2).[185,193]

Spherical nucleic acids have been reported as being both highly immunostimulatory[194] and completely immune-inactive.[180] In part, this can be controlled by the nature of both the core and the bound oligonucleotides.

1.6.3.4 Nuclease Stability

Small oligonucleotide dendrimers (2-9 arms) have displayed greater nuclease resistance than their corresponding monomeric oligonucleotides.[165,174] A fully phosphorothioate-modified monomer was 90% degraded in FBS after 24 h, while the corresponding nonamer showed no observable degradation.[174] This is presumed to be as a result of the increased steric congestion not allowing for nuclease enzymes to bind and cleave as readily as they can do for single oligonucleotides. This increase in serum stability extends to spherical nucleic acids, where the more densely packed strands show a greater serum stability than less densely packed analogues.[195]

1.7 Synthesis of Oligonucleotides

Oligonucleotides are synthesised by use of a solid support. This provides several advantages over using a solution phase technique: it becomes possible to use a large excess of reagents ensuring that the reactions are quickly driven to completion, it is possible to wash away impurities and excess reagents after each reaction and the process

becomes amenable to automation.[196] The solid support is usually controlled pore glass (CPG) or polystyrene, functionalised with some linker (typically succinic acid with an initial nucleoside monomer attached at the 3′ position on the sugar). Each synthetic cycle attaches the next nucleoside and eventually the entire strand is deprotected and removed from the solid support by reaction with an appropriate cleavage solution (Fig. 1.27).

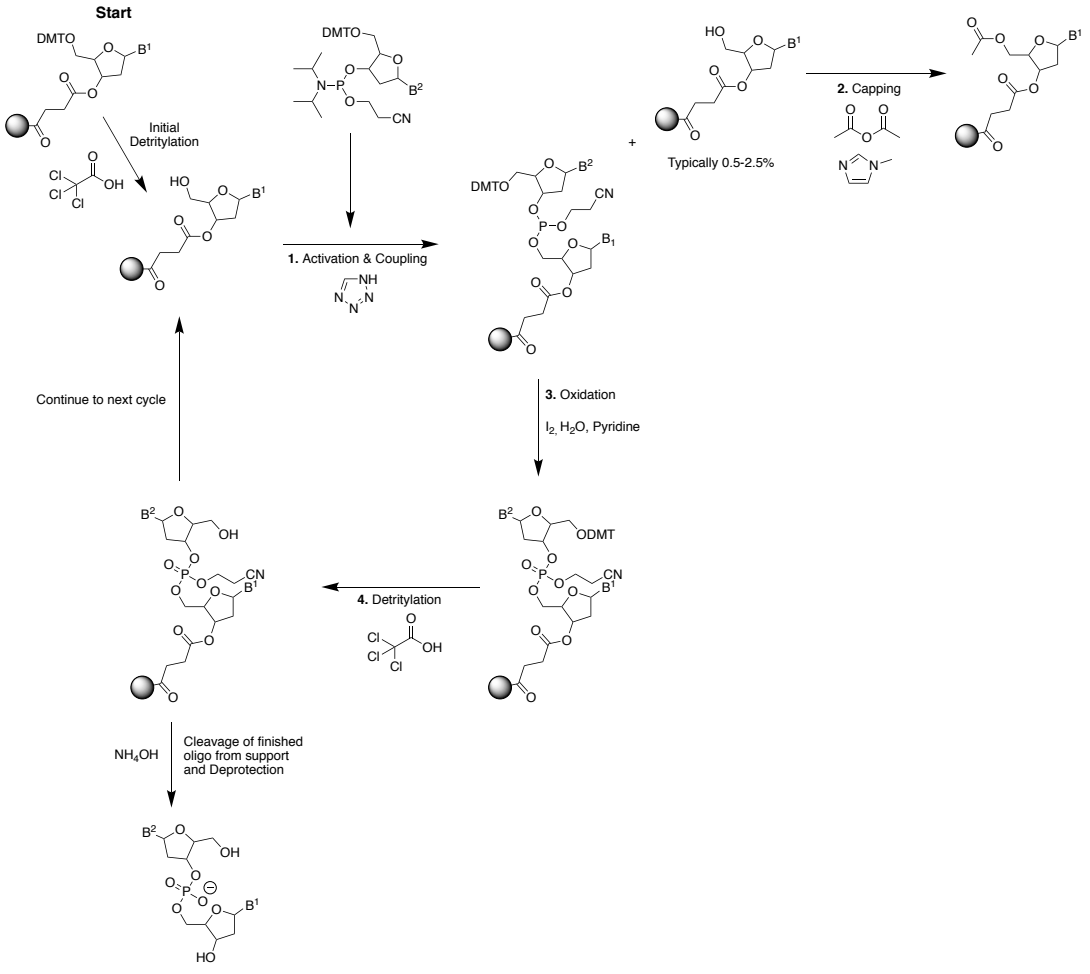


Figure 1.27: Solid phase oligonucleotide synthesis.

In Step 1, TCAA removes the DMT protecting group from the resin-bound oligomer, unmasking the 5′ OH. In step 2, the alcohol is allowed to undergo nucleophilic substitution with the tetrazole-activated incoming phosphoramidite. Capping (step 3) using Ac_2O

and *N*-methylimidazole blocks any unreacted oligo from reacting in the next cycle by acetylating the nucleophilic OH, preventing extension of base-deleted oligonucleotides and allowing for easier removal of such failure sequences from the final product. Oxidation with iodine and water (step 4) stabilises the acid-sensitive phosphite triester by making it into a phosphate triester. Instead of oxidising the phosphite triester, it can be sulfurised to produce the phosphorothioate-modified derivative. This cycle continues until the required oligonucleotide has been synthesised. Typical stepwise yields are often ca. 98% for RNA with ca. 99.5% possible for DNA under optimum conditions. The cumulative yield of a 20 mer oligonucleotide with each step yielding 98.5% is 0.985^{19}= 75%.

1.7.1 Purification and Quantification of Oligonucleotides

1.7.1.1 Deprotection

Once the synthesis is complete, cleavage from the solid support, as well as phosphate and base deprotection is accomplished typically by reaction with concentrated ammonium hydroxide solution or a 1:1 mixture of concentrated ammonium hydroxide and 40% $MeNH_{2(aq)}$ (AMA) giving the product oligomer in solution. Excess base is removed by rotary evaporation and the resulting oligomer purified either by precipitation into 70% $EtOH_{(aq)}$ or by size-exclusion chromatography using Sephadex.

AMA has become a ubiquitous cleavage/deprotection reagent in recent years owing to its enhanced activity relative to conc. ammonia facilitating much a faster reaction. AMA does however induce transamination in N^{Bz} protected C (Fig. 1.28), however, necessitating the use of conc. ammonia for oligonucleotides containing this protecting group.

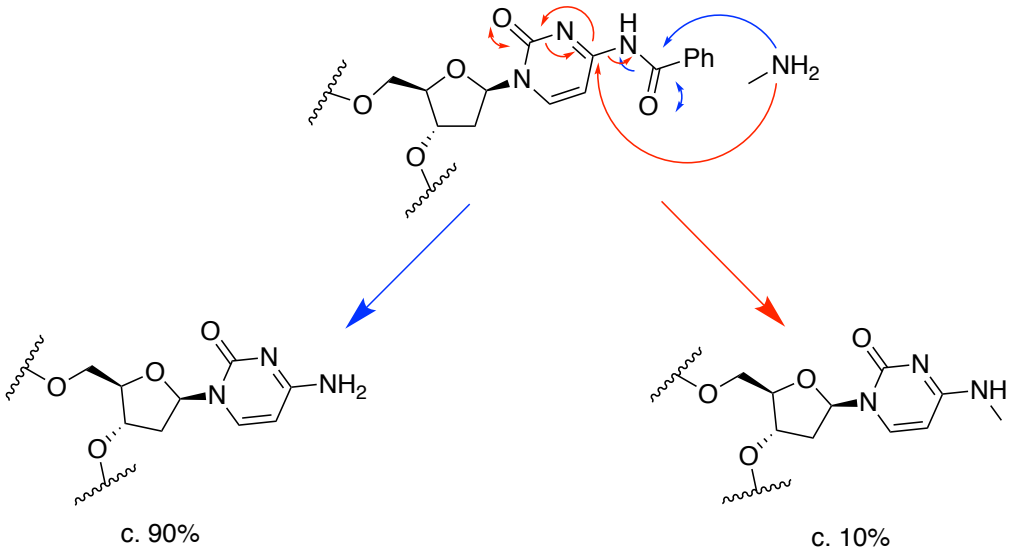


Figure 1.28: AMA induced transamination of N^{Bz} protected C

In order to facilitate the use of AMA with oligonucleotides containing C, the exocyclic amine can be protected with an acetyl group which is more readily removed and hence precludes formation of the transaminated product (Fig. 1.29)

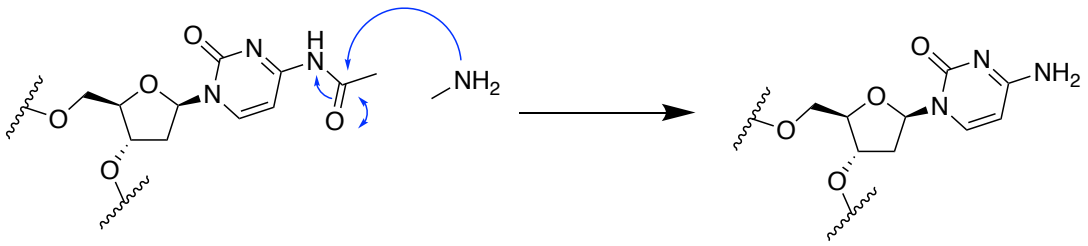


Figure 1.29: Use of AMA with N^{Ac} protected C

K_2CO_3/MeOH is sometimes used as an 'ultramild' deprotection system, intended for use when a modification has been introduced that would degrade with the ammonia/AMA deprotection techniques (e.g. CyDyes).

1.7.1.2 Purification

Oligonucleotides are often not pure immediately after cleavage from the solid support. Despite the synthesis cycle being extremely effective there will invariably be n-1 deletions present in the final product; the capping step is intended to reduce this as much as possible, but is not completely effective in doing so. Reversed-phase HPLC is an effective method used to purify shorter and more hydrophobic oligonucleotides, while PAGE (polyacrylamide gel electrophoroesis) purification can be better suited to longer and more hydrophilic oligonucleotides. Ion-exchange HPLC is an effective method for the purification of oligonucleotides up to 30 bases in length. HPLC methods will typically give an oligo in >85% purity while PAGE will often give up to 90%.[197]

1.7.1.3 Quantitation and QC

Once purified, the product is quantified using UV-vis spectrophotometry, applying the Beer-Lambert law:

$$A = \epsilon c l$$

and solving for c:

$$c = \frac{A}{\epsilon l}$$

Where A is the absorbance measured, l is the path length and ϵ is the molar extinction coefficient (which can be found by entering the target sequence into an online calculator[2]). These calculators work by one of two main methods; summing the absorbance values of each individual base and subtracting 10 % for the effects of hypochromicity, or by summing the absorbance values of every dinucleotide pair, having accounted for their specific, empirical hypochromicity[198] and dividing by two.

[2] Such as the one by IDT: https://www.idtdna.com/calc/analyzer

Following quantification, LC-MS is performed in order to confirm the identity of the oligonucleotide.

Chapter 2

Development of Tools for Studying Biodistribution

2.1 Declaration of Work Done

I synthesised all fluorophores, fluorescent ASOs and siRNAs, though amine-terminal siRNAs were prepared by Dimas Escherriva of the Khvorova lab. I did all of the hands-on mouse work, though live-animal imaging was done in collaboration with Maire Osborn and Andrew Coles of the Khvorova Lab and the UMMS small-animal imaging core. I did all of the presented fluorescence microscopy and staining for immunofluorescence and immunohistochemistry. All of the animal use protocols were written by me and are appended to this thesis. I did all of the rabbit work, immunogen design, preparation and compounding. Rabbit bleeds were assisted by Elizabeth Cusson or Maryellen Blombach Berry of the UMMS Department of Animal Medicine.

2.2 Introduction

Biodistribution is the study of how a molecule of interest distributes in an organism following administration. Biodistribution is a key parameter to understand when developing a potential drug candidate because often the molecule's efficacy is dependent on its effective accumulation at the anatomic region of interest. It is important when assaying biodistribution that whichever technique is employed accurately monitors biodistribution without perturbing it.

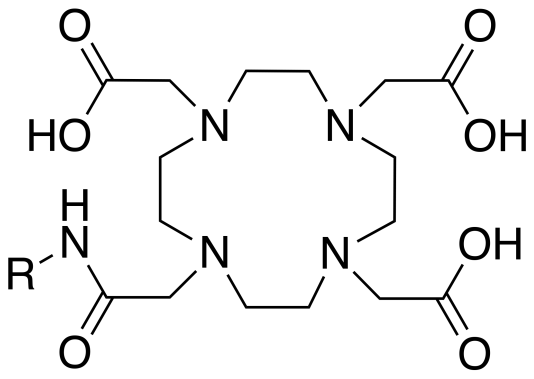


Figure 2.1: Structure of the metal chelator 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) modified as attached to a protein biologic drug.

Small molecule biodistribution is often assayed using a radioisotope-labelled analogue of the drug.[199] Radioisotope biodistribution relies on the incorporation of a radionuclide, commonly ^{11}C, ^{15}O or ^{18}F, into the molecule with biodistribution revealed using SPECT or PET imaging modalities.[199] Radioisotope tagging of biologics is also commonly done, especially in the case of antibody therapeutics.[200] In the instance where a large protein therapeutic is being assayed, the radionuclides are more commonly metal ions such as ^{68}Ga, ^{64}Cu or ^{89}Zr.[201] The reason for this is that large proteins are amenable to conjugation to a metal chelating moiety (such as DOTA, Fig. 2.1) which will chelate a radioactive metal atom in a more synthetically reliable manner than trying to prepare a radioactive protein.[201]

Clearly, working with radioactive materials is a challenge from both a regulatory and a health and safety perspective. As such other modalities for assaying biodistribution have been developed such as fluorescence[202] and MRI[203].

Much as for other drugs, a key question in the development of therapeutic oligonucleotides is how structure and modification patterns affect biodistribution. With the discovery of GalNAc conjugation to therapeutic siRNA and ASO molecules and its profound effect on hepatocyte uptake, there is a need to be able to rapidly screen for cell-specific uptake changes of novel oligonucleotides.[204] In similar fashion to other drugs, there have been a number of technologies utilised to assay for oligonucleotide biodistribution such as radioisotope labelling, fluorophore conjugation and MRI contrast labelling. Analysis of these oligonucleotides can be complicated by the fact that the presence of a fluorophore or other modification allowing for labelling could affect the biodistribution of the oligonucleotide, misrepresenting the distribution obtained when an unconjugated oligonucleotide is used. Indeed, when assaying the biodistribution of therapeutic antibodies, changes in the conjugated fluorophore have been shown to affect the nature of the observed biodistribution.[205] Ionis pharmaceuticals have developed an antibody, discussed in section 2.4, which allows for excellent visualisation of oligonucleotides.

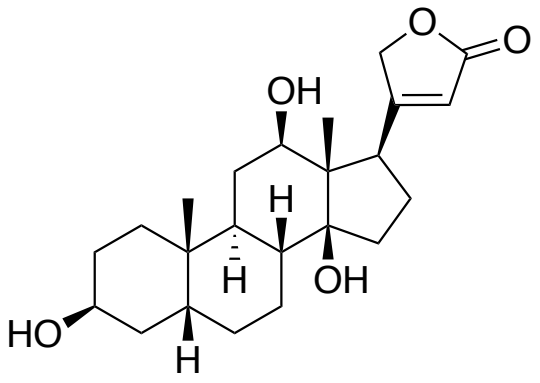


Figure 2.2: Structure of the steroid digoxygenin (DIG).

In addition to assaying qualitative oligonucleotide biodistribution by SPECT/MRI or microscopy, it is essential to be able to quantitate the degree of accumulation of an oligonucleotide in a given tissue of interest. There have been a plethora of assays developed for assaying oligonucleotide accumulation in tissues, so-called 'hybridisation assays' can serve this function.[206] In this approach, a PNA probe complementary to the stand of interest is synthesised and fluorophore-labelled. Tissue homogenate or plasma of treated animals is allowed to

hybridise with the probe, and the solution is then passed through an HPLC and detected by fluorescence.[207]

Another assay that has been described relies on the digoxygenin (DIG) label (Fig. 2.2).[208,209] Tissue lysate or serum of a treated animal is applied to a well with a pre-adsorbed high-affinity nucleic acid probe that is complimentary to part of the sequence of the injected oligonucleotide. A DIG-tagged, high affinity probe is added that binds to the rest of the sequence and is ligated. An anti-DIG antibody is used to detect this binding probe and following binding of an HRP-conjugated secondary antibody and the plate is read in a luminometer (Fig. 2.3).

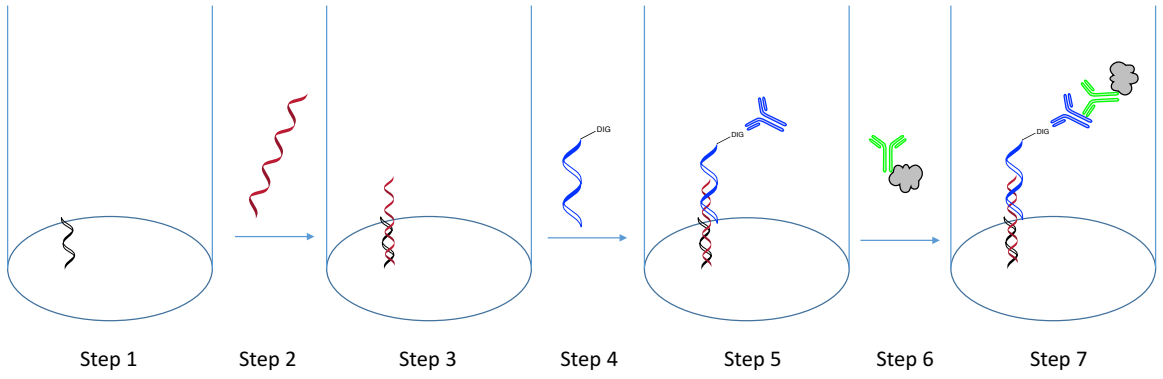


Figure 2.3: Schematic representation of the DIG-labelled plate assay.

These assays generally require instrumentation that is uncommonly available, and all require reagents that are challenging to obtain or rely on a modified oligonucleotide being injected in the first instance. It was desired to design an assay that is high throughput, doesn't require instrumentation beyond the reach of most pharmacology laboratories and that requires relatively easily synthesised or obtained reagents.

2.3 Fluorophores in Oligonucleotide Biodistribution

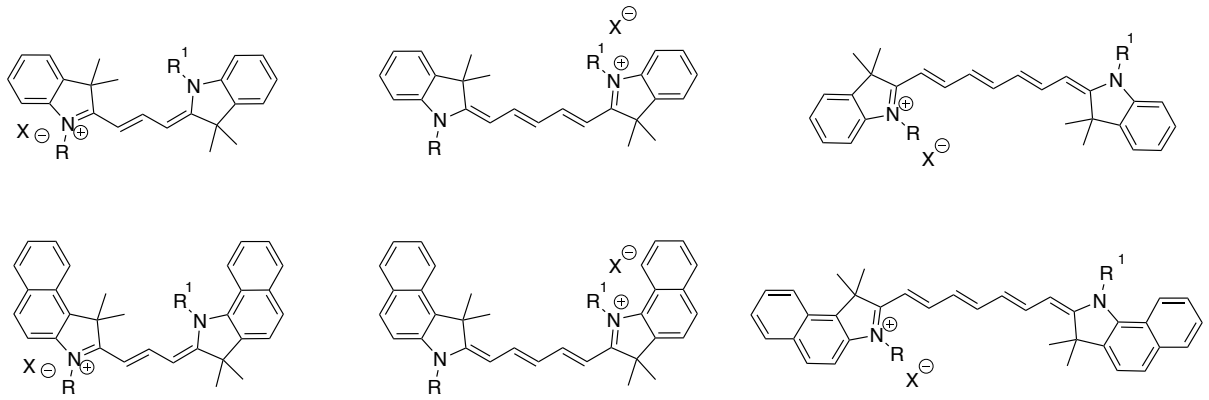


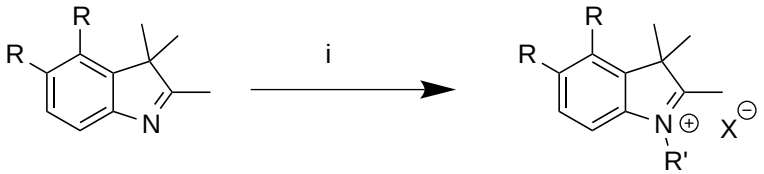
Figure 2.4: Cyanine fluorophores used in fluorescent labelling of oligonucleotides. Top: Cy3, Cy5, Cy7. Bottom: Cy3.5, Cy5.5, Cy7.5

Cyanine dyes (CyDyes, Fig. 2.4) are a class of polymethine compounds that exhibit intense fluorescence. Structurally, they consist of a polymethine chain with either an indolenine or benzo[*e*]indolenine end. CyDyes are incredibly versatile owing to the variety of emission/absorbance characteristics that are accessible by varying the length of the polymethine chain. CyDye naming is based on the number of carbon atoms constituting the central chain of the dye, while X.5 dyes have an additional phenylene ring annulated to each aromatic end (Fig. 2.4). For biological use, cyanine dyes are often asymmetrical. One of the R groups is typically an alkyl group and the other a carboxylic acid-derivitised alkyl group. The carboxylic acid is usually subsequently modified to bear an alkyne, azide or active ester for attachment to a biomolecule of interest. The cyanine dyes and their sulphonated AlexaFluor analogues have been used for years as fluorescent tags in biochemistry. They are extremely bright,[210] relatively photostable,[211] and synthetically accessible.[212,213] Other fluorophores, such as the fluorescein and rhodamine family molecules are also used, though they are less bright and stable than the CyDyes. The family of CyDyes covers a significant range of the visible to near-IR region of the electromagnetic spectrum and as a result one can choose which dye to use based on the intended readout. There are two routes to obtaining

dye-conjugates of oligonucleotides. The first and most technically simple is to purchase the dye as a phosphoramidite and include it as a part of the synthesis cycle. The second is to purchase or synthesise a synthetically amenable fluorophore and then to purchase or synthesise an appropriately modified oligonucleotide. The dye can, for example, be purchased as an N-hydroxysuccinimide (NHS) ester and the oligonucleotide modified to include a terminal primary amine substituent. The advantage of the phosphoramidite method is that it saves time and maximises yield, but is expensive and is limited in the fluorophores that can be used. CyDyes larger than Cy5, for example are sensitive to base and make deprotection a technical challenge. The post-synthetic method allows for a greater diversity of fluorophores, but requires the additional synthetic effort as well as requiring a further purification step to remove unreacted oligonnucleotide and unreacted or hydrolysed fluorophore.

2.3.1 Synthesis of Cyanine Dyes

CyDyes are commercially available with a large variety of click handles and other functionalities, but are extremely expensive. It was felt that as a significant amount of these compounds would be used, it would be more cost effective to synthesise a variety of such materials.[1] A body of literature concerning the preparation of these compounds exists, but perhaps surprisingly includes relatively little detail on the preparation of the target compounds for this work.[214]



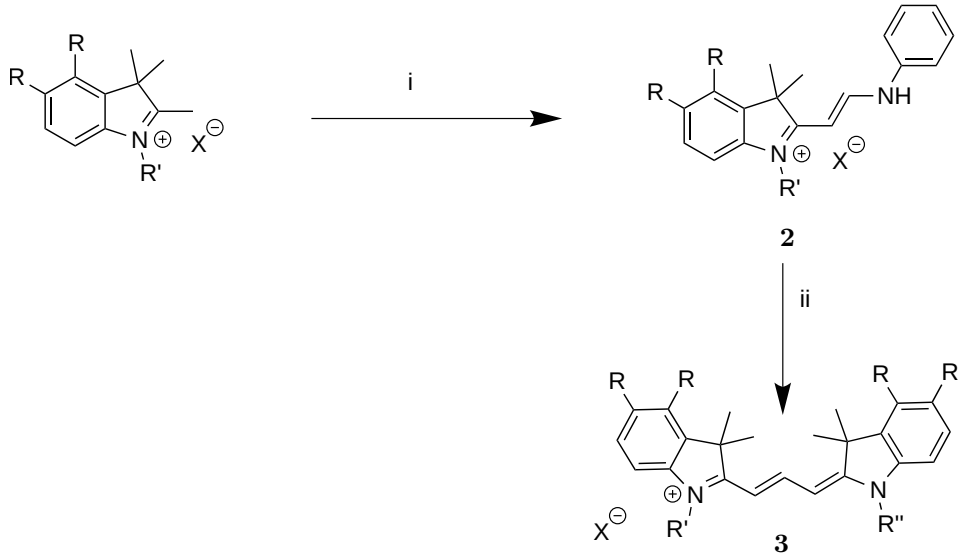
1

Scheme 2.1: Synthesis of quarternary indolinium precursors.
R= H or o-phenylene i) R'-X, Where R'= Me, Et or hexanoic acid and X=Br or I (**80%+**)

[1] £200 consumables and 2 weeks of work (£500) yielded ca. 2 g of various CyDyes (ca. £18,000)

Each of the cyanine dyes requires broadly the same starting materials, quaternised indolenines (for Cy 3/5/7) and quarternised benzo[*e*]indolenines (for X.5 dyes). Preparation of these precursors is by S_N2 reaction of the heterocycle with an appropriate alkyl halide to give the corresponding salt **1**.[214]

2.3.2 Cy3

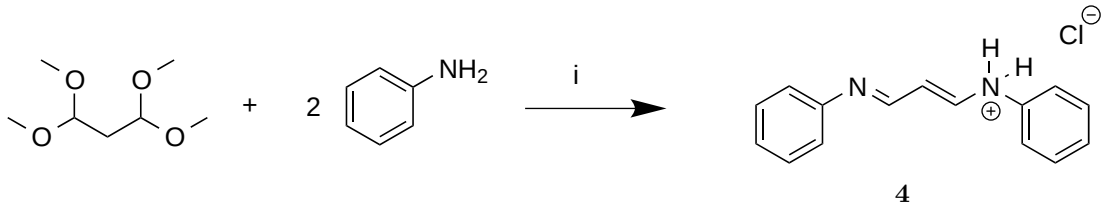


Scheme 2.2: Synthesis of Cy3 *via* hemicyanine precursor.
R= H or o-phenylene i) Diphenylformamidine, $(EtO)_3CH$, EtOH, reflux 6h ii) **1**, Ac_2O, pyridine, 50°C, 18 hours
Where R=H or o-phenylene, R'/R"= Me, Et or hexanoic acid and X=Br or I and where R' $\neq R''$**>80%**

Synthesis of the Cy3/3.5 dyes requires preformation of a hemicyanine dye by reaction of one of the dye termini with diphenylformamidine and triethylorthoformate to give **2**. Reaction with the desired **1** under activation with Ac_2O in pyridine gives the target **3** in a typically poor yield (15% in the instance of Cy3.5) after purification.[214]

2.3.3 Cy5

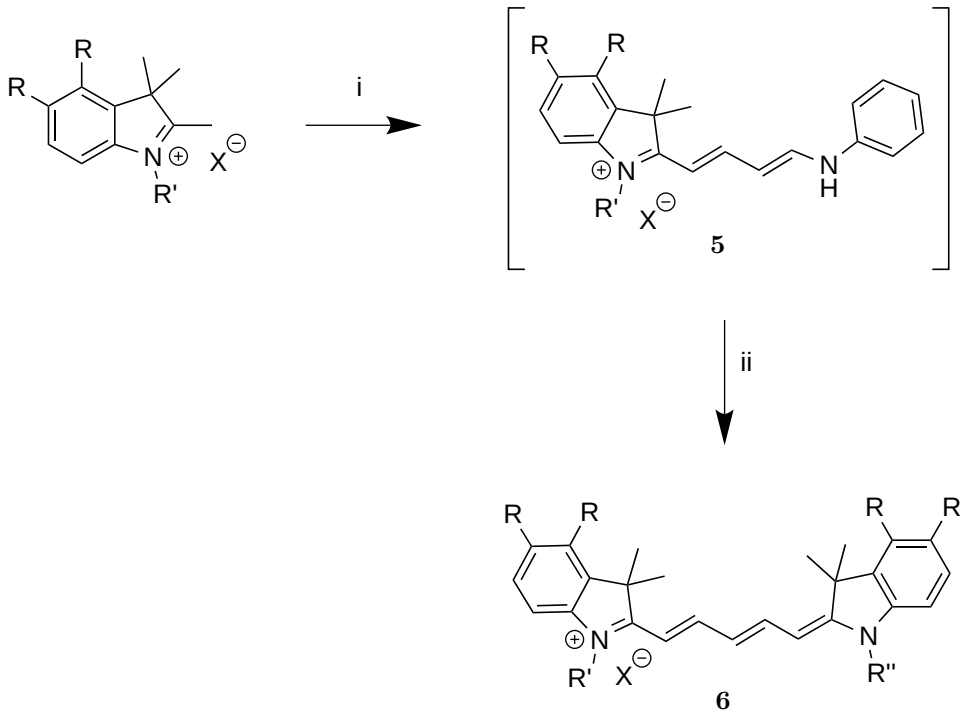
The Cy5 dyes require the preparation of a suitable linker compound; whereas for Cy3 diphenylformamidine is commercially available, the trimethine analogue, malondialdehyde dianil is not.



4

Scheme 2.3: Synthesis of malondialdehyde dianil HCl
i) $HCl_{(aq)}$ **60%**

Preparation of **4** is straightforward by reaction of malondialdehyde tetramethyl acetal with aniline under acid conditions, giving the target compound as a pure solid after filtration albeit in moderate yield (60%).[214]



Scheme 2.4: Synthesis of the Cy5 family.
i) **4**, Ac_2O, reflux ii) **1**, pyridine, rt, overnight
Where R=H or o-phenylene, R'/R"= Me, Et or hexanoic acid and X=Br or I and where R' $\neq R''$

In contrast to the Cy3 family, Cy5 dyes are usually prepared in a one pot reaction without isolation of the associated hemicyanine compound **5**. Preparation of the final dye **6** is facile from **5**, requiring addition of the desired indolenine in pyridine.[214] This is typically accompanied by a vivid colour change indicating reaction procession.

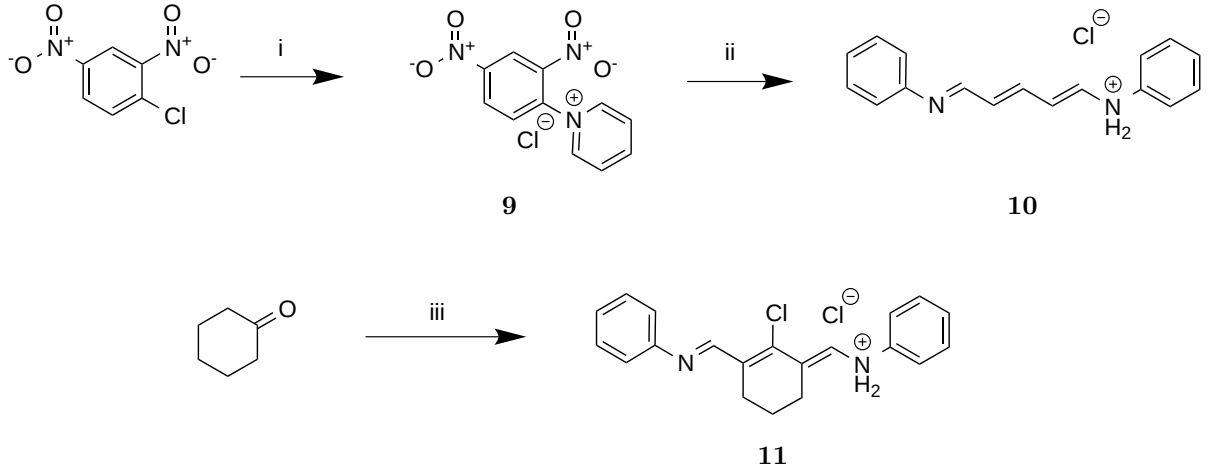
2.3.4 Cy7



7 **8**

Scheme 2.5: Examples of Cy7 dyes, showing the different linker structures commonly found.

The Cy7 family differ from both the Cy3 and Cy5 families in that there are two commonly available varieties of the dyes, one with an unrigidified linker (**7**) and one in which the linker is partially rigidified by a cyclohexene ring (**8**).



9 **10**

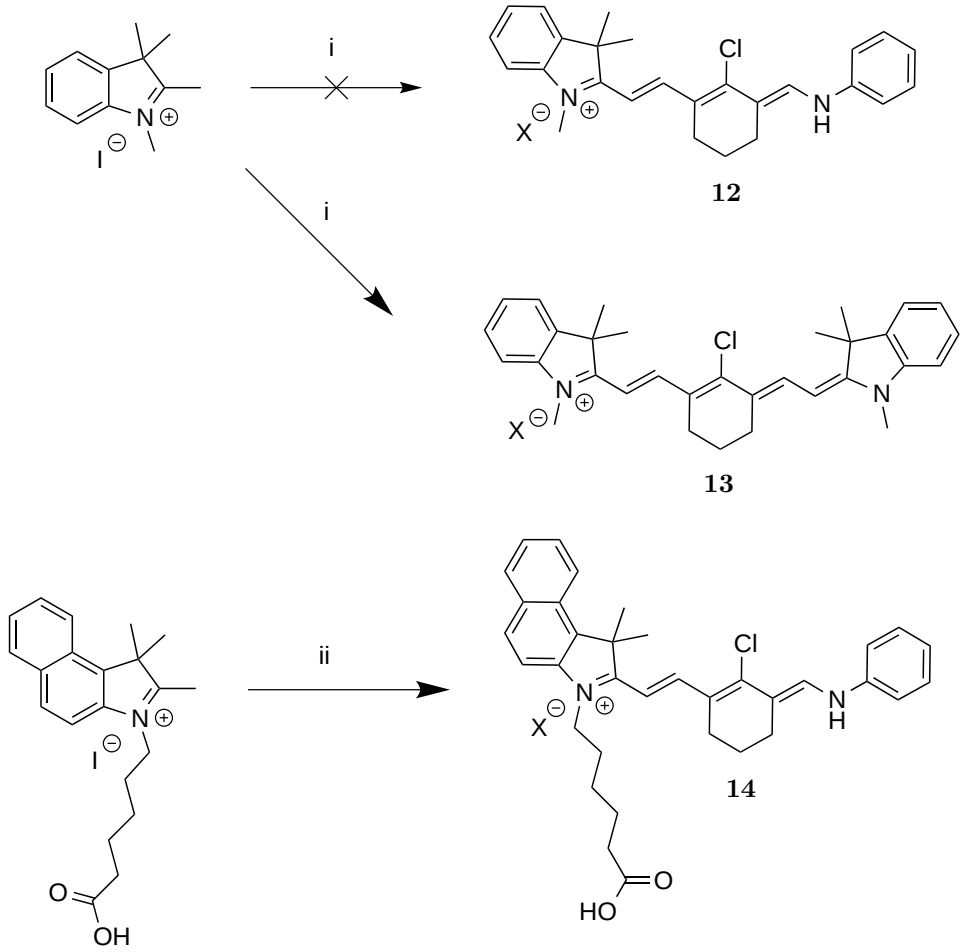
11

Scheme 2.6: Synthesis of Cy7 linkers
i) Pyridine, acetone, reflux 18 h **95%**, ii) Aniline, EtOH, H_2O, rt **38%** iii) $POCl_3$, DMF reflux, then aniline/EtOH **65**%

Syntheses of the associated linkers are reported in the literature and were reproduced to facilitate access to these compounds.[215,216]

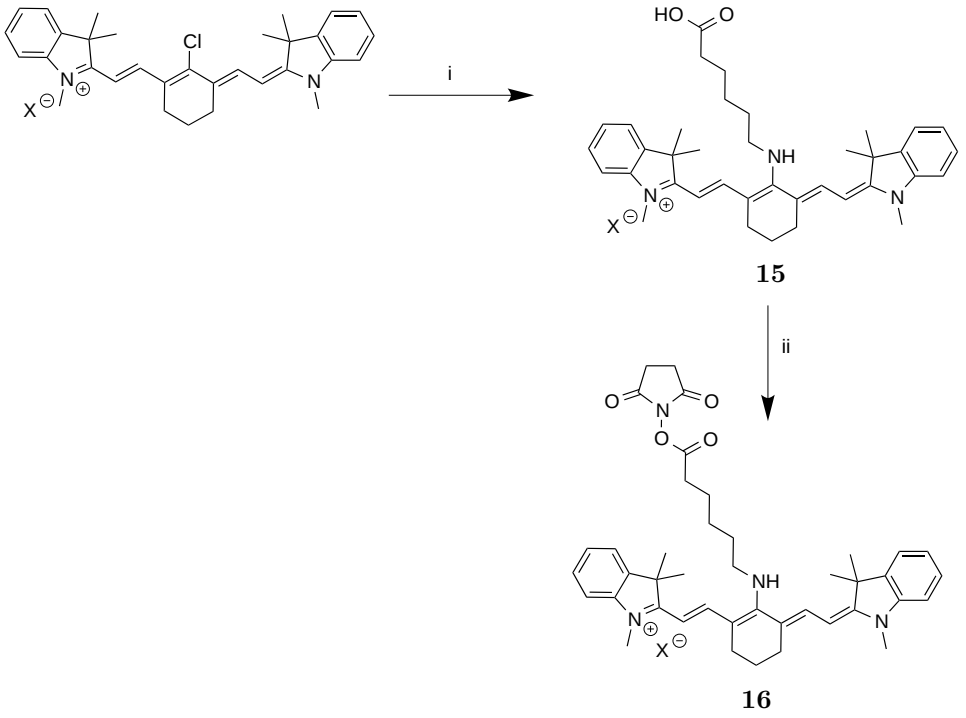
9 reacts with aniline to give **10** in a poor yield. The rigidified analogue **11** is prepared by reaction of cyclohexanone with the Vilsmeier reagent, followed by aminolysis of the resultant bis-iminium compound with aniline. The central Cl presumably results from a substitution reaction of the activated cyclohexenone with chloride.

Both the rigidified and non-rigidified Cy7 syntheses were attempted; they follow Cy3 in that the hemicyanine intermediate is isolated and purified before reaction to give the final dye. Synthesis of the rigidified dye posed some unexpected challenges.



Scheme 2.7: Proposed synthesis of Cy7 (rigidified)
i) **11**, **1**, NaOAc, EtOH reflux 6h ii) **11**, NaOAc, EtOH **21%**

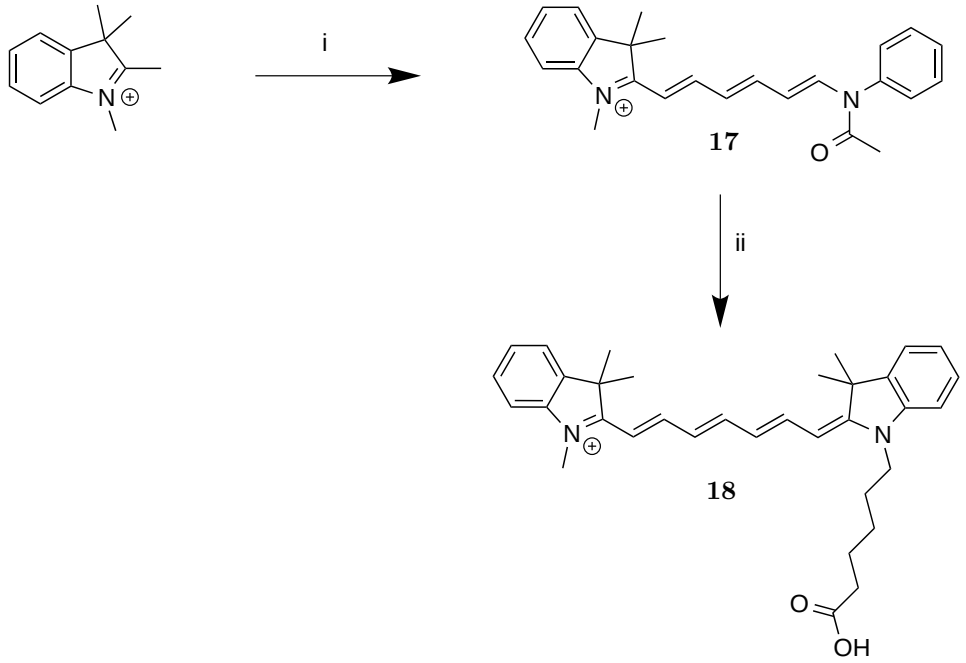
Instead of giving the intended hemicyanine **12**, the reaction gave **13**. Peculiarly, when a similar synthesis for Cy7.5 was undertaken as per the literature procedure by Shimizu *et al.* the hemicyanine **14** was isolated in a moderate yield.[216] Subsequent reaction of **14** with the N-Me indolenine gave a mixture of the symmetrical N-Me dye, the intended product and the symmetrical N-hexanoic acid product that were inseparable by both HPLC and silica gel chromatography. The reason for this is not known, or reported in the literature, though speculatively it must be the case that the symmetrical product is thermodynamically favoured, as the reaction was run at RT. Shimizu *et al.* subsequently reported that the asymmetric compound with N-CH_2CH_2OH was prepared, implying that the cause of the observed symmetrisation may be the N-Me.[216] Rivera *et al.* acknowledge the formation of some symmetrical dyes, but their proposed method of purification was ineffective when tested.[217]



Scheme 2.8: Synthesis of Cy7 NHS-ester.
i) 6-aminohexanoic acid, TEA, DMF 85°C, 3 hours **33%** ii) NHS, HATU, DIPEA, DMF **99%**

In the instance of Cy7s, symmetrical dyes with substitution at the central carbon of the heptamethine chain is common.[218–220] This may be as a result of the observed tendency

for these compounds to symmetrise or as a result of the synthetic route to the rigidified linker leaving it amenable to nucleophilic attack at the centrally chlorinated carbon. Given that the unexpected compound **13** still had desirable fluorescent properties, a centrally substituted derivative **15** was prepared in good yield, and then its NHS activated ester **16** using the procedure of Lu *et al.*[221]



Scheme 2.9: Synthesis of Cy7 (unrigidified)
10, Ac_2O, pyridine, rt, 45 min, **47%** ii)**1**, pyridine, 50°C, 1 hour **54%**

The unrigidified Cy7 was synthesised in analogy with the method developed by Berezin *et al.*; the N-acylated, activated hemicyanine **17** was prepared in 47% yield and subsequently reacted with **1** to give **18** in 54% yield.

Conjugation of NHS-modified fluorophores to primary amine-labelled oligonucleotides was effected using a published protocol (see 2.5),[222] in which a solution of oligonucleotide in bicarbonate buffer is reacted with a solution of NHS-modified fluorophore in DMSO and the product purified by HPLC.

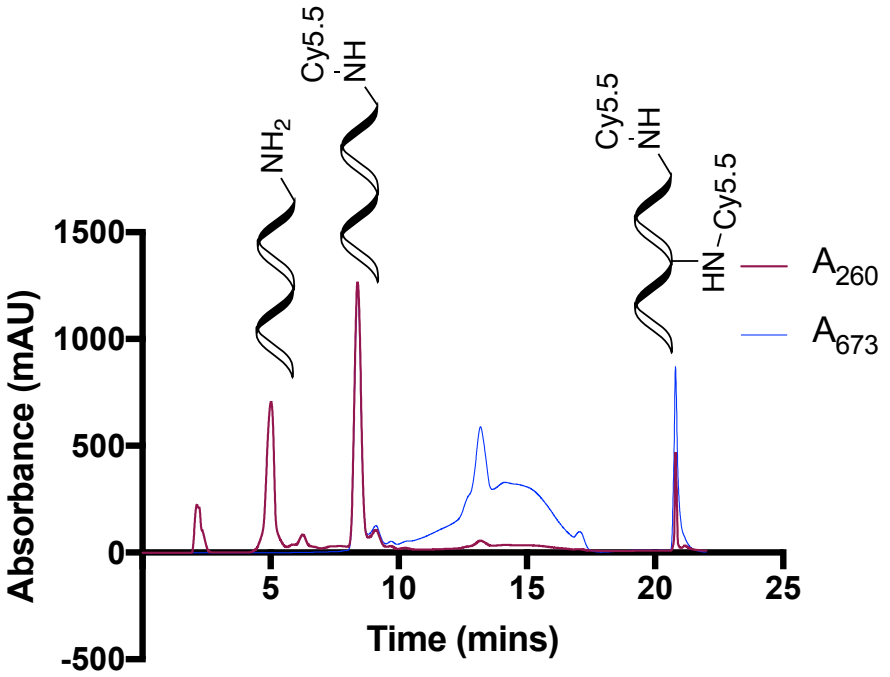


Figure 2.5: Reversed-phase preparative HPLC profile of the crude reaction mixture between an amino-terminated hsiRNA molecule and Cy5.5NHS. The fluorophore tagged oligonucleotide elutes at ca. 8 min, while the unconjugated oligonucleotide comes at 5 minutes. Also visible is a double-Cy5.5 tagged species at ca. 21 min.

2.3.5 Dye Conjugation Increases Hydrophobicity

Figure 2.5 demonstrates one property of significance regarding the conjugated oligonucleotides (eluting at ca. 8 minutes), that is that they are significantly more hydrophobic than the parent, unconjugated oligonucleotide (eluting at ca. 5 minutes). Hydrophobic conjugates have been used in a variety of iterations to alter the cell uptake properties and biodistribution of oligonucleotides.[152,223–225] This effect of hydrophobic conjugation presents a confounding variable when considering the data produced by tagged oligonucleotides.

2.3.6 *In Vivo* Imaging

Light penetrates tissue most effectively between 650-950 nm,[226] thus the more red-shifted cyanine fluorophores can be used in live-animal imaging studies to track the distribution of attached biomolecules without having to euthanise animals for biodistribution timepoints. This reduces the number of animals used for a study which is both ethically and financially beneficial. As such, a series of Cy-conjugated hsiRNAs were prepared in collaboration with Anastasia Khvorova's lab, and sought to examine the effect on measured intensity and signal to noise of the different Cyanine dye conjugates prepared (Fig. 2.6). Full experimental details are available in section 2.5.6.

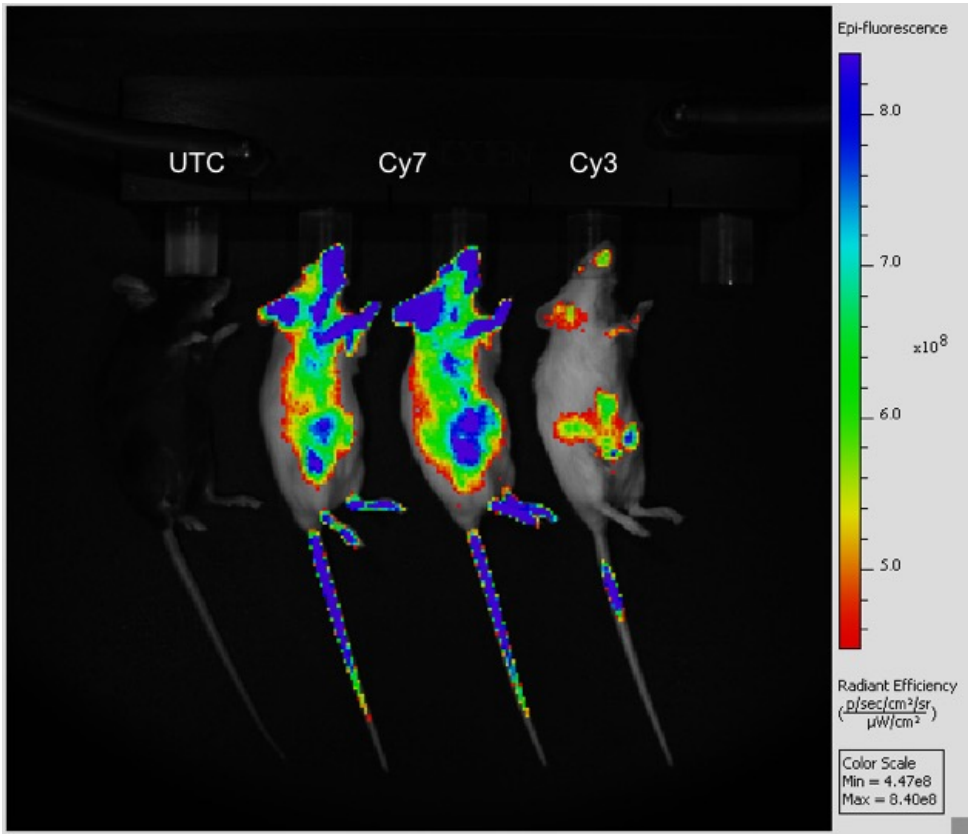


Figure 2.6: L to R, untreated mouse, two mice treated with Cy7-conjugated oligonucleotide, mouse treated with Cy3-conjugated oligonucleotide.
Fluorescence image of animals treated with the same oligonucleotide conjugated to different fluorophores. Each animal was injected intravenously with 20 mg/kg oligonucleotide (in saline) at 48 and 24 hours before imaging.

The Cy7 oligonucleotide is more clearly visible than the Cy3 oligonucleotide owing fluorescence of the dye at the stimulant wavelength (750 nm). Interestingly, the Cy3 conjugated oligonucleotide had been originally included as a negative control and was anticipated not to generate any signal, however it is the case that Cy3 exhibits some absorption at the interrogative wavelength and this is sufficient to show signal at the most intensely bioaccumulated regions (liver and kidney). Signal is also observed around the regions of the mouse that are less obscured by fur (tail, ears and mucus membranes), but this is presumably as a result of haem fluorescence.

While certainly an interesting proof of concept experiment, the data provided are clearly crude in their nature and one would struggle to obtain meaningful information on a comparative basis. Curious to see whether the resolution could be improved by removing skin and fur, the animal was euthanised and dissected to remove the tissues of interest (Fig. 2.7).

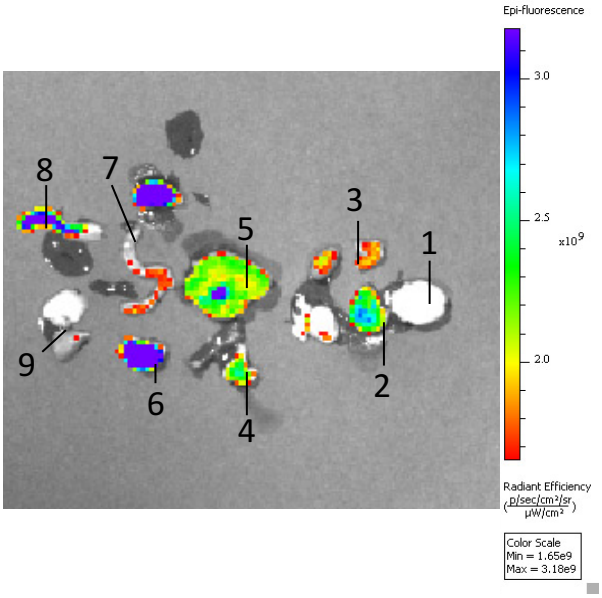


Figure 2.7: Tissues excised from a mouse treated with Cy7 conjugated oligonucleotide 1. Brain, 2. Salivary gland, 3. Cervical lymph nodes, 4. Spleen and pancreas, 5. Liver, 6. Kidney, 7. Intestine, 8. Fallopian tube, 9. Adipose fat and skeletal muscle

The resulting image is marginally more clear, showing the distinction in uptake between

liver, kidney, intestines, fallopian tubes and other viscera, however it remains a very crude assessment of tissue accumulation. It also negates one of the main values of live-animal imaging in that it requires the euthanasia of the animal. The other clear issue with this imaging modality is that the resolution is remarkably limited. Being able to bluntly articulate tissues in which bioaccumulation occurs is of limited use; far more relevant is examination of the cell-type specificity obtained. Techniques with greater resolution are needed.

2.3.7 Fluorescent Microscopy

Fluorescent microscopy is a useful technique for interrogating the distribution of fluorophore-tagged oligonucleotides both *in vitro* and *in vivo*. Once injected animals have reached their intended timepoint, they are euthanised, perfused transcardially with PBS and then NBF until fixed, tissues of interest are excised, post-fixed in NBF and then paraffin embedded and sectioned before rehydration, DAPI nuclear counterstaining and mounting.

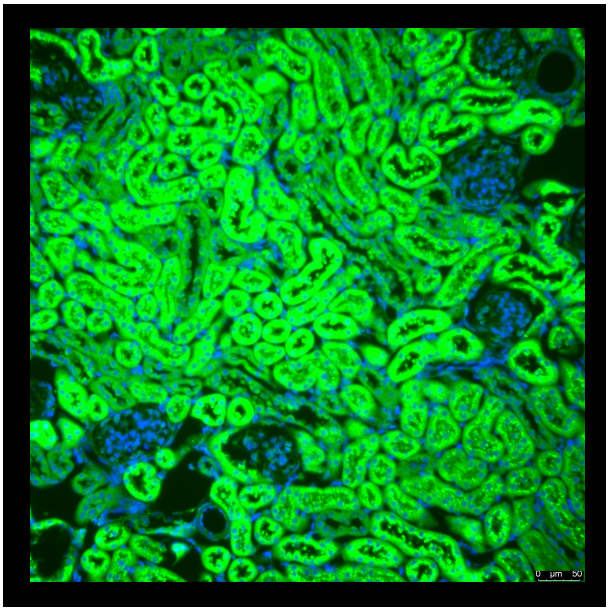


Figure 2.8: Cortical kidney biodistribution of a Cy5 tagged ASO injected intravenously Blue: DAPI, Green: Cy5 ASO, each animal was injected intravenously with 10 mg/kg oligonucleotide (in saline) and euthanised at 24 hours post-injection.

2.4 Development of a Polyclonal Antibody Directed Against Phosphorothioate

Dissatisfied with the use of fluorophore tagged oligonucleotides, and indeed the use of any imaging modality that requires chemically modifying the oligonucleotide from its intended final form, a way to image and quantify the distribution of native oligonucleotides was sought. It was rapidly realised that there were only two practical options. The first, to radioactively label the oligonucleotide (such as with ^{3}H or ^{32}P), which was quickly discounted owing to the administrative, safety, and financial challenges associated with the use of radioisotopes.[227] Moreover, radioactive oligonucleotides can be used to track biodistribution in the living animal but are not suitable for microscopy. Thus, while they provide a detailed picture of the gross biodistribution in the animal, they give no indication of cell-type specificity. The second option was to generate an antibody that would recognise some aspect of the injected oligonucleotide; this enables microscopic evaluation following suitable immunohistological staining either by fluorescence or light as well as being a versatile reagent for the development of other assays. While antibodies against the different nucleobases have been reported, we wanted to avoid these specifically because of their interaction with normal DNA representing a potentially significant source of aberrant staining in the nuclei of cells.[228,229] As such it was desired to raise antibodies against the phosphorothioate backbone modification in the hopes that it would be able to detect the vast majority of oligonucleotides of interest. Such an antibody has been described in the literature by Ionis pharmaceuticals, but is not made available to investigators that are not working as direct collaborators.[124,230–232] The methods used to generate an antibody with the proposed specificity are not disclosed in the literature.

2.4.1 Antibody Basics

When an immune response occurs and B cells start producing antibodies, each cell will present a single version of the antibody on its cell surface. These go through a self-check process to ensure that the generated antibody does not bind to 'self' (preventing autoimmune disease).[233] B cells that pass this initial screen divide and mature into secretory plasma cells once they bind the target antigen. Those that bind will be prompted to proliferate, undergo a class switch (from IgM to IgG) which is secreted into the blood stream, with higher affinity antibodies eventually being selected for (termed affinity maturation). Many different antibodies that each bind different regions of the target antigen will be produced by this process.[234,235] Blood drawn from an animal that has been repeatedly immunised yields antisera containing this mixture of different antibodies.

2.4.2 Monoclonal or Polyclonal?

One of the first questions when it came to approaching making an antibody was whether a monoclonal or polyclonal antibody should be sought. Each has distinct benefits and drawbacks.

Monoclonal antibodies (mAbs) are typlically sold by antibody vendors, contributing to their prevalence in research. mAbs have the benefit of being very specific in their epitope recognition because they comprise one single clone, which can decrease off-target interactions. Their specificity can also be a disadvantage however, if the specific epitope is rendered inaccessible by fixation, or damaged in the process of antigen retrieval the mAb will fail to recognise its target and no labelling will be seen. mAbs are typically purified from cell-culture supernatant, hence all impurities can be excluded by choosing reagents appropriately. While mAbs are appropriate on an industrial scale, lab-scale production of mAbs is a technical challenge because it requires the fusion of harvested B cells from spleens of immunised animals with myeloma cells,

facilitating the perpetual growth of antibody expressing cells.[236] Each individual fused hybridoma needs to be expanded and titrated against the antigen to determine which clones are the most potent (see section 2.5.8 for details on antibody titration). Once the ideal clones are found, they can be expanded to generate thousands of liters of antibody containing supernatant, however the antibody concentration is typically low ($20\,\mu g\,mL^{-1}$) and clearly very large volumes of supernatant are cumbersome to work with.[237] A smaller scale option for generation of mAbs is to inject hybridoma cells intraperitoneally into mice, to stimulate the production of ascites fluid, which is extremely antibody rich.[238] This is considered to be a painful procedure, however and the animals involved are placed into an IACUC 'use category' that requires special approval given the degree of distress.

Polyclonal antibodies (pAbs) are less common in research because by their nature they are an undefined, heterogeneous mixture of antibodies targeting different epitopes of the same immunogen. This promiscuity is both an advantage, in that contrary to the mAb situation an imperfect epitope presents less of an issue as there will likely be an antibody that recognises the available fragments, and a disadvantage in that there is a greater risk of off-target binding given the diversity of F(ab) arrangements. pAbs are generally purified from the serum of immunised laboratory animals, which is easier for a research lab than working with liters of cell culture medium. Antiserum (the serum of an immunised animal) can be quite rich in antibodies allowing for large amounts of antibody to be collected with each bleed.

Because of the additional work required to make a mAb, and the fact that a nontrivial quantity of antibody was required to optimise future assays with, production of a pAb was chosen.

2.4.3 Choice of Host Species

Having made the choice to make a pAb, the next decision was which animal to use as the host species. The two most logical animals are the mouse and the rabbit because IgG from these animals is well characterised and there are well categorised secondary antibodies available against these host species. Aware that a significant quantity of the antiserum was required, and that this is obtained by serial bleeding of the immunised animal, rabbits were chosen because of their greater circulating blood volume (safely able to draw up to 20 mL in a rabbit, vs 200 µL in a mouse) and relative ease of manipulation. The rabbit also has the advantage of being often used for raising antibodies and as such protocols are established for boost/bleed schedules.

2.4.4 Immunogen Selection and Adjuvant Choice

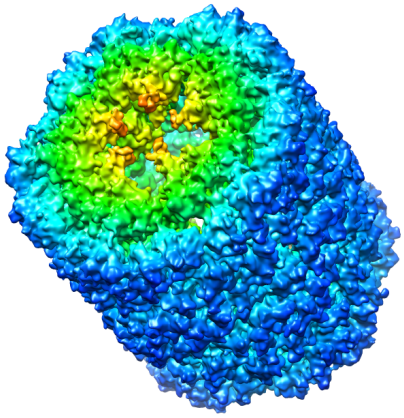


Figure 2.9: Structure of keyhole limpet hemocyanin.
Used under creative commons license.[239]

As our host organism and clonality had been decided, an appropriate immunogen was required. This represented a non-trivial challenge owing to the relative lack of immunogenicity of nucleic acids, certainly compared to the proteins more commonly used for antibody generation.[234] One of the tools available for the generation of antibodies against relatively non-immunogenic haptens, is covalent conjugation of the small molecule to an immunogenic protein. Immunogenic proteins typically espouse a number of properties – they are large, complex and phylogenetically different to the host.[235] There are several commercial protein kits available for conjugation of small molecule

molecules. The most commonly used carrier proteins are ovalbumin, bovine serum albumin, blue carrier (Concholepas concholepas hemocyanin) and keyhole limpet hemocyanin (KLH, Fig. 2.9).[240] KLH was chosen as our immunogenic protein for several reasons: KLH is readily available and reported to elicit a very strong immune response. More importantly, KLH is phylogenetically very different to both the cells and the tissues that the eventual antiserum would be used to stain. This is of particular importance because following immunisation with KLH conjugated ASOs, antibodies are raised against both the ASO and the carrier protein. If the carrier protein is very phylogenetically disparate from the target tissue the chance of anti-carrier antibodies being cross-reactive with the target is diminished.

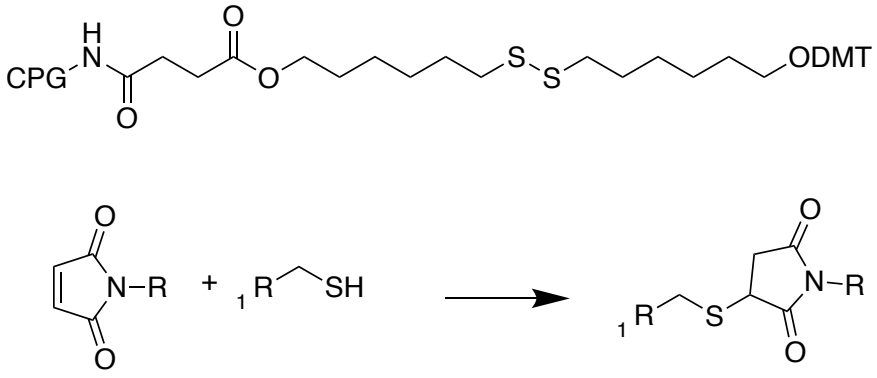


Figure 2.10: Top: Disulphide CPG used to synthesise oligonucleotides
Bottom: Thiol Maleimide reaction

KLH is available functionalised with maleimide. In the preparation of the immunogen, it was decided an immunising antisense oligonucleotide would be synthesised as a disulphide conjugate (Fig. 2.10) and reduced just prior to conjugation to the maleimide tagged KLH. The thiol-maleimide reaction (Fig. 2.10) has been extensively documented as a fast and reliable process for attaching two biomolecules.[241,242] A sequence and modification pattern, if any, also had to be chosen to use for the hapten oligonucleotide.

A non-targeting sequence **(ASO 82)** that we had used to excellent non-effect in gene-silencing experiments previously was chosen in order to ensure no abberent silencing in the immunsed animals.

Oligonucleotide Name	**Sequence (5′ – 3′)**
ASO 82- Disulphide	+A*+A*+C*A*^{Me}C*G*T*^{Me}C*T*A*T*A*+C*+G*+C-C_6SSC_6OH

Table 2.1: Sequence of the Immunogen ASO.
'+' indicates and LNA inclusion, '*' indicates a phosphorothioate inclusion.

Finally, the adjuvant had to be chosen. Adjuvants are necessary in ensuring a robust immune response to an injected immunogen. They come in several forms but in the laboratory setting are most often oils that can be emulsified with the immunogen of choice. The mechanism of action of adjuvants is not clearly delineated, though it is presumed that they act via a 'depot' effect, ensuring the slow and consistent release of immunogen to the immune system, prolonging the response.[243] Very commonly in the laboratory setting, researchers conduct their first immunisation using Freund's Complete Adjuvant (FCA) – a paraffin oil containing heat-killed *Mycobacterium tuberculosis*. FCA is known for producing an extremely robust immune response, but its use is associated with unpleasant side effects including granulomas at the site of injection, hepatic and renal granuloma formation and necrotising dermatitis, among others.[244] The propensity for FCA to cause profoundly unpleasant lesions in animals caused us to seek out less toxic adjuvants for use in this project. Fortunately, the subject of raising antibodies in rabbits has been addressed in the prior art with attention paid towards the adjuvant.[245] On balance of the available options, Montanide ISA 70 VG, a proprietary blend of oils produced by Seppic, was chosen.

2.4.5 Vaccination and Measuring Antibody Titer

The final step was to establish a logical immunisation and bleed schedule. ThermoFisher offer a custom antibody service and have published their protocol. Following discussion

with the UMMS institutional veterinary surgeons the following recommendations were used.[246]

Day 0: Pre-Immune bleed

Day 1: Immunisations

Day 14: 1st booster and small bleed (3.5 mL)

Day 28: 2nd booster and small bleed (3.5 mL)

Day 35: Production Bleed (10 mL - 10% cbv)

Day 42: 3rd booster

Day 56: Production Bleed (10 mL - 10% cbv)

Day 60: decide to continue or terminate, if to continue- boost every 1-3 months and bleed 10 days after the booster (10 mL - 10% cbv) and a maximum of every 2 weeks thereafter depending on the lab's need for antibody.

The rabbits were each immunised subcutaneously and had blood drawn in accordance with the protocol (detailed procedures in section 2.5). It was clear that there was a need to assay the serum collected to see whether antibodies were indeed being produced to our hapten-conjugated carrier protein. This is typically assayed using an enzyme-linked immunosorbent assay (ELISA, Fig. 2.11) or a derivative thereof.

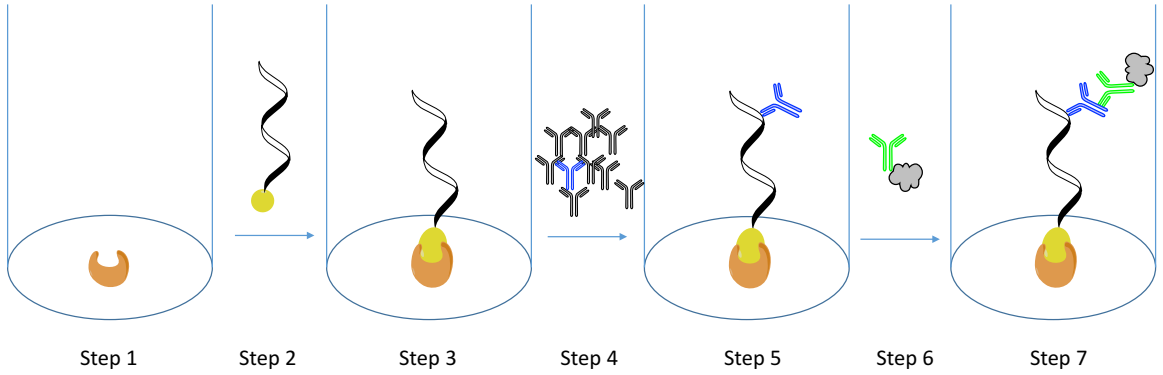


Figure 2.11: Schematic of the ELISA design Used to titrate antibody production.

In the first step a blank ELISA plate is coated with neutravidin, a modified, more soluble form of the protein avidin that exhibits very strong binding to the small

molecule biotin.[247] This is followed by incubation in a solution containing a high percentage of goat serum to block nonspecific binding of the detection antibody in step 7. In the second step, a biotinylated form of the oligonucleotide used to immunise the animals is added to the wells of the plate and in step 3, allowed to bind to the adsorbed avidin. Step 4 sees the addition of a series of dilutions of the collected serum to the plate and step 5 allows for the antibodies present to bind their target. In step 6, an anti-rabbit horseradish peroxidase (HRP) conjugated antibody is added to bind the rabbit antibodies that are binding their target. In the final step, a suitable substrate is added to the plate and luminescence read using a luminometer.

Typically, the different dilutions of antiserum added in step 4 are twofold, starting at approximately a 1:250 dilution. Halving the concentration in each sequential well for 10 further wells across a standard 96 well plate sees a dilution range from 1:250 to 1:256,000. Plotting the luminescence at these values yields a dose response curve, the EC50 of which is a useful measure of the amount of antibody present at a given bleed.

2.4.5.1 Titration Results

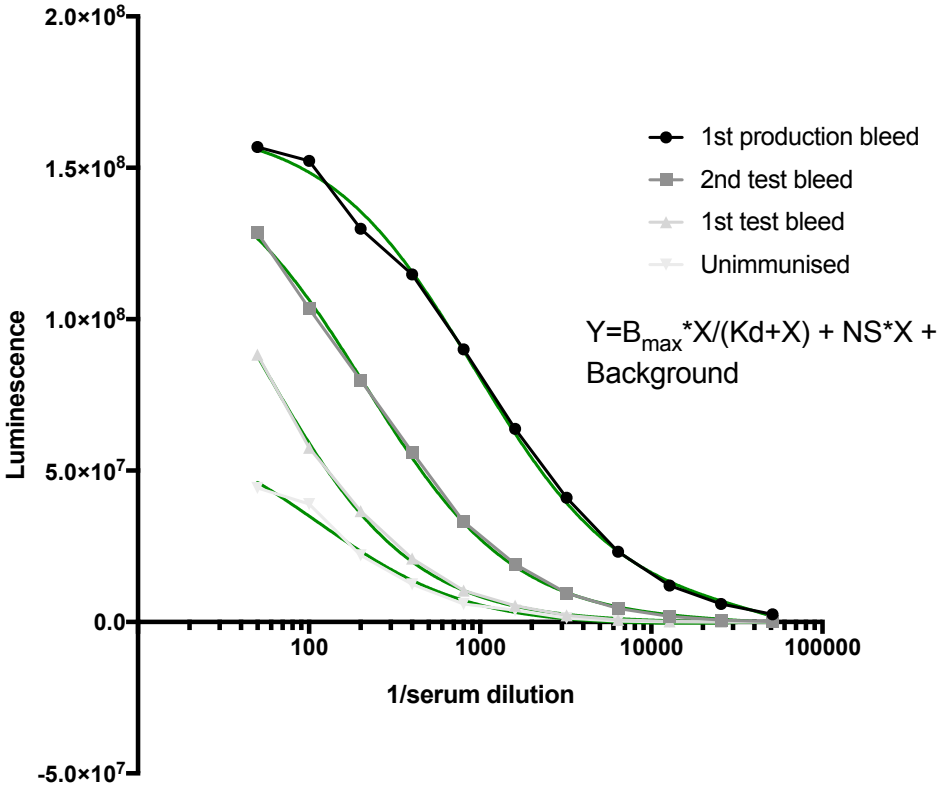


Figure 2.12: Early titration results from one experimental rabbit. Best fit lines shown in green.

Parameter	Unimmunised	1st Test Bleed	2nd Test Bleed	Production Bleed
B_{max}	-68392040 ±5884606	-168985138 ±6675619	-156413442 ±2734656	-160480024 ±3433469
Kd	113.7 ±26.16	54.33 ±4.119	212.1 ±13.1	921.9 ±83.87
NS	30.74 ±47.57	0.2676 ±21.07	-17.24 ±41.19	-92.68 ±97.19
Background	67003865 ±6355353	168688632 ±6918930	156485910 ±3029365	164221500 ±2561496
R^2	0.9905	0.9994	0.9992	0.9984

Table 2.2: Values for the data presented generated by ELISA Figure 2.12

Figure 2.12 demonstrates the intended effect; with time the drawn blood shows an increasing luminescence corresponding to more binding at lower dilutions.

2.4.6 Specificity of Antisera

It was next tested whether the antibody was cross-reactive to non-phosphorothioate-modified DNA. As such, oligonucleotides **ASO 387- no PS**, **ASO 132- 1 PS** and **ASO 139- fully PS** (Table 2.3) were prepared and subjected to the same titration process as described in 2.4.5 in order to determine the degree of avidity between the antiserum and the differently phosphorothioated oligonucleotides.

Oligonucleotide Name	**Sequence (5′ – 3′)**
ASO 387- no PS	AACACGTCTATACGC-Biotin
ASO 132- 1 PS	A*ACACGTCTATACGC-Biotin
ASO 139- fully PS	A*A*C*A*C*G*T*C*T*A*T*A*C*G*C-Biotin

Table 2.3: Sequence of ASOs containing various phosphorothioate inclusions. '*' indicates a phosphorothioate inclusion.

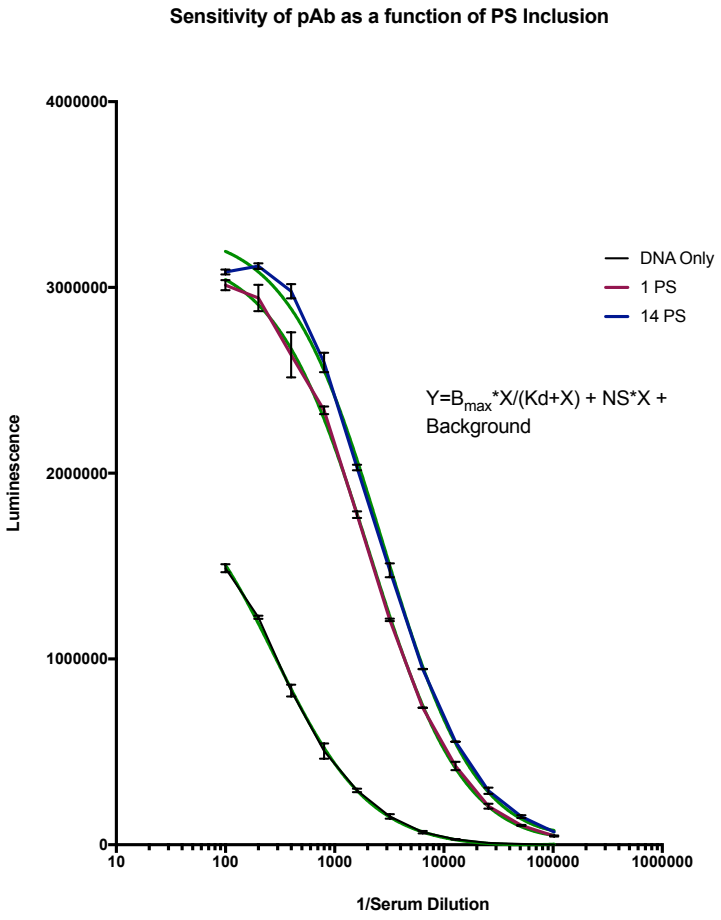


Figure 2.13: The effect of phosphorothioate inclusion on antibody binding. Best fit lines shown in green.

Parameter	DNA Only	1 PS Inclusion	14 PS Inclusion
B_{max}	-2059593 ±34865	-3235116 ±38390	-3364901 ±56831
Kd	285.4 ±14.35	2076 ±101.9	2741 ±176.4
NS	0.1673 ±0.1802	0.3003 ±0.541	0.4005 ±0.775
Background	2039719 ±38140	3191040 ±26549	3312616 ±32226
R^2	0.9987	0.9988	0.998

Table 2.4: Values for the data presented generated by ELISA Figure 2.13

Figure 2.13 shows that the antiserum does indeed bind to some extent to non-phosphorothioate containing DNA, however to a significantly lower degree when

compared to an oligonucleotide containing even one phosphorothioate inclusion. Somewhat unexpectedly, there seems to be a small difference between the oligonucleotide containing one phosphorothioate (**ASO 132- 1 PS**) and the oligonucleotide that is fully phosphorothioated (**ASO 139- fully PS**). One possible explanation for this is the steric effects of one single antibody binding a short DNA sequence may preclude further antibody molecules from binding.

2.4.7 Applications: Immunohistochemistry

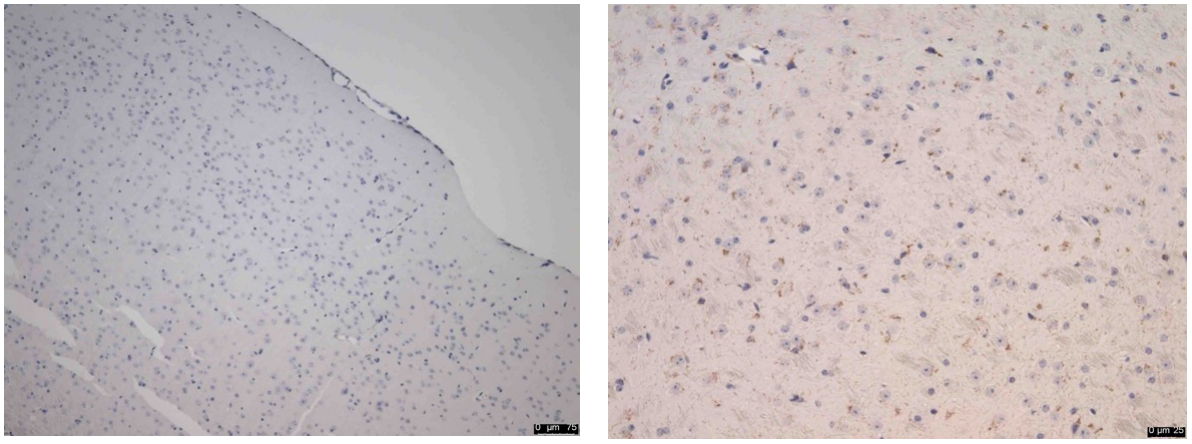


Figure 2.14: Photomicrographs of IHC-stained mouse cortex. Left: PBS treated animal, Right: ASO treated animal (intracerebroventricular injection). Nuclei are stained with Haematoxylin (blue), ASO is detected using the anti-ASO pAb with a poly-HRP secondary and revealed with DAB (brown)

Immunohistrochemistry is a routine technique in the examination of tissues. Traditionally, a tissue section is stained with haematoxylin and eosin (termed H&E staining). Haematoxylin gives the nucleus a blue/violet colour and eosin stains the cytoplasm a pink colour; this allows for tissue slides to be imaged using visible light, avoiding the need for a specialised fluorescence microscope.

It is common to extend this H&E process by the inclusion of antibody staining; an antibody directed against a given antigen can be added to a tissue section, followed by an HRP-conjugated secondary antibody to recognise the primary. This HRP

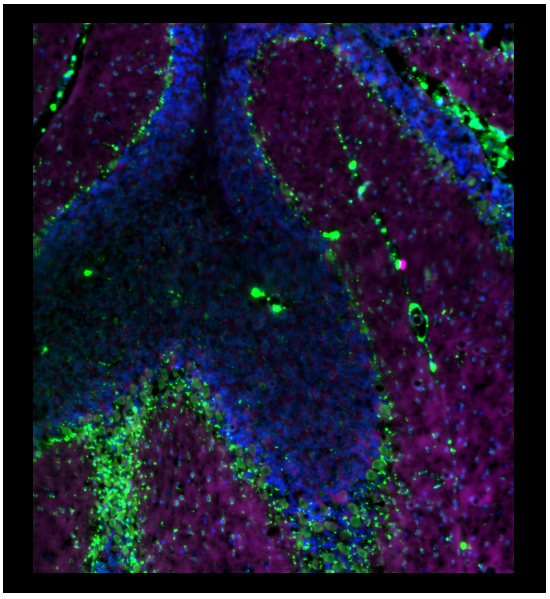
conjugation can be used to reveal the location of a given antigen by adding one of several commercially available reagents (such as 3,3′-diaminobenzidine, (DAB)) with H_2O_2 to induce the precipitation of a pigment revealing the location of the antigen.

One of the main benefits to using immunohistochemistry is that the staining is permanent (in contrast to photobleachable fluorophores used in immunofluorescence). Immunohistochemical staining is also higher-resolution than immunofluorescent staining because one is able to use a visible light microscope, avoiding electronic noise that can present a challenge with fluorescence microscopes. However, only one antigen can be detected in a given tissue section, because the process of revealing staining (addition of H_2O_2 and DAB) will occur at all HRP-tagged secondary antibodies and hence each antigen will be revealed in the same way.

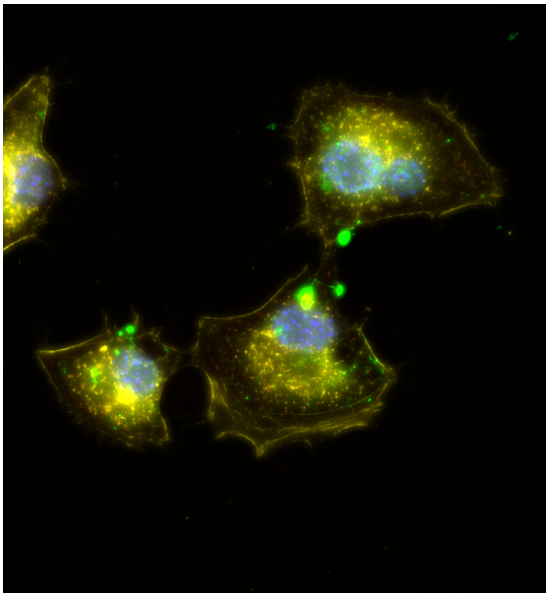
2.4.8 Applications: Immunofluorescence

One of the applications of this pAb that was most potentially exciting was its use in immunofluorescence. Immunofluorescence is typically used for detection of proteins in a given sample (usually cultured cells or animal tissue). The primary antibody has an affinity to the protein of interest and once it is bound, a fluorophore-labelled secondary antibody (with affinity to the Fc of the primary antibody) is added to enable detection of the protein of interest by fluorescence. In this specific instance ASOs were the intended target as opposed to proteins, but the principle remains the same.

One of the benefits of immunofluorescence in contrast to immunohistochemistry, is the ability to detect multiple antigens simultaneously, or indeed to stain with multiple different fluorophores.



(a) Immunofluorescence image of the cerebellum of a mouse injected with a Cy5-tagged ASO intracerebroventricularly.
Blue: DAPI, Purple: Huntingtin Protein, Green: Cy5-ASO



(b) Immunocytochemical image of MEF cells that have taken up a tetrameric oligonucleotide (see section 3.3.1).
Blue: DAPI, Green: anti-ASO pAb, Red: Lysotracker, Yellow: Phalloidin

Figure 2.15: Immunofluorescence in tissue and cultured cells, showing multiple labels

It was extremely pleasing that the generated antibody was able to be used for immunofluorescence as evidenced in figure 2.15b.

2.4.9 Applications- Flow Cytometry

One tissue that remains challenging to assess by microscopy is the blood. Specifically, the population of haematopoietic cells in the spleen and bone marrow- while these generally look quite similar morphologically, they serve very different functions. Commonly, the haematopoietic cells are analysed using flow cytometry, a technique that uses fluorophore-labelled antibodies to specifically label antigens either on the surface of, or within the body of a cell. The benefit of using flow cytometry is that you can label multiple antigens with different fluorophore-conjugated antibodies to distinguish between cells of a B cell

population that are very developmentally close and are perhaps partially only expressing a developmentally-specific antigen.

Injection of a Cy5 labelled antisense oligonucleotide allows for visualisation of oligonucleotide in haematopoietic cells, however as has been mentioned earlier, the effects of the hydrophobic fluorophore may be impacting this distribution. As such, a FITC conjugated antibody was prepared for use in flow cytometric analysis. Unfortunately, a high background was observed in the FITC channel owing to the use of fixed and permeabilised cells which rendered signal indistinguishable from noise.

2.4.9.1 Purification of Antibodies

In order to label the generated pAb, it needed to separated from the remaining proteins in serum. There are several ways to do accomplish this, but by far the most common is to use immobilised Protein A, a protein produced by Staphylococcus aureus that has high affinity to the Fc of antibodies at a neutral pH.[248] At low pH (typically pH 2.8 glycine buffer) the interaction between antibody and Protein A decreases and the antibody is eluted from the column. Typically the eluate is immediately neutralised so as to prevent denaturation of the antibody. The resulting purified antibody is dialysed to remove salts and subsequently quantitated using UV spectrophotometry.

2.4.10 Applications- Plate Assay for Oligonucleotide Quantitation

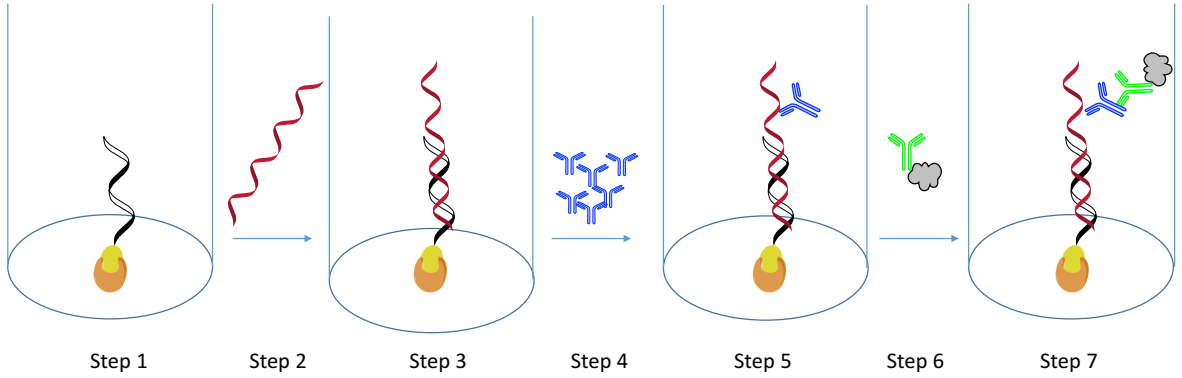


Figure 2.16: Schematic of the intended plate based assay for quantitation of oligonucleotide in tissue homogenate.

One of the applications that was considered for the pAb was in quantitation of oligonucleotides from tissue homogenate. In brief, as shown in Figure 2.16, a short, high affinity (LNA) oligonucleotide is synthesised to be complimentary to one end of an injected oligonuclotide of interest, tagged with biotin and bound to a neutravidin coated plate. This probe contains no phosphorothioate so as not to cross-react with the antibody. A homogenate of tissue from an animal treated with the oligonucleotide of interest is added to the well and allowed to bind. The pAb is added and then detected using an anti-rabbit HRP-conjugated antibody with the signal detected using a suitable substrate and read on a luminometer. In this way, and with an appropriate standard curve of oligonucleotide added to each plate it should be possible to quantitate the amount of oligonucleotide per milligram of homogenised tissue.

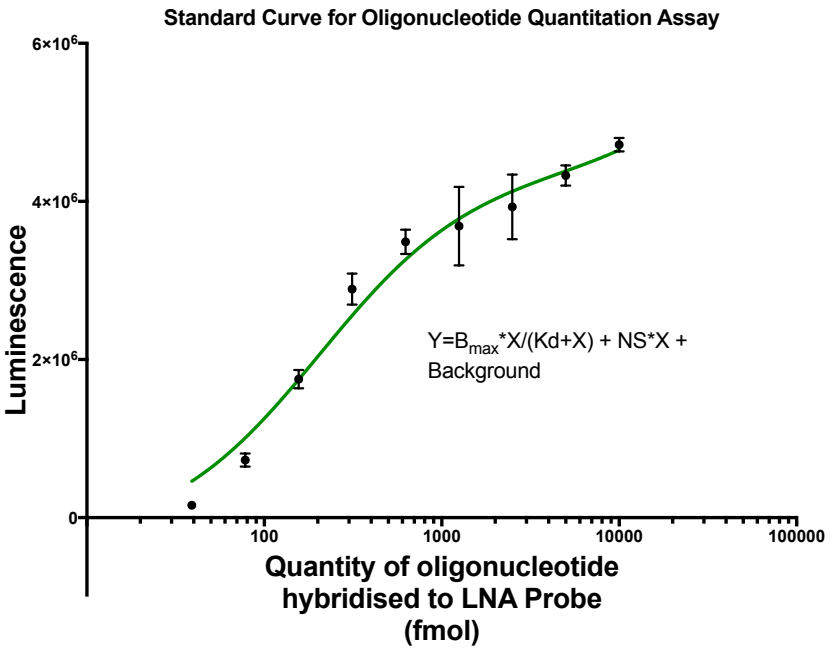


Figure 2.17: A standard curve obtained from a range of dilution of test oligonucleotide. Best fit lines shown in green.

Parameter	Value
B_{max}	4686728 ±368909
Kd	203 ±58.35
NS	35.28 ±49.67
Background	-296302 ±247450
R^2	0.9831

Table 2.5: Values for the data presented generated by the plate assay Figure 2.17

Oligonucleotide Name	**Sequence (5′ – 3′)**
LNA Probe	+T+A+G+A+C+G+T+G+T+T-Biotin
Test Oligonucleotide	+A*+A*+C* A*C*G* T*C*T* A*T*A* +C*+G*+C

Table 2.6: Sequences of the LNA probe and test oligonucleotide employed for the plate assay. '+' indicates and LNA inclusion, '*' indicates a phosphorothioate inclusion.

Our preliminary tests were designed to find the linear range of the assay. This was performed by adding decreasing quantities of oligonucleotide as a solution in PBS to the fully bound probe (Fig. 2.17). Pleasingly, the limit of detection was below 50 fmol, though the linear range of approximately 80 fmol - 800 fmol only represents a small window of accurate quantitation. A sigmoidal curve can be fitted to the data however, with R^2= 0.9919. One may be able to extend the utility of this assay though judicial section of enzyme immunoassay plates, perhaps by employing pre-adsorbed avidin at a given quantity per well.

2.4.11 Summary of Available Techniques

Table 2.7 highlights the main approaches that have been attempted in biodistribution and quantitation of oligonucleotides. Radioisotope labelling has been used to assay biodistribution of therapeutic oligonucleotides *in vivo*[227] however requires the use of particularly hazardous substances and is not suitable for sub-cellular examination of distribution. While NIR fluorophores can be used to examine both whole-animal and sub-cellular accumulation of oligonucleotide it requires that the oligonucleotide be fuorophore-modified, which may affect biodistribution properties. Techniques that require very specialised equipment such as MRI/SPECT, score poorly in the throughput category while scarcely providing more information than a fluorophore tag when coupled with *in vivo* imaging.

The only methods that allow for accurate quantitation mandate the use of an native oligonucleotide and by their plate-based nature will have a moderate throughput. HPLC assays that require 'pulling down' oligonucleotide from tissue are the most-time consuming because each sample has to be run sequentially on an HPLC instrument.

Method	Throughput	Special Reagents/ Equipment	Native ASO	Quantitative
Whole animal imaging: Radiolabel	++	Radioactive Oligonucleotide	Yes	Potentially
Whole animal imaging: Fluorophore tag	++	Labelled Oligonucleotide	No	No
Whole animal imaging: MRI	++	Labelled Oligonucleotide	Yes (generally)	No
Tissue section imaging: Fluorophore tag	++++	Labelled Oligonucleotide	No	NA
Tissue section imaging: Anti-ASO antibody	+++	Anti-ASO Antibody	Yes	NA
HPLC assay: fluorescent hybridization probe	+	HPLC and fluorescent probe	Yes	Yes
Plate-based assay: DIG probe	+	DIG probe, ligase, anti-DIG antibody	Yes	Yes
Plate-based assay: Fluorescent probe	++	HPLC and fluorescent probe	Yes	Yes
Plate-based assay: Anti-ASO antibody	++	Anti-ASO Antibody, capture probe	Yes	Yes

Table 2.7: Available techniques for assaying oligonucleotide biodistribution.

2.5 Experimental

2.5.1 General Methods

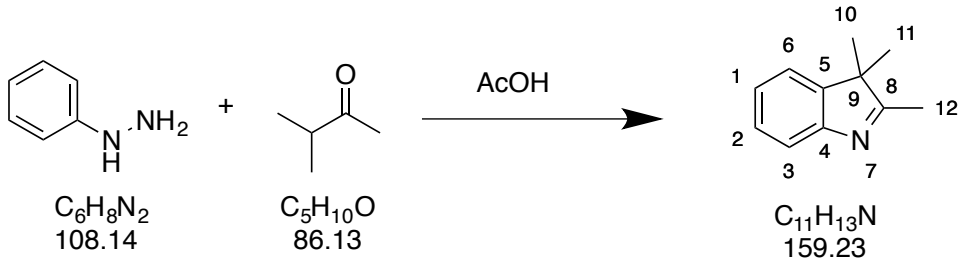
All reagents were purchased from Aldrich, Fisher, Alfa or Fluorochem and used without further purification unless otherwise stated. TLC was conducted on fluorescent silica plates from Merck and visualised with both U.V. and $KMnO_4$ or vanillin stains. Column Chromatography was conducted using Silica purchased from Merck or on a Biotage isolera 4 automated column chromatography instrument using either SNAP or SNAP ULTRA pre-packed cartridges with either a linear or step gradient.

NMR spectra were recorded on Bruker AVII400, Bruker AVIIIHD400 or Bruker AVIIIHD500 FT-NMR spectrometers in the indicated solvent at 298 K. Chemical shifts for proton and carbon spectra are reported on the delta scale in ppm.

Samples were analysed using a Waters (Manchester, UK) TQD mass spectrometer equipped with a triple quadrupole analyser. Samples were introduced to the mass spectrometer via an Acquity H-Class quaternary solvent manager (with TUV detector at 254nm, sample and column manager). Ultra-performance liquid chromatography was undertaken via a Waters BEH C18 column (50 mm x 2.1mm 1.7µm). Low resolution

mass spectra were recorded using positive ion electrospray ionisation, using a gradient of 20% acetonitrile (0.2% formic acid) to 100% acetonitrile (0.2% formic acid) in 5 min at a flow rate of 0.6 mL/min. Alternatively, low-resolution mass spectra were recorded using negative ion electrospray ionisation using a gradient of 20% acetonitrile (0.2% ammonium hydroxide) to 100% acetonitrile (0.2% ammonium hydroxide) in 5 min at a flow rate of 0.6 mL/min.

2.5.1.1 2,3,3-Trimethyl-3*H*-indole



AcOH (250 mL), Phenyhydrazine (29.5 mL, 300 mmol) and 3-methyl-2-butanone (50 mL, 360 mmol) were combined in a flask and bought to reflux (ca. 130 °C) for 5 h. The reaction was then cooled to RT and the volatiles removed *in vacuo*. The remaining red oil was partitioned between Et_2O (200 mL) and H_2O (200 mL), the aqueous phase was re-extracted with a further 50 mL Et_2O and the organics pooled and treated with $NaHCO_3$ and water in order to remove any residual AcOH. Once effervescence ceased, the organic phase was separated, washed with water and brine and dried (Na_2SO_4) and the solvents removed *in vacuo*.

Yield: 40.1 g (252 mmol, 84%)

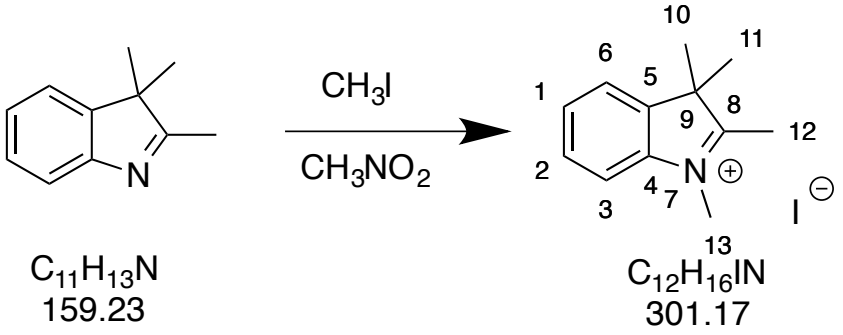
^{1}**H NMR** (400 MHz, $CDCl_3$) δ 7.53 (1H, d, J = 7.6 Hz, H-6), 7.29 (1H, td, J = 7.6 Hz, J = 1.4 Hz, H-3), 7.27 (1H, d, J = 7.0 Hz, H-1), 7.19 (1H, td, J = 7.4 Hz, J = 1.0 Hz, H-2), 2.27 (3H, s, H-12), 1.29 (6H, s, H-11, 10)

^{13}**C NMR** (100 MHz, $CDCl_3$) δ 188.0 (C-8), 153.7 (C-4), 145.6 (C-5), 127.6 (C-2), 125.1 (C-1), 121.3 (C-3), 119.9 (C-6), 53.6 (C-9), 23.1 (C-11, 10), 15.4 (C-12)

LRMS (ESI) m/z= 160 $[M+H]^+$

The above analytical data are in agreement with the literature[249]

2.5.1.2 1,2,3,3-Tetramethyl-3*H*-indol-1-ium iodide



2,3,3-trimethyl-3*H*-indole (7.96 g, 50 mmol) was dissolved in $MeNO_2$ (30 mL) and purged with argon. Iodomethane (4.65 mL, 50 mmol) was added and the mixture allowed to stir for 20 h, following which a thick crystalline precipitate was found. Et_2O (200 mL) was added, inducing further precipitation and all was filtered to yield an off-white crystalline solid.

Yield: 12.43 g (41.3 mmol, 83%)

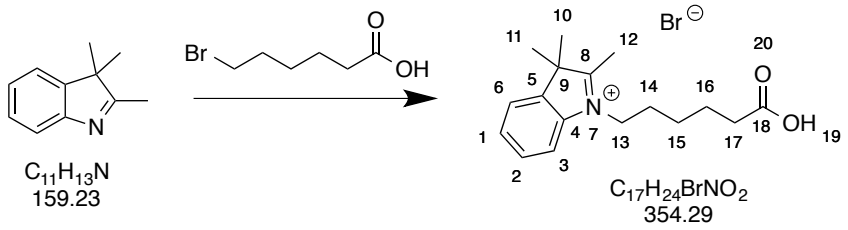
^{1}**H NMR** (400 MHz, $CDCl_3$) δ 7.89 - 7.96 (1H, m), 7.81 - 7.86 (1H, m), 7.59 - 7.67 (2H, m, H-2, 1), 3.99 (3H, s, H-13), 2.78 (3H, s, H-12), 1.54 (6H, s, H-10, 11)

^{13}**C NMR** (100 MHz, $CDCl_3$) δ 196.5 (C-8), 142.6, 142.1, 129.8, 129.3, 123.8 (C-6), 115.6 (C-3), 54.4 (C-9), 35.2 (C-13), 22.2 (C-11, 10), 14.7 (C-12)

LRMS (ESI) m/z= 174.1 $[M]^+$

The above analytical data are in agreement with the literature[250]

2.5.1.3 1-(5-carboxypentyl)-2,3,3-Trimethyl-3*H*-indol-1-ium bromide



2,3,3-trimethyl-3*H*-indole (1.611 g, 10.1 mmol) and 6-bromohexanoic acid (1.579 g, 8.1 mmol) were mixed neat and heated to 110 °C for 20 h with stirring. This formed an extremely thick, red gum which on cooling semi-solidified. EtOAc (8 mL) was added and the mixture bought to reflux, colouring the solvent. This was repeated 6 times with EtOAc, 2 times with acetone (8 mL) and a further 2 times with EtOAc (16 mL), finally producing a slightly pink, crystalline solid.

Yield: 1.445 g (4.1 mmol, 51%)

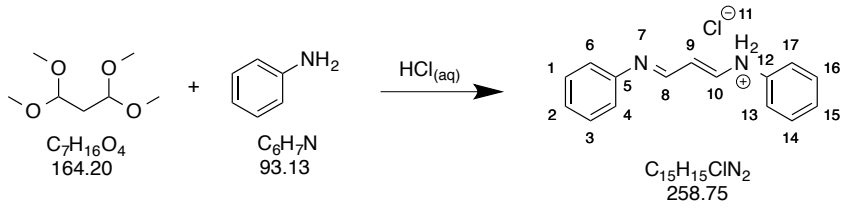
^{1}**H NMR** (400 MHz, DMSO-d_6) δ 7.97 - 8.02 (1H, m), 7.84 - 7.88 (1H, m), 7.60 - 7.66 (2H, m, H-2, 1), 4.47 (2H, br t, J = 7.7 Hz, H-13), 2.86 (3H, s, H-12), 2.24 (2H, t, J = 7.2 Hz, H-17), 1.85 (2H, quin, J = 7.7 Hz, H-14), 1.50 - 1.63 (8H, m, H-16), 1.35 - 1.49 (2H, m, H-15)

^{13}**C NMR** (100 MHz, $CDCl_3$) δ 197.0 (C-8), 174.8 (C-18), 142.3, 141.5, 129.9, 129.4, 124.0 (C-6), 116.0 (C-3), 54.6 (C-9), 47.9 (C-13), 33.8 (C-17), 27.4 (C-14), 25.9 (C-16), 24.5 (C-15), 22.5 (C-11, 10), 14.5 (C-12)

LRMS (ESI) m/z= 274.1 $[M]^+$

The above analytical data are in agreement with the literature[251]

2.5.1.4 *N*-((1*Z*,3*E*)-3-(phenylimino)prop-1-en-1-yl)benzenaminium chloride



To H_2O (171 mL) was added concentrated aqueous HCl (8.5 mL). 1,1,3,3-tetramethoxypropane (10.5 mL, 60 mmol) was added at 50 °C forming a colourless solution. A separate solution of aniline (11.1 mL, 120 mmol) and concentrated aqueous HCl (15 mL) in H_2O (210 mL) was prepared and added portionwise to the solution of tetramethoxypropane at 50 °C. A deep red solution was formed initially, followed by the precipitation of lustrous red crystals. After 4 h stirring at this temperature, the mixture was cooled to RT and filtered. The red/orange solid was dried under high vacuum for 2 d.

Yield: 9.5 g (37 mmol, 60%)

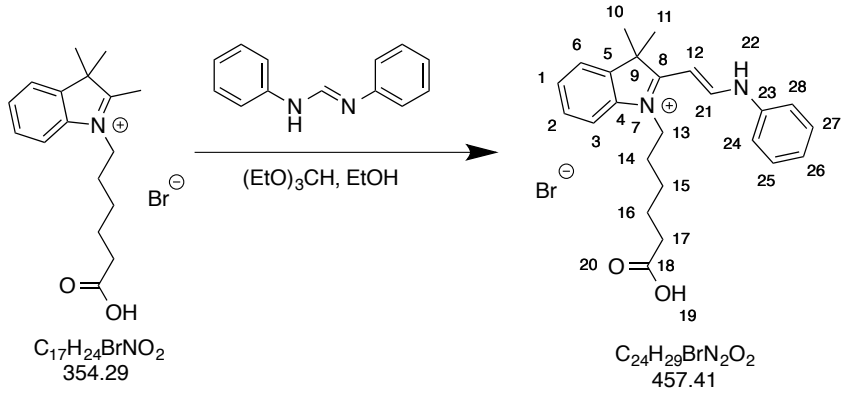
^{1}**H NMR** (400 MHz, DMSO-d_6) δ 12.69 (1H, br d, J = 13.8 Hz), 8.90 (1H, dd, J = 13.7 Hz, J = 11.7 Hz), 7.38 - 7.55 (6H, m), 7.19 - 7.35 (2H, m), 6.51 (1H, t, J = 11.6 Hz)

^{13}**C NMR** (100 MHz, DMSO-d_6) δ 58.9, 139.2, 130.4), 126.4, 117.9, 99.1

LRMS (ESI) m/z= 223.0 $[M]^+$

The above analytical data are in agreement with the literature[250]

2.5.1.5 (*E*)-1-(5-carboxypentyl)-3,3-dimethyl-2-(2-(phenylamino)vinyl)-3*H*-indol-1-ium bromide



1-(5-carboxypentyl)-2,3,3-trimethyl-3H-indol-1-ium bromide (979 mg, 3 mmol) was slurried into EtOH (16 mL) with diphenylformamidine (648 mg, 3.3 mmol). Triethylorthoformate (548 μL, 3.3 mmol) was added and the mixture bought to reflux for 6 h. The reaction was cooled to RT and added to Et_2O before being left for 20 h. This produced a mass of red crystals with a purple glint.

Yield: 1.181 g (2.6 mmol, 85%)

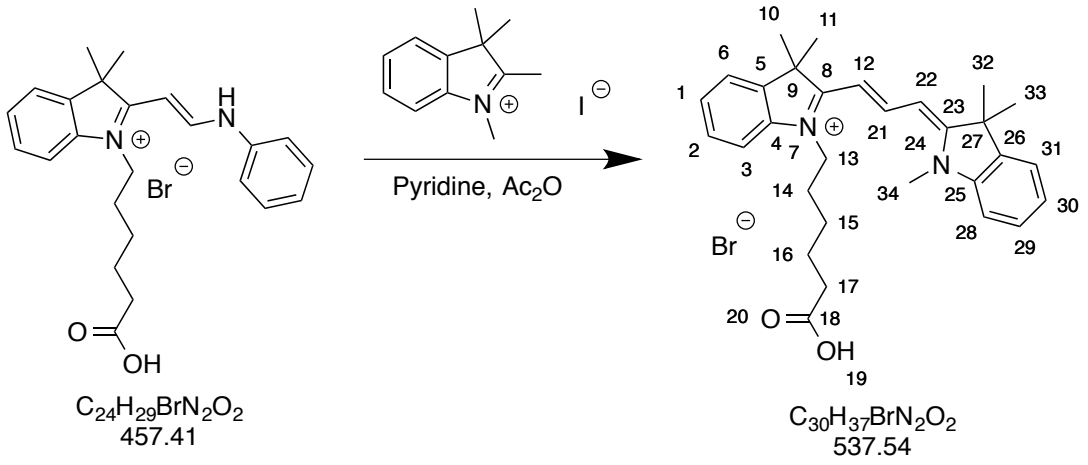
^{1}**H NMR** (400 MHz, $CDCl_3$) δ 8.63 - 8.75 (1H, m, H-21), 7.24 - 7.73 (10H, m, H-25, 24, 26, 27, 28, 3, 2, 1, 6), 6.33 - 6.45 (1H, m, H-12), 4.03 - 4.17 (2H, m, H-13), 2.23 (1H, s, H-17), 2.19 - 2.27 (2H, m, H-14), 1.71 (8H, s, H-14, 10, 11), 1.53 - 1.63 (2H, m, H-16), 1.37 - 1.50 (2H, m, H-15)

^{13}**C NMR** (100 MHz, $CDCl_3$) δ 177.9 (C-18), 174.8, 152.2, 142.0, 141.5, 138.9, 130.2, 129.0, 126.5, 126.0, 123.0, 118.7, 112.2, 91.7, 50.0 (C-9), 44.5 (C-13), 34.0 (C-17), 28.2 (C-10, 11), 26.9 (C-14), 26.3 (C-16), 24.7 (C-15)

LRMS (ESI) m/z= 377.1 $[M]^+$

The above analytical data are in agreement with the literature[252]

2.5.1.6 1-(5-Carboxypentyl)-3,3-dimethyl-2-((1*E*,3*E*)-3-(1,3,3-trimethylindolin-2-ylidene)prop-1-en-1-yl)-3*H*-indol-1-ium bromide



(E)-1-(5-carboxypentyl)-3,3-dimethyl-2-(2-(phenylamino)vinyl)-3*H*-indol-1-ium bromide (1.1425 g, 2.5 mmol) and 1,2,3,3-tetramethyl-3*H*-indol-1-ium iodide (1.1256 g, 3.75 mmol) were dissolved in anhydrous pyridine (12.5 mL). Ac_2O (2.35 mL) was added and the pink mixture stirred at 50 °C for 20 h, subsequently, all was poured into Et_2O and the precipitate purified by column chromatography (5% MeOH in EtOAc) giving a green-gold iridescent pink solid.

Yield: 550 mg (1.02 mmol, 41%)

^{1}**H NMR** (400 MHz, $CDCl_3$) δ 8.42 (1H, t, J = 13.4 Hz, H-21), 7.31 - 7.44 (4H, m), 7.12 - 7.26 (5H, m, H-12), 7.07 (1H, d, J = 13.4 Hz, H-22), 4.20 (2H, br t, J = 7.5 Hz, H-13), 3.78 (4H, s), 2.45 (2H, br t, J = 6.4 Hz, H-17), 1.82 - 1.94 (2H, m, H-16), 1.75 (2H, br d, J = 7.5 Hz, H-15), 1.64 (3H, br s, H-14), 1.71 (11H, d, J = 2.1 Hz, H-32, 33, 11, 10)

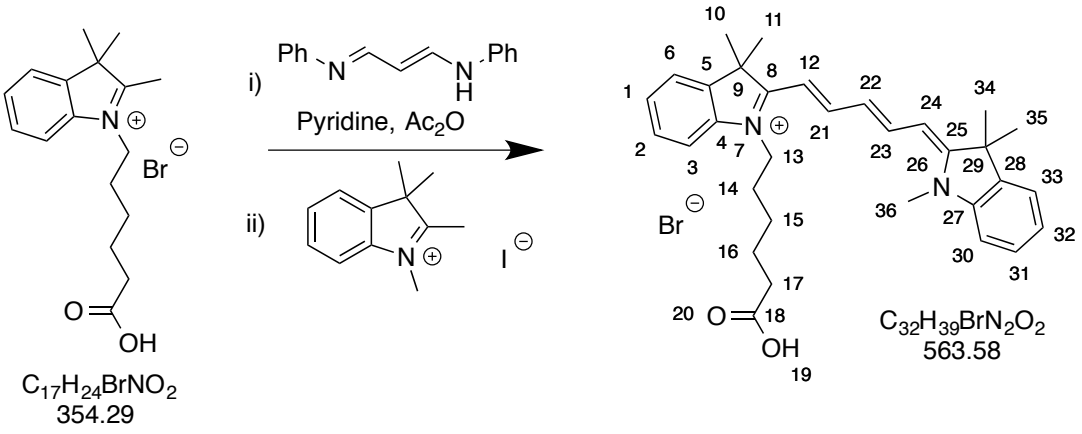
^{13}**C NMR** (100 MHz, $CDCl_3$) δ 174.2, 173.7, 150.7 (C-21), 142.7, 142.0, 140.6, 140.5, 129.0, 128.9, 125.4, 125.3, 124.7, 122.1, 122.0, 111.2, 111.0, 104.7 (C-12), 104.2 (C-22),

49.0, 48.9, 44.9, 44.8 (C-13), 32.9, 28.2, 28.1 (C-32, 33, 11, 10), 27.3 (C-16), 26.4 (C-14), 24.8 (C-15)

LRMS (ESI) m/z= 457.2 $[M]^+$

The above analytical data are in agreement with the literature[253]

2.5.1.7 1-(5-Carboxypentyl)-3,3-dimethyl-2-((1*E*,3*E*,5*Z*)-5-(1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3*H*-indol-1-ium bromide



(E)-1-(5-carboxypentyl)-3,3-dimethyl-2-(2-(phenylamino)vinyl)-3H-indol-1-ium bromide (354.7 mg, 1 mmol) and N-((1Z,3E)-3-(phenylimino)prop-1-en-1-yl)benzenaminium chloride (336.2 mg, 1.3 mmol) were refluxed together in Ac_2O (3 mL) under argon for 30 min. The mix was cooled to RT and a solution of 1,2,3,3-tetramethyl-3H-indol-1-ium iodide (600 mg, 2 mmol) in pyridine (4 mL) was added. The turquoise solution was allowed to stir at RT for 20 h and was subsequently poured into rapidly stirring Et_2O. The light green solution was poured away from the intensely blue solid and the solid was taken up into DCM and purified by column chromatography (10% MeOH in DCM + 2.5% AcOH) to provide a golden-green-red iridescent blue solid.

Yield: (NMR integration determining AcOH impurity) 398 mg (706 μmol, 71%)

^{1}**H NMR** (400 MHz, $CDCl_3$) δ 8.09 (2H, td, J = 13.0 Hz, J = 2.9 Hz, H-23, 21), 7.74 (1H, tt, J = 7.7 Hz, J = 1.8 Hz), 7.32 - 7.40 (6H, m), 7.22 (2H, t, J = 7.3 Hz), 7.12 (1H, d, J = 7.9 Hz), 7.08 (1H, d, J = 8.1 Hz,), 6.90 (1H, br t, J = 12.5 Hz, H-22), 6.39 (1H, d, J = 13.6 Hz, H-24), 6.34 (1H, d, J = 13.6 Hz, H-12), 4.07 (2H, t, J = 7.6 Hz, H-13),

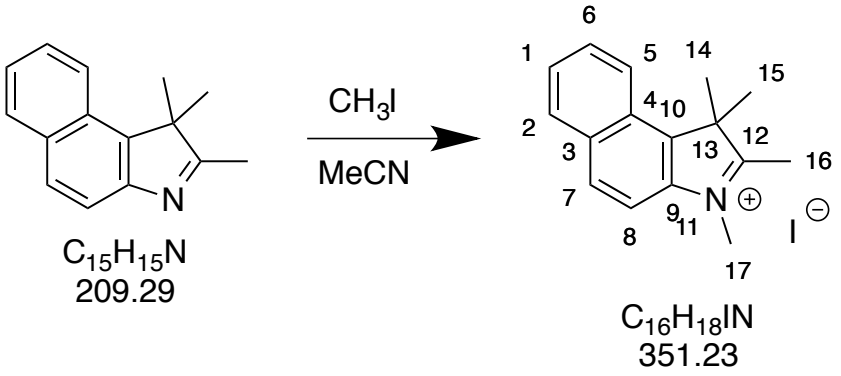
3.70 (3H, s, H-36), 2.42 (2H, t, J = 7.2 Hz, H-17), 1.81 - 1.91 (2H, m, H-16), 1.71 - 1.78 (2H, m, H-15), 1.74 (11H, d, J = 9.3 Hz, H-35, 34, 10, 11), 1.50 - 1.62 (2H, m, H-14)

^{13}C NMR (100 MHz, $CDCl_3$) δ 176.0 (C-18), 173.4, 172.8, 153.6, 153.6 (C-23, 21), 142.8, 142.0, 141.3, 141.0, 136.8, 128.8, 128.6, 126.6 (C-22), 125.1, 125.1, 124.1, 122.3, 122.2, 120.0, 110.5, 104.3 (C-24), 103.8, 49.4, 49.3, 44.2 (C-13), 34.0, 28.2, 28.0 (C-35, 34, 10, 11), 27.0 (C-16), 26.9, 26.3, 24.3 (C-15)

LRMS (ESI) m/z= 483.3 $[M]^+$

The above analytical data are in agreement with the literature[253]

2.5.1.8 1,1,2,3-Tetramethyl-1*H*-benzo[e]indol-3-ium iodide



1,1,2-Trimethylbenz[*e*]indole (5.00 g, 23.89 mmol) was dissolved in MeCN (25 mL), MeI (1.79 mL, 28.7 mmol) was added and all heated to reflux. After 15 min at reflux the whole reaction mixture set up to an olive-green mass, which was broken up, filtered and washed with Et_2O to yield the product as an olive-green solid.

Yield: 7.28 g (21 mmol, 88%)

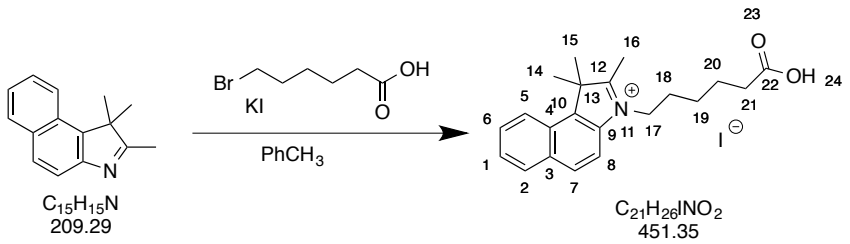
^{1}**H NMR** (400 MHz, DMSO-d_6) δ 8.43 (1H, d, J = 8.0 Hz, H-5), 8.35 (1H, d, J = 8.9 Hz, H-7), 8.28 (1H, d, J = 7.9 Hz, H-2), 8.17 (1H, d, J = 8.9 Hz, H-8), 7.85 (2H, ddd, J = 8.2 Hz, J = 7.0 Hz, J = 1.3 Hz, H-6), 7.78 (1H, ddd, J = 8.4 Hz, J = 7.0 Hz, J = 1.6 Hz, H-1), 4.16 (3H, s, H-17), 2.94 (3H, s, H-16), 1.81 (6H, s, H-14, 15)

^{13}**C NMR** (100 MHz, DMSO-d_6) δ 196.4 (C-12), 139.9 (C-9), 137.0 (C-10), 133.5 (C-3, 4), 131.0 (C-7), 130.2 (C-2), 128.9 (C-6), 127.6 (C-1), 123.9 (C-5), 113.6 (C-8), 55.7 (C-13), 35.6 (C-17), 21.8 (C-14, 15), 14.5 (C-16)

LRMS (ES) *m/z*= 224 $[M]^+$

The above analytical data are in agreement with the literature[250]

2.5.1.9 3-(5-Carboxypentyl)-1,1,2-trimethyl-1*H*-benzo[e]indol-3-ium iodide



1,1,2-Trimethylbenz[*e*]indole (3.00 g, 14.34 mmol), KI (3.6 g, 23 mmol) and 6-bromohexanoic acid (4.20 g, 21 mmol) were added to a flask with toluene (2.5 mL) and bought to reflux. Toluene (2 mL) was added to help remove sublimated 6-bromohexanoic acid from the condenser and all left to reflux for 20 h. The formed mass was cooled to RT, broken up and washed with THF, cold water and $CHCl_3$ before being dried under high vacuum to yield an off-white solid.

Yield: 5.00 g (11 mmol, 77%)

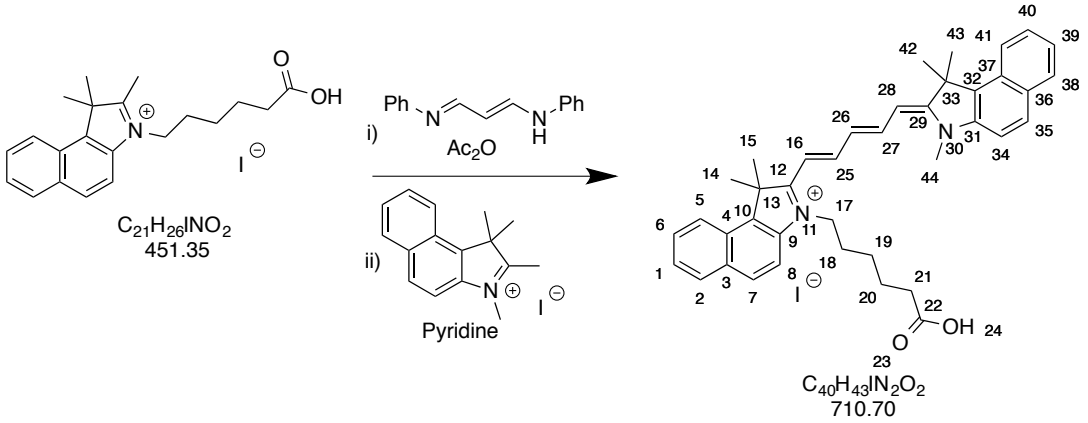
^{1}**H NMR** (400 MHz, $CDCl_3$) δ 8.37 (1H, d, J = 8.3 Hz, H-5), 8.29 (1H, d, J = 8.9 Hz, H-7), 8.22 (1H, d, J = 7.9 Hz, H-2), 8.16 (1H, d, J = 8.9 Hz, H-8), 7.76 - 7.83 (1H, m, H-6), 7.70 - 7.77 (1H, m), 4.59 (2H, br t, J = 7.6 Hz, H-17), 2.95 (3H, s, H-16), 2.48 - 2.52 (1H, m, H-18), 2.23 (2H, t, J = 7.2 Hz, H-21), 1.91 (2H, quin, J = 7.6 Hz, H-18), 1.76 (6H, s, H-15, 14), 1.52 - 1.63 (2H, m, H-20), 1.41 - 1.52 (2H, m, H-19)

^{13}**C NMR** (100 MHz, $CDCl_3$) δ 196.8 (C-12), 174.8 (C-22), 139.0 (C-10), 137.4 (C-9), 133.5 (C-3), 131.2 (C-7), 130.2 (C-2), 128.9 (C-6), 127.8 (C-4), 127.7 (C-1), 123.9 (C-5), 113.8 (C-8), 56.0 (C-13), 48.2 (C-17), 33.8 (C-21), 27.6 (C-18), 22.1 (C-15, 14), 14.3 (C-16)

LRMS (ESI) m/z= 324.1 $[M]^+$

The above analytical data are in agreement with the literature[253]

2.5.1.10 3-(5-Carboxypentyl)-1,1-dimethyl-2-((1*E*,3*E*,5*E*)-5-(1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1*H*-benzo[e]indol-3-ium iodide



3-(5-carboxypentyl)-1,1,2-trimethyl-1*H*- benzo[e]indol-3-ium iodide (900.6 mg, 2 mmol) was mixed with N-((1Z,3E)-3-(phenylimino)prop-1-en-1- yl)benzenaminium chloride (621 mg, 2 mmol) in Ac_2O (8 mL). The mixture was bought to reflux for 30 min forming a brown solution. All was cooled to RT and 1,1,2,3-tetramethyl-1H-benzo[e]indol-3-ium iodide (737.6 mg, 2.1 mmol) was added with pyridine (10 mL) forming a deep blue solution. The mixture was allowed to stir at RT under Argon overnight and was subsequently poured into rapidly stirring Et_2O. The precipitated solid was taken into DCM and purified by column chromatography (10% MeOH in DCM).

Yield: 602 mg (42%)

^{1}**H NMR** (400 MHz, $CDCl_3$) δ 8.38 (2H, br t, J = 13.1 Hz, H-25, 27), 8.16 (2H, t, J = 8.3 Hz), 7.91 (4H, dd, J = 8.7 Hz, J = 2.4 Hz), 7.74 (2H, tt, J = 7.7 Hz, J = 1.8 Hz), 7.55 - 7.62 (2H, m), 7.42 - 7.48 (2H, m), 7.39 (3H, d, J = 8.8 Hz), 7.36 - 7.41 (2H, m), 7.31 - 7.37 (3H, m), 7.24 - 7.29 (1H, m), 6.84 (1H, br t, J = 12.4 Hz, H-26), 6.31 (2H, t, J = 14.5 Hz, H-16), 4.16 (2H, br t, J = 7.5 Hz, H-17), 3.81 (3H, s), 2.42 (2H, t, J = 7.2

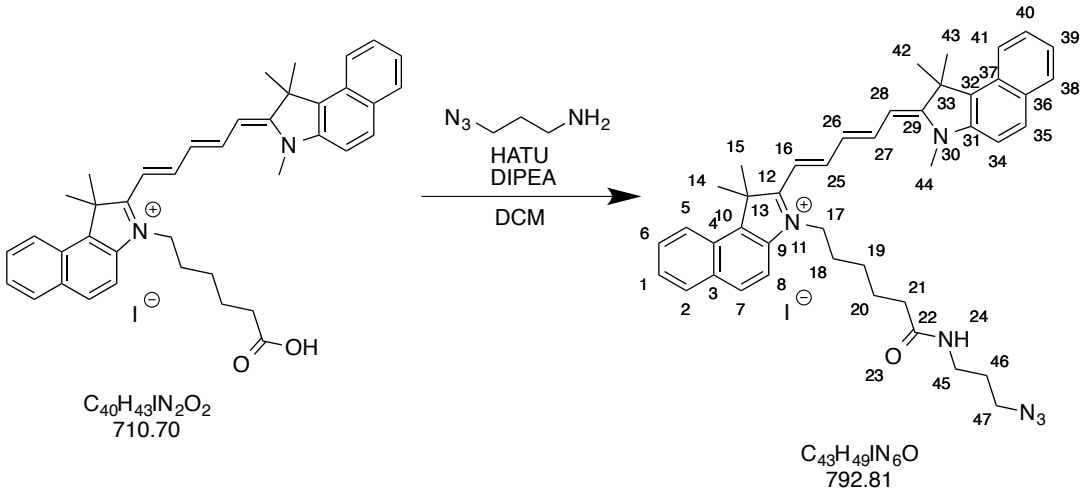
Hz, H-21), 2.25 (1H, s), 2.00 - 2.13 (15H, m, H-43, 42, 14, 15), 1.84 - 1.95 (2H, m, H-18), 1.76 (2H, quin, J = 7.4 Hz, H-20), 1.53 - 1.65 (2H, m, H-19)

^{13}C NMR (100 MHz, $CDCl_3$) δ 177.3 (C-22), 174.8 (C-12), 174.3 (C-29), 174.3, 152.9 (C-25, 27), 140.0, 139.3, 136.9, 134.2, 133.7, 131.8, 130.5, 130.0, 128.8, 128.2, 128.2, 127.7, 126.3 (C-26), 125.0, 124.1, 122.3, 110.4, 103.6 (C-16), 103.1, 51.3, 51.2, 44.3 (C-17), 34.0 (C-21), 27.7 (C-43, 42, 14, 15), 27.3 (C-18), 26.3 (C-19), 24.4 (C-20), 21.0

LRMS (ESI) m/z= 583.2 $[M]^+$

The above analytical data are in agreement with the literature[254]

2.5.1.11 3-(6-((3-Azidopropyl)amino)-6-oxohexyl)-1,1-dimethyl-2-((1*E*,3*E*,5*E*)-5-(1,1,3-trimethyl-1*H*-benzo[e]indol-2(3*H*)-ylidene)penta-1,3-dien-1-yl)-1*H*-benzo[e]indol-3-ium iodide



3-(5-carboxypentyl)-1,1-dimethyl-2-((1E,3E,5E)-5-(1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1H-benzo[e]indol-3-ium iodide (178 mg, 250 μmol) was added as a solution in DCM (5 mL) to HATU (114 mg, 300 μmol) under Argon. DIPEA (104 μL, 500 μmol) was added and all was allowed to stir at RT for 15 min. 3-azidopropylamine (50 mg, 500 μmol) as a solution in DCM (5 mL) was added and all was allowed to stir at RT overnight. The following morning the reaction mixture was washed with citric acid (10% aq), $NaHCO_3$ (sat.) and brine and the solvent dried (Na_2SO_4) before being concentrated (in vacuo). The produced blue oil was purified by column chromatography (5% MeOH in DCM).

Yield: 148.5 mg (75%)

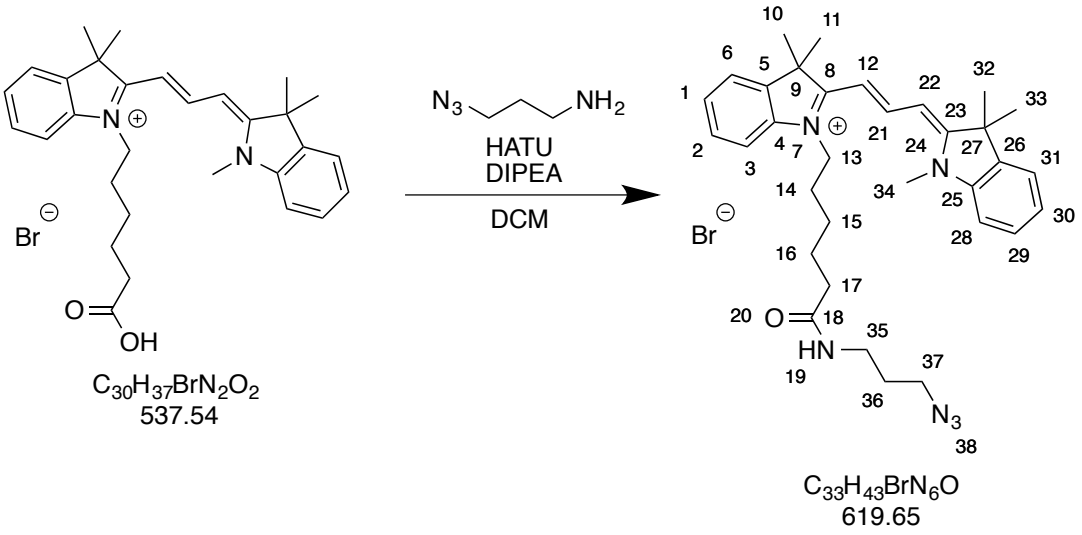
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 (2H, br d, J = 8.6 Hz), 7.97 - 8.04 (2H, m), 7.93 - 7.96 (4H, m), 7.59 - 7.65 (2H, m), 7.33 - 7.39 (2H, m), 7.25 - 7.68 (2H, m), 6.82 (1H, t, J = 12.5 Hz), 6.31 (2H, br d, J = 13.6 Hz), 6.25 (1H, d, J = 13.7 Hz), 4.10 (2H, br t, J = 7.5 Hz), 3.72 (3H, s), 3.19 - 3.36 (4H, m), 2.29 (2H, t, J = 7.2 Hz), 1.99 - 2.07 (12H, m), 1.84 - 1.94 (2H, m), 1.69 - 1.82 (4H, m), 1.48 - 1.61 (3H, m)

^{13}C NMR (100 MHz, $CDCl_3$) δ 173.5, 130.7, 130.6, 130.1, 128.2, 127.8, 125.0, 122.1, 110.5, 110.3, 103.6, 51.1, 51.0, 49.3, 36.8, 36.1, 28.8, 27.6, 27.4, 26.5, 25.2

LRMS (ESI) m/z= 665.3 $[M]^+$

Novel compound, no data available

2.5.1.12 1-(6-((3-Azidopropyl)amino)-6-oxohexyl)-2-((1*E*,3*E*)-3-(1,3,3-trimethylindolin-2-ylidene)prop-1-en-1-yl)-3*H*-indol-1-ium bromide



1-(5-carboxypentyl)-3,3-dimethyl-2-((1E,3E)-3-(1,3,3-trimethylindolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium bromide (134.4 mg, 250 μmol) was added as a solution in DCM (5 mL) to HATU (114 mg, 300 μmol) under Argon. DIPEA (104 μL, 500 μmol) was added and all was allowed to stir at RT for 15 min. 3-azidopropylamine (50 mg, 500 μmol) as a solution in DCM (5 mL) was added and all was allowed to stir at RT overnight. The following morning the reaction mixture was washed with citric acid (10% aq), $NaHCO_3$ (sat.) and brine and the solvent dried (Na_2SO_4) before being concentrated (*in vacuo*). The residue was purified by column chromatography (5% MeOH in DCM).

Yield: 88.3 mg, 54%

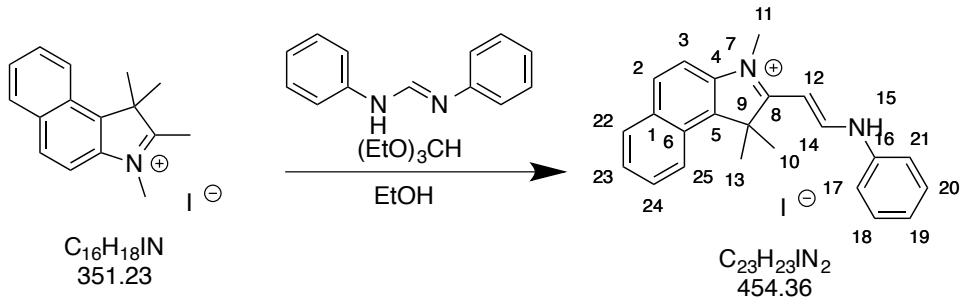
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.41 (1H, t, J = 13.5 Hz), 7.34 - 7.44 (4H, m), 7.23 - 7.32 (3H, m), 7.08 - 7.19 (2H, m), 6.58 (1H, d, J = 13.5 Hz), 6.48 (1H, d, J = 13.5 Hz), 6.30 (1H, br t, J = 5.5 Hz), 3.98 - 4.07 (2H, m), 3.68 (3H, s), 3.27 - 3.38 (4H, m), 2.28 (2H, t, J = 7.3 Hz), 1.72 - 1.75 (15H, m), 1.51 - 1.91 (8H, m)

^{13}C NMR (100 MHz, $CDCl_3$) δ 174.7, 173.9, 173.8, 150.7, 142.6, 141.9, 140.5, 129.0, 125.5, 122.0, 110.9, 103.9, 103.4, 49.2, 49.1, 44.6, 36.4, 31.4, 28.8, 28.1, 28.1, 27.1, 26.3, 25.4

LRMS (ESI) m/z= 539.3 $[M]^+$

Novel compound, no data available

2.5.1.13 (*E*)-1,1,3-Trimethyl-2-(2-(phenylamino)vinyl)-1*H*-benzo[e]indol-3-ium iodide



1,1,2,3-tetramethyl-1H-benzo[*e*]indol-3-ium iodide (2.50 g, 7.11 mmol) and diphenylformamidine (1.67 g, 8.54 mmol) were suspended in EtOH (25 mL). Triethylorthoformate (1.42 mL, 8.54 mmol) was added and all allowed to reflux for 23 h. The reaction mixture was cooled to RT and the solvents removed *in vacuo*, the residue was dissolved in DCM and precipitated into Et_2O producing a green solid.

Yield: 2.92 g (92%)

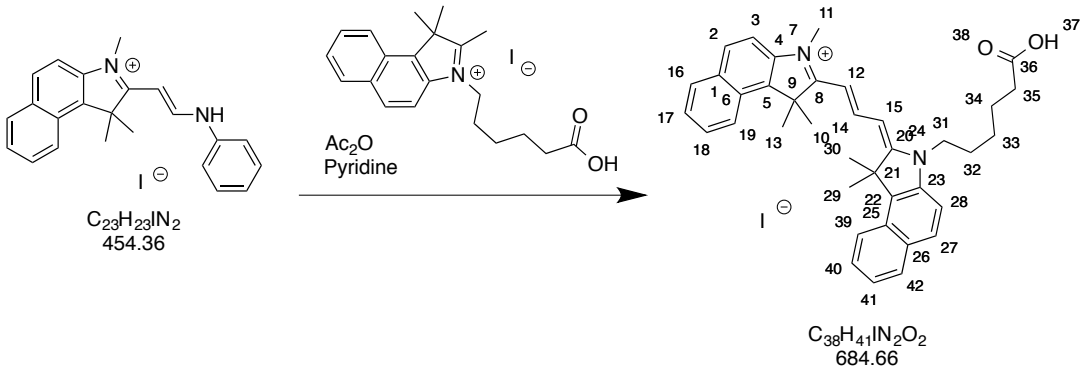
^{1}**H NMR** (400 MHz, $CDCl_3$) δ 8.64 (1H, d, J = 12.6 Hz, H-14), 8.07 - 8.13 (1H, m), 7.95 - 8.02 (2H, m), 7.65 (1H, ddd, J = 8.4 Hz, J = 7.0 Hz, J = 1.2 Hz), 7.45 (3H, br s), 7.39 - 7.56 (4H, m, H-3, 12), 7.22 - 7.28 (1H, m), 3.82 (3H, s, H-11), 1.99 (6H, s, H-10, 13)

^{13}**C NMR** (100 MHz, $CDCl_3$) δ 179.2 (C-8), 149.9 (C-14), 139.5 (C-4), 138.4 (C-16), 133.3 (C-5), 132.2, 131.1, 130.3, 130.0, 128.2, 127.8, 126.7, 125.6, 121.8, 117.9, 110.6 (C-3), 91.6 (C-12), 51.2 (C-9), 32.6 (C-11), 28.8 (C-10, 13)

LRMS (ESI) m/z= 327 $[M]^+$

The above analytical data are in agreement with the literature[250]

2.5.1.14 2-((1*E*,3*Z*)-3-(3-(5-Carboxypentyl)-1,1-dimethyl-1*H*-benzo[e]indol-2(3*H*)-ylidene)prop-1-en-1-yl)-1,1,3-trimethyl-1*H*-benzo[e]indol-3-ium iodide



(E)-1,1,3-trimethyl-2-(2-(phenylamino)vinyl)-1H-benzo[*e*]indol-3-ium iodide (1 g, 2.2 mmol) and 3-(5-carboxypentyl)-1,1,2-trimethyl-1H- benzo[e]indol-3-ium iodide (1.22 g, 2.64 mmol) were suspended in pyridine (10 mL). Ac_2O (2 mL) was added and the mixture stirred at 50°C under Argon. After 19 h, the mixture was cooled to RT and precipitated into Et_2O. the solid was purified by column chromatography (10% MeOH in DCM) to yield the product as a purple solid.

Yield: 208 mg (15%)

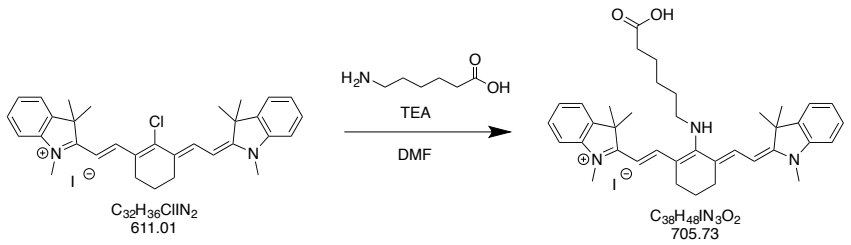
^{1}**H NMR** (400 MHz, $CDCl_3$) δ 8.58 - 8.74 (2H, m), 8.12 (3H, d, J = 8.5 Hz), 7.95 (6H, br d, J = 8.6 Hz), 7.59 - 7.67 (3H, m), 7.38 - 7.56 (7H, m), 7.17 - 7.33 (2H, m), 4.34 (2H, br t, J = 7.6 Hz), 3.93 (4H, s), 2.46 (2H, br t, J = 6.7 Hz), 2.05 (15H, d, J = 1.2 Hz), 1.66 - 1.83 (5H, m)

^{13}**C NMR** (100 MHz, $CDCl_3$) δ 177.5, 175.6, 175.0, 149.2, 140.1, 139.5, 133.3, 133.2, 131.9, 130.9, 130.8, 130.2, 128.1, 128.0, 127.9, 125.1, 121.9, 110.8, 103.8, 50.7, 50.6, 44.8, 34.1, 33.2, 27.8, 27.8, 27.5, 26.2, 24.5

LRMS (ESI, MeOH) m/z= 557.3 $[M]^+$

The above analytical data are in agreement with the literature[254]

2.5.1.15 Symmetrical Cy7COOH



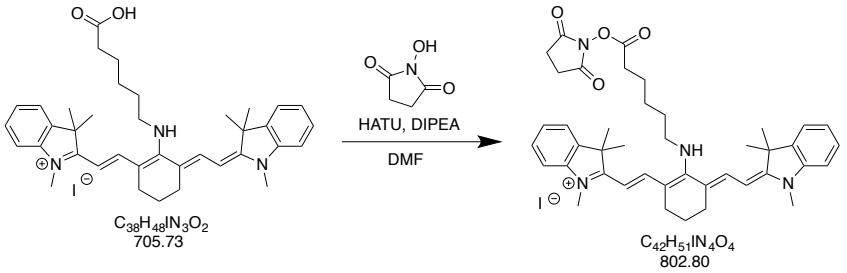
The symmetrical dye compound (137 mg, 283 μmol) was dissolved in DMF (10 mL) and TEA (198 μL, 1.42 mmol) was added. The mixture was heated to 85° C for 3 h and then cooled and the solvent was removed *in vacuo*. The mixture was purified by column chromatography (10 to 100% MeOH in DCM) to give the product as an iridescent blue solid.

Yield: 65 mg (92 μmol, 33%)

^{1}**H NMR** (400 MHz, d6-Acetone) δ 7.92 - 8.09 (1H, m), 7.73 - 7.89 (2H, m), 7.35 - 7.47 (2H, m), 7.23 - 7.34 (2H, m), 6.98 - 7.13 (4H, m), 5.62 - 5.93 (2H, m), 3.87 (2H, br t, J = 6.1 Hz), 3.41 - 3.57 (6H, m), 2.57 (4H, s), 2.23 - 2.38 (2H, m), 1.88 - 1.98 (2H, m), 1.77 - 1.87 (2H, m), 1.65 (15H, s), 1.43 - 1.56 (2H, m)

LRMS (ESI) m/z= 578 $[M]^+$

2.5.1.16 Symmetrical Cy7CONHS



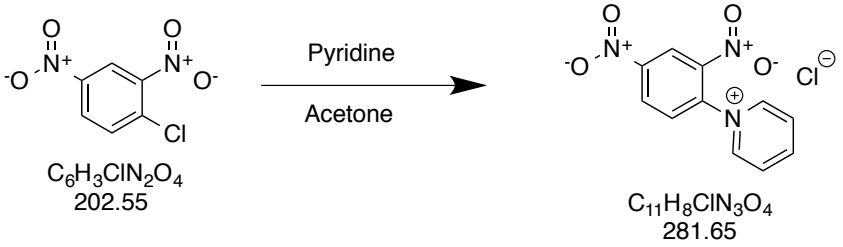
Symmetrical Cy7COOH (65 mg, 92 μmol) was dissolved in DMF (2 mL). HATU (58 mg, 100 μmol), DIPEA (50 μL, 300 μmol) and NHS (11.5 mg, 100 μmol) were added and the mixture allowed to react overnight. The following morning, all was dumped into DCM (10 mL) and washed with water (2 x 10 mL) and brine (10 mL). The organics were dried (Na_2SO_4) and the solvent removed *in vacuo* to give the product as a blue iridescent solid.

Yield: 74 mg (9.2 μmol, quant)

^{1}H NMR (400 MHz, d6-Acetone) δ 7.96 (1H, s), 7.86 (3H, br d, J = 13.0 Hz), 7.42 (2H, d, J = 7.2 Hz), 7.26 - 7.36 (2H, m), 7.04 - 7.16 (4H, m), 5.86 (2H, br d, J = 13.1 Hz), 3.52 (5H, s), 2.94 (4H, s), 2.80 - 2.90 (8H, m), 2.63 - 2.71 (2H, m), 2.53 - 2.61 (4H, m), 1.96 (2H, dt, J = 14.5 Hz, J = 7.2 Hz), 1.75 - 1.89 (4H, m), 1.56 - 1.74 (14H, m), 1.40 - 1.53 (2H, m)

LRMS (ESI) m/z= 675 $[M]^+$

2.5.1.17 Dinitrophenylpyridinium chloride



Pyridine (1 eq., 3.00 g, 3.07 mL, 37.9 mmol) was added to acetone (17 mL) with 2,4-dinitrochlorobenzene (1 eq., 7.67 g, 37.9 mmol) and the mixture was bought to reflux overnight. The following morning the solid mass was broken up with a spatula, cooled to RT and filtered, the retentate was washed with hexane and dried under high vacuum.

Yield: 9.82 g (34.87 mmol, 92%)

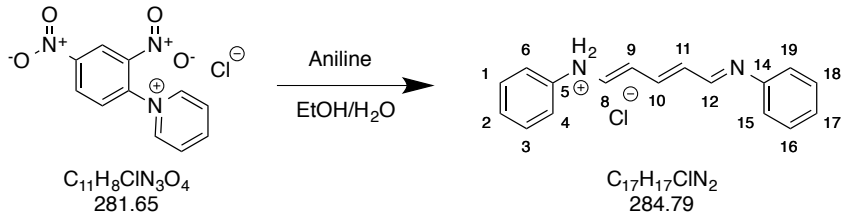
^{1}**H NMR** (400 MHz, d6-DMSO) δ 9.45 (2H, d, J = 6.0 Hz), 9.12 (1H, d, J = 2.4 Hz), 8.96 - 9.01 (1H, m), 8.87 - 9.04 (1H, m), 8.48 (1H, d, J = 8.4 Hz), 8.37 - 8.53 (2H, m)

^{13}**C NMR** (100 MHz, d6-DMSO) δ 149.6, 149.3, 146.6, 143.6, 139.2, 132.4, 130.7, 128.5, 121.9

LRMS (ESI) m/z= 246 $[M]^{+}$

The above analytical data are in agreement with the literature[255]

2.5.1.18 Glutacodianil



Aniline (2.11 eq., 3.9 g, 3.82 mL, 41.9 mmol) in a mixture of ethanol (37.6 mL) and water (10 mL) was added dropwise to a solution of 1-(2,4-dinitrophenyl)-1-pyridin-1-ylium chloride (1 eq., 5.6 g, 19.9 mmol) in ethanol (37.6 mL) and water (10 mL). After 20 min, red crystals of product were filtered from the flask and every 10 min thereafter the mix was filtered until yellow crystals began to separate, which were discarded.

Yield: 2.132 g (4.786 mmol, 38%)

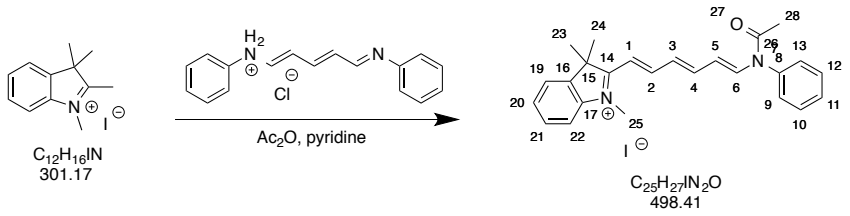
^{1}**H NMR** (400 MHz, d6-DMSO) δ 8.60 (2H, br d, J = 10.8 Hz, H-8, 12), 7.84 (1H, br t, J = 13.0 Hz, H-10), 7.40 - 7.53 (10H, m), 6.39 (2H, br t, J = 12.3 Hz, H-11)

^{13}**C NMR** (100 MHz, d6-DMSO) δ 155.9 (C-8, 12), 150.3 (C-1, 14), 130.2, 129.8, 118.0, 109.9 (C-11, 9)

LRMS (ESI) m/z= 249 $[M]^{+}$

The above analytical data are in agreement with the literature[256]

2.5.1.19 Cy7 Hemicyanine N-Ac



1,2,3,3-tetramethyl-3*H*-indol-1-ium iodide (1.0 g, 3.32 mmol) and N-[(1E,3E,5E)-5-(phenylimino)penta-1,3-dien-1-yl]anilinium chloride (0.9 g, 3.16 mmol) were dissolved in Ac_2O (13 g, 12 mL, 127 mmol) and AcCl (1.1 g, 1 mL, 14 mmol) was added. The mixture was heated to 120°C for 30 min under argon and then the solvent was removed *in vacuo*. The residue was taken up in DCM (7 mL) and precipitated into vigorously stirring Et_2O (200 mL). The produced solid was filtered off and purified on a Biotage Isolera 4 instrument using a gradient elution of methanol in DCM to give the product.

Yield: 1.0 g (2.01 mmol, 64%)

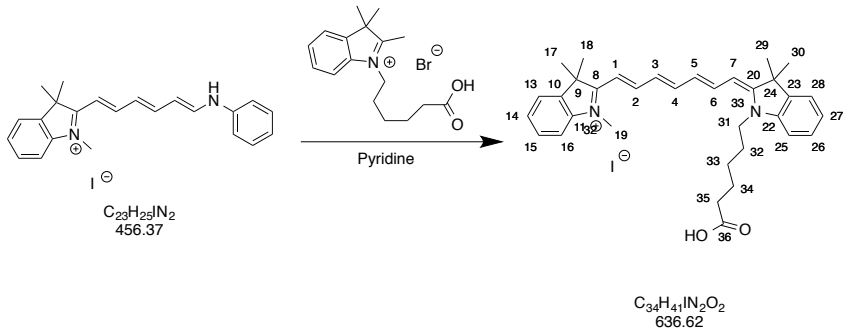
^{1}**HNMR** (400 MHz, $CDCl_3$) δ 8.17 (1H, d, J = 13.7 Hz, H-6), 7.90 (1H, dd, J = 14.9 Hz, J = 11.4 Hz, H-2), 7.43 - 7.61 (7H, m, H-1), 7.24 - 7.32 (1H, m, H-4), 7.17 (2H, dd, J = 8.0 Hz, J = 1.3 Hz), 6.85 (1H, dd, J = 14.2 Hz, J = 11.4 Hz, H-3), 5.38 (1H, dd, J = 13.7 Hz, J = 11.5 Hz, H-5), 4.20 (3H, s, H-25), 1.97 (3H, s, H-28), 1.73 (6H, s, H-24, 23)

^{13}**C NMR** (100 MHz, $CDCl_3$) δ 80.6 (C-14), 169.5 (C-27), 156.0 (C-2), 151.7 (C-4), 142.6 (C-16), 141.2 (C-6), 130.1 (C-3), 130.0, 128.1, 122.4, 113.9 (C-1), 113.6 (C-5), 51.5 (C-15), 36.1 (C-25), 27.2 (C-24, 23), 23.4 (C-28)

LRMS (ESI) m/z= 371 $[M]^+$

The above analytical data are in agreement with the literature[257]

2.5.1.20 Cy7 COOH



1,3,3-trimethyl-2-[(1E,3E,5E)-6-(N-phenylacetamido)hexa-1,3,5-trien-1-yl]-3H-indol-1-ium iodide (1 eq., 175 mg, 0.351 mmol) was added to a solution of 1-(5-carboxypentyl)-2,3,3-trimethyl-3H-indol-1-ium bromide (1 eq., 124 mg, 0.351 mmol) in pyridine (3.73 mL) and all was heated to 50°C for 1 h. The green solution was allowed to cool to RT and precipitated into Et_2O (100 mL). The mixture was filtered and the green, iridescent solid was purified using a Biotage Isolera 4 with an EtOAc flush, followed by a 0-10% MeOH in DCM gradient.

Yield: 120 mg (189 μmol, 54%)

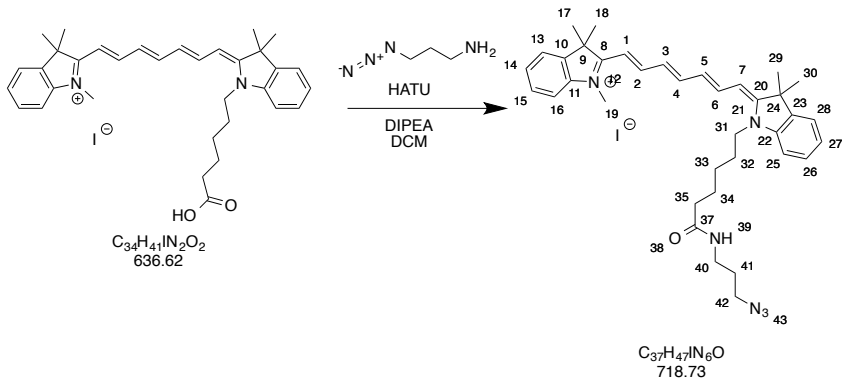
^{1}**H NMR** (400 MHz, $CDCl_3$) δ 7.77 - 7.92 (2H, m, H-2, 6), 7.60 - 7.71 (1H, m, H-4), 7.29 - 7.35 (2H, m), 7.29 - 7.39 (3H, m), 7.15 - 7.22 (2H, m), 7.06 - 7.13 (2H, m), 6.67 (2H, br t, J = 11.5 Hz, H-3, 5), 4.04 (2H, br t, J = 6.6 Hz, H-7, 1), 4.04 (2H, br t, J = 6.6 Hz, H-31), 3.64 (3H, s, H-19), 2.43 (2H, t, J = 7.2 Hz, H-35), 1.78 - 1.88 (2H, m, H-32), 1.69 - 1.78 (3H, m, H-34), 1.67 (12H, s, H-18, 17, 29, 30), 1.50 - 1.61 (2H, m, H-33)

^{13}**C NMR** (100 MHz, $CDCl_3$) δ 177.3 (C-36), 171.7 (C-20), 156.7 (C-4), 151.4, 151.3, 142.8, 142.1, 140.7, 128.5, 126.5, 126.4, 124.8, 122.2, 122.1, 110.3, 103.7, 49.0 (C-24), 48.9 (C-9), 44.2 (C-31), 33.9 (C-35), 32.1 (C-19), 27.9 (C-18, 17, 29, 30), 27.0 (C-32), 26.2 (C-33), 24.3 (C-34)

LRMS (ESI) m/z= 510 $[M]^+$

The above analytical data are in agreement with the literature[257]

2.5.1.21 Cy7 Azide



2-[(1E,3E,5E)-7-[(2E)-1-(5-carboxypentyl)-3,3-dimethyl-2,3-dihydro-1H-indol-2-ylidene]hepta-1,3,5-trien-1-yl]-1,3,3-trimethyl-3H-indol-1-ium iodide (1 eq., 120 mg, 0.188 mmol) was dissolved in DCM (2 mL) and HATU (1.2 eq., 86 mg, 0.226 mmol) and DIPEA (2 eq., 48.7 mg, 0.0623 mL, 0.377 mmol) were added. The mixture was allowed to stir for 20 min under argon before a solution of 3-azidopropylamine (2 eq., 37.7 mg, 0.377 mmol) in DCM (1 mL) was added. After 40 min, TLC showed completion of the reaction. The mixture was washed once with 1M HCl (20 mL) and concentrated *in vacuo*, before the residue was purified on a Biotage Isolera 4 using a 0-20% gradient of MeOH in DCM.

Yield: 127 mg (176.7 μmol, 94%)

^{1}**H NMR** (400 MHz, $CDCl_3$) δ 7.76 - 7.84 (1H, m, H-2), 7.69 - 7.88 (1H, m), 7.45 - 7.60 (1H, m, H-4), 7.29 - 7.42 (5H, m), 7.15 - 7.25 (1H, m), 7.15 - 7.25 (1H, m), 7.15 - 7.25 (1H, m), 7.00 - 7.11 (2H, m), 6.63 (1H, br t, J = 12.2 Hz, H-3), 6.44 - 6.55 (1H, m, H-5), 6.26 (1H, br t, J = 5.5 Hz, H-39), 6.17 (1H, br d, J = 13.7 Hz, H-6, 7), 6.02 (1H, br d, J = 13.8 Hz, H-1), 3.98 (2H, br t, J = 7.5 Hz, H-31), 3.52 (3H, s, H-19), 3.36 (2H, s, H-42), 3.29 - 3.34 (2H, m, H-40), 2.26 (2H, t, J = 7.3 Hz, H-35), 1.76 - 1.86 (4H, m, H-41, 32), 1.61 - 1.75 (18H, m, H-29, 30, 17, 18, 34), 1.44 - 1.56 (2H, m, H-33)

^{13}**C NMR** (100 MHz, $CDCl_3$) δ 173.4 (C-37), 142.9, 142.0, 141.1, 140.7, 128.8, 128.6, 125.3, 124.6, 122.2, 122.1, 110.7, 109.8, 49.3, 49.3 (C-42), 48.8, 44.4 (C-31), 38.6, 36.9

(C-40), 36.1 (C-35), 31.0 (C-19), 28.8 (C-32), 27.8, 27.8 (C-29, 30, 17, 18), 27.7, 27.1 (C-41), 26.4 (C-33), 25.2 (C-34)

LRMS (ESI) m/z= 591 $[M]^+$

Novel compound, no data available

2.5.2 Oligonucleotide Synthesis

The LNA gapmer used for immunisation was synthesized on disulfide CPG (Chemgenes N-9987-10) using an AKTA Oligopilot 10 (GE Life Sciences) at 10 µmol scale using 0.1M DDTT (Chemgenes RN-1689) as the sulfurising reagent and BTT (AIC AB3953) as activator. The oligonucleotide was deprotected using concentrated ammonia at 55 for 18 h and purified using ion-exchange chromatography (eluting with 30% acetonitrile in water containing increasing gradients of $NaClO_4$.) The purified oligonucleotide was desalted with Sephadex G25 and concentrated.

All other oligonucleotides were synthesized on ABI 394 or Dr. Oligo 48 oligonucleotide synthesizers using standard methods. Biotin CPG (Chemgenes N-9162-10, for the biotinylated oligonucleotides) was used at 1 µmol scale with 10 minute coupling times for the modified phosphoramidites and 1 min coupling times for standard DNA, or on a Dr. Oligo 48 synthesiser using Biotin CPG (Chemgenes N-9162-10) at 200 nmol scale with a custom synthesis cycle.

2.5.3 Fluorophore-Conjugated Oligonucleotides

Following the formula:

$$\text{NHS ester mass (mg)} = \frac{8 \times \text{amino compound mass (mg)} \times \text{NHS ester Mr}}{\text{Amino compound Mr}}$$

Oligonucleotide was solubilised in 0.1 M $NaHCO_3$ solution, while the fluorophore was solubilised in 10% of the oligonucleotide solution volume of DMSO. The fluorophore solution was added to the oligonucleotide solution and incubated at 4°C overnight before passing through a NAP-25 sephadex column. The resulting conjugated oligonucleotide solution was evaporated to dryness in a speedvac and purified by RP-HPLC.

2.5.4 Preparation of the Immunogenic Protein Conjugate

Lyophilized disulphide-containing oligonucleotide **ASO 82- Disulphide** (5.5 mg) was treated with 50 mM aqueous DTT containing 2% TEA (500 µL) at RT for 30 min. Reduction was monitored by LCMS to ensure full conversion of the disulphide to the thiol. The resulting solution was desalted using 2 separate NAP-25 (GE Life Sciences) columns, then immediately frozen and lyophilised.

The lyophilised thiol-oligonucleotide was dissolved in 400 μL of conjugation buffer (pH 7.4 PBS) and added to a suspension of mc-KLH (2.2 mg) in water (200 μL) resulting in the formation of a turbid solution. The reaction was continued at RT for 2 hours before centrifugation. The supernatant was passed through a desalting column (and quantitated using the absorbance at 260 nm (intrinsic to the oligonucleotide and as opposed to the intrinsic protein A280, which was dwarfed by the oligonucleotide absorbance). The conjugate was stored in 40-μL aliquots in Protein LoBind tubes (Eppendorf) at -80°C until use.

2.5.5 Animal Work

All animal work was undertaken in accordance with the University of Massachusetts Medical School Institutional Animal Care and Use Committee (protocols 2591 (Rabbit) and 2551 (Rodent)). Institutional Biosafety Committee approval was also granted under protocol I-656-16.

2.5.5.1 Rabbit Work

Two female New Zealand White rabbits were provided as a kind gift from Charles River laboratories and were maintained on grain with *ad libitum* hay and water (details of light dark cycle, enrichment). Following arrival, the rabbits were acclimatised to gentle restraint and to the investigator for 2 weeks before any procedures were undertaken.

Rabbits were sedated with an intramuscular (epaxial muscles) injection of acepromazine maleate (1 mg/kg) or midazolam (1 mg) and were given topical lidocaine/prilocaine cream (EMLA) over the central auricular artery 30 minutes before blood collection. An initial pre-immune bleed (7 mL) was obtained from each rabbit to provide background sera for ELISA titration. Immunisations were given by subcutaneous injection at four sites across each animal's back (250 μL each). All blood obtained was from the central auricular artery into BD SST tubes and allowed to clot at RT for 30 minutes before serum separation (2000 RPM for 15 minutes at 4°C). Serum, including antiserum, was stored in Protein LoBind tubes (Eppendorf) at -80°C.

2.5.5.2 Mouse Work

Female FVB/NCr mice were obtained from Charles River Laboratories at 8 weeks of age and were maintained on standard rodent chow and acidified water *ad libitum*. Sodium pentobarbital (>800 mg/kg as Fatal Plus) was administered intraperitoneally as a dilution in saline for terminal anaesthesia. Oligonucleotides were administered intravenously using the lateral tail vein, or subcutaneously over the flank as dilutions in saline.

2.5.6 Live Animal Imaging

Treated animals were anaesthetised with isoflurane and imaged using an IVIS live-animal imaging system set to examine with light of wavelength 750 nm.

2.5.7 Immunofluorescence and Immunohistochemistry

Terminally anaesthetised mice were perfused transcardially with PBS (pH 7.4) at 4 °C until the perfusate was clear and the liver was completely blanched. Following PBS perfusion, 10% NBF (Sigma) was perfused until fixation was complete (complete muscular

rigidity and cessation of twitching). Tissues of interest were excised and post-fixed in NBF at 4 °C for at least 24 h.

2.5.7.1 IF-F

Tissues were removed from formalin and cryo-protected in 30% sucrose until the tissues sank, embedded in OCT media, cryo-sectioned to 20 μm and mounted onto charged slides (UMMS Morphology Core). The slides were hydrated in PBS (5 min) before application of Cyto-Q Background Buster (Innovex Biosciences) for 30 minutes. After washing twice, the diluted antisera (1:500-1:10,000 in 2% BSA 10% normal goat serum in PBS with 0.025% Triton X-100) was applied. The slides were incubated with diluted antisera overnight at 4 °C, washed 3 times for 5 min each with PBS, AF488 goat anti-rabbit IgG (CST) at 1:500 in PBS containing 2% BSA, 10% normal goat serum and 0.025% Triton X-100 for 1 h at RT. The slides were then washed 3 times for 5 min each with PBS. Sections were covered with SignalStain Boost with DAPI and coverslipped with Leica Surgipath glass slides.

2.5.7.2 FFPE-IF

Once the tissue was removed from NBF, it was placed in a cassette, paraffin embedded and sectioned to 4 µm before mounting to charged slide and dewaxing at 60 °C for 1 h. Slides were then deparaffinized and rehydrated (two washes in xylene, two washes in 100% ethanol, two washes in 95% ethanol, one wash in water). Endogenous peroxidases were quenched by treating in 3% H_2O_2 in methanol for 10 min followed by two washes in TBST. Sections were treated with Proteinase K (Dako) for 10 min (Dako) then washed twice with TBST. Sections were treated with Cyto-Q Background Buster (Innovex Biosciences) then washed twice. The diluted antisera was applied (1:500 in 2% BSA 5% normal goat serum in TBST). The slides were incubated with antisera overnight at 4 °C, washed 3 times for 5 min each with TBST, AF488 goat anti-rabbit IgG (CST) at 1:500 in 2% BSA

5% normal goat serum in TBST For 1 h at room temp. The slides were then washed 3 times for 5 min each with TBST. Sections were covered with SignalStain Boost with DAPI and coverslipped with Leica Surgipath glass slides.

2.5.7.3 FFPE-IHC

Slides were deparaffinized and rehydrated (two washes in xylene, two washes in 100% ethanol, two washes in 95% ethanol, one wash in water). Endogenous peroxidases were quenched by treating in 3% H_2O_2 in methanol for 10 min followed by two washes in TBST. Sections were treated with Proteinase K for 10 min (Dako) then washed twice with TBST. Sections were treated with Cyto-Q Background Buster (Innovex Biosciences) then washed twice. The diluted antisera was applied (1:10,000 in 2% BSA 5% normal donkey serum in TBST). The slides were incubated with antisera overnight at 4 °C, washed 3 times for 5 min each with TBST and covered in SignalStain Boost IHC Detection Reagent (CST) for 30 min at RT, slides were washed 3 times (TBST) and SignalStain DAB reagent was applied. After staining was sufficient (approximately 10 min at RT), the slides were washed in water and counterstained with haematoxylin before being covered with SignalStain Boost and coverslipped with Leica Surgipath glass slides.

2.5.7.4 Microscopy

Fluorescent images were obtained with a Leica DMi8 inverted fluorescence microscope equipped with a DAPI, GFP, Y3 and Y5 filter cube and HC PL FLUOTAR 5x/0.15, HC PL FL 10x/0.3 PH1, HC PLAN APO 20x/0.7 PH2, HC PL FL 20x/0.4 CORR PH1, HCX PL FL L 40x/0.6 CORR PH2, HCX PL APO 40x/1.3 OIL, HCX PL APO 63x/1.4-0.6 OIL objectives.

2.5.8 ELISA Titrations

In order to assess the production of antibodies, we utilised an ELISA approach. EIA plates were coated with neutravidin (100 μL of a 2 μg/mL solution per well) overnight at 4 °C. Plates were washed 3x (PBST) and stored for 1-30 days in blocking solution (1% BSA, 0.05% Tween 20 and 0.1% normal goat serum in PBS).The blocking solution was removed and the plates washed 3x (PBST) before being coated with hapten by the addition of 100 uL/well of 10 nM biotinylated oligonucleotide (i.e., 1 pmol oligonucleotide/well). The biotin/neutravidin complex was allowed to form for 1 h at RT before the plates were washed 3x. Antiserum was serially diluted (in blocking solution) in technical duplicate across the plate and allowed to bind at RT for 1 h. Plates washed 3x with PBST. Anti-rabbit HRP secondary antibody (CST, diluted 1:10,000 in blocking solution) was then allowed to bind for 1 h at RT and the plates were washed 3x. Finally, ELISA Pico-Substrate (Thermofisher) was applied and the luminescence was quantitated using a Veritas Luminometer (0.5 s integration). Data was analysed using Graphpad Prism 7.

Chapter 3

Dendrimeric Antisense Oligonucleotides: Synthesis and Biological Properties

3.1 Declaration of Work Done

I synthesised all of the small molecules and oligonucleotides. The tissue culture work was a joint effort between Pranathi Meda and myself: Pranathi was responsible for plating cells for the microscopy work and transfecting the cells for the silencing experiments while I did all of the *in vitro* microscopy, staining, image processing, silencing assays and data processing. I conducted all of the animal work and subsequent assays (microscopic and silencing).

3.2 Introduction

As has been described in Section 1.6, multimeric oligonucleotides demonstrate enhanced stability in serum, enhanced endocytosis and improved ability to silence target genes. While dimeric, trimeric, nonameric and polymeric structures had been synthesised using one chemistry or another; there had never been a well-controlled *in vitro* or *in vivo* comparison between a well-defined and systematic series of multimeric oligonucleotides.

This work was intended to explore the effect of size/valency, shape, polarity and arm 'density' on silencing, immunogenicity and biodistribution in order to determine whether there is an optimum size for any specific function. Reflecting on the uptake and distribution properties of SNAs and other multimeric structures, this work was intended to explore whether these properties could be recapitulated using smaller structures and a more accessible synthetic methodology.

3.2.1 Post-synthetic Dendrimer Assembly

As discussed broadly in Section 1.6.2, the post-synthetic approach to oligonucleotide assembly was our first attempted route. In this approach, a small molecule bearing several reactive functionalities is transformed to contain some number of click-amenable functionalities that are in turn reacted with oligonucleotides bearing the other click moiety to give a covalently bound multimer. Work surrounding this area, particularly concerning carbohydrate starting materials, was the topic of my Masters thesis (Fig. 3.1).[258]

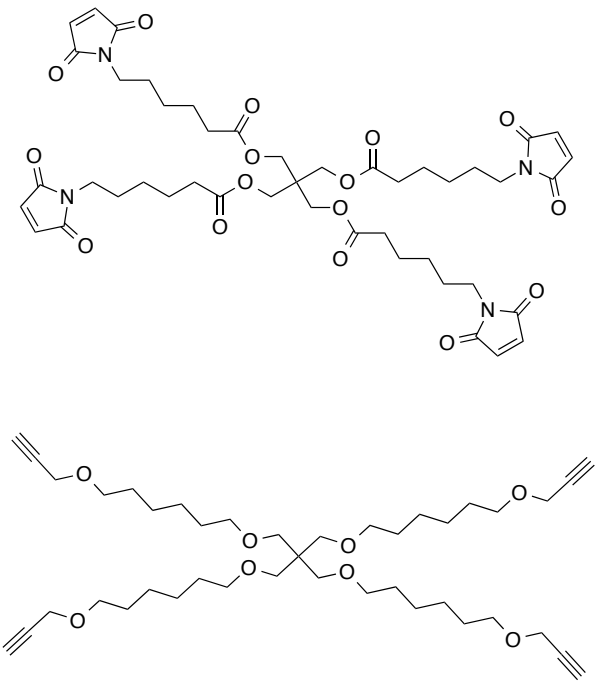
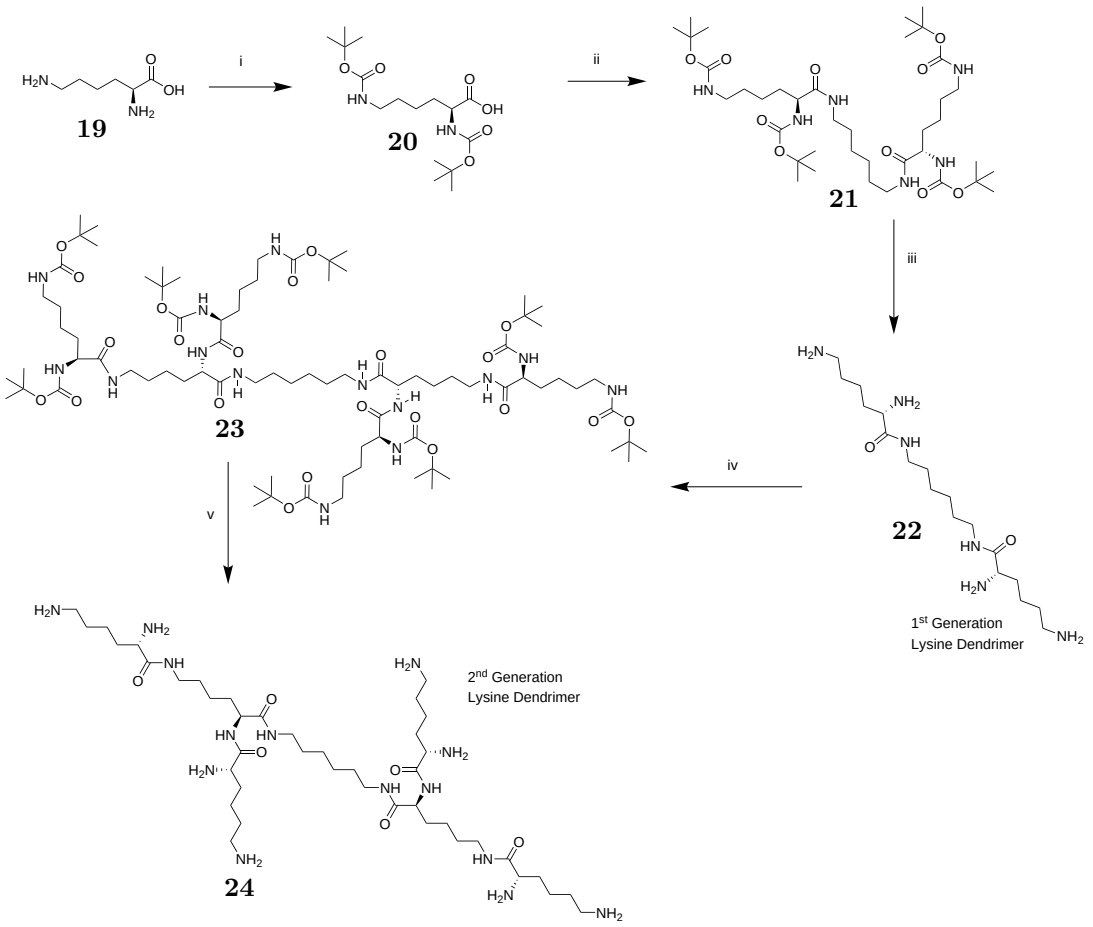


Figure 3.1: Two of the target pentaerythritol cores from previous group work.

3.2.1.1 Lysine Dendrimers

Continuing the post-synthetic approach lysine dendrimers were chosen as scaffolds, owing to their ease of synthesis and the presence of their analytical information in the literature. In brief, **19** is protected with Boc_2O to give **20**. **20** is reacted with hexanediamine to give the protected dendrimer **21**. This can be deprotected to give **22** which can be subjected to the same reaction with **20**, yielding **23**. The benefit of working with this type of dendrimer is that the reactions are all facile, high-yielding amide bond formation reactions or facile deprotections. Yields of dendrimers and of the Boc-protected dendrimers were not generally quantified owing to the intensely hygroscopic nature of the compounds. **22** and **24** were deliquescent, which would pose storage problems should the dendrimers be considered for further exploration. Both LC-MS and NMR data was obtained for all of the compounds, indicating that conversion was complete and that products were obtained in pure form. Of most interest to this project were generation 1 or 2 dendrimers, bearing 4 (**22**) or 8 (**24**) free amine groups respectively (Scheme 3.1).



Scheme 3.1: Synthesis of generation 1 and 2 lysine dendrimers
i) Boc_2O, $NaHCO_3$, THF/H_2O **67%** ii) 1,6-hexanediamine, HATU, DIPEA, DCM iii) TFA iv) (Boc)-lys(Boc)-OH, HATU, DIPEA, DMF v) TFA, Et_3SiH

It was intended that these reactive amines could then be used to form an amide with maleimidohexanoic acid (Fig. 3.2), or its active ester derivatives (Fig. 3.3) forming either a tetra or octa-maleimido dendrimer. These compounds could in turn react under maleimide-thiol click conditions with a thiol-modified oligonucleotide to provide the desired oligonucleotide dendrimer.

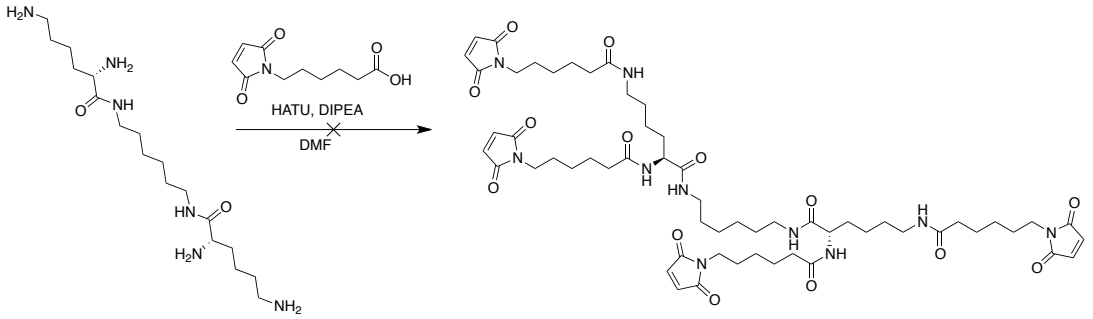


Figure 3.2: Attempted reaction of generation 1 dendrimer with MCA.

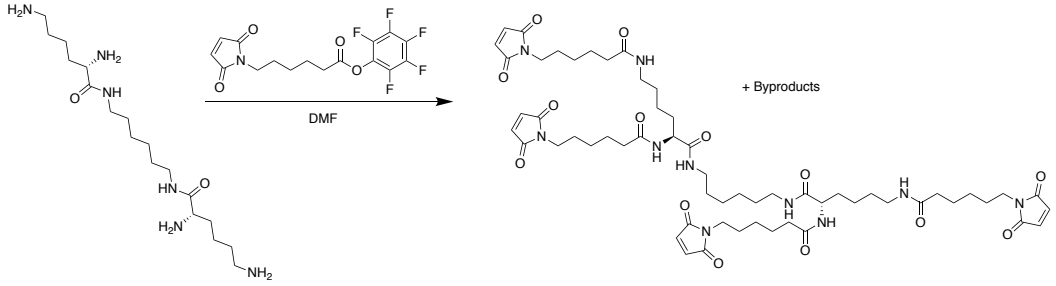


Figure 3.3: Attempted reaction of generation 1 dendrimer with MCAPFP.

The attempted reaction with HATU (Fig. 3.2) failed with complete recovery of starting material, while the reaction with the pre-prepared active ester seemed more promising (Fig. 3.3) from analysis LC-MS of the crude reaction mixture, however the pure product was not isolated from the reaction.

What was also evident from the analytical data (Figs. 3.4, 3.5, 3.6, 3.7) is that the reaction does not yield the tetra-maleimide selectively. Figures 3.5 and 3.6 show the mass spectra obtained for the tricoupled product, while figure 3.7 shows the mass spectra obtained for the intended tetracoupled product. These results may reflect the intrinsic nature of lysine dendrimers to have a mixture of primary and secondary amines; the primary amines are significantly less sterically hindered than their secondary counterparts and hence are more able to react with the active ester leading to a mixture of products.

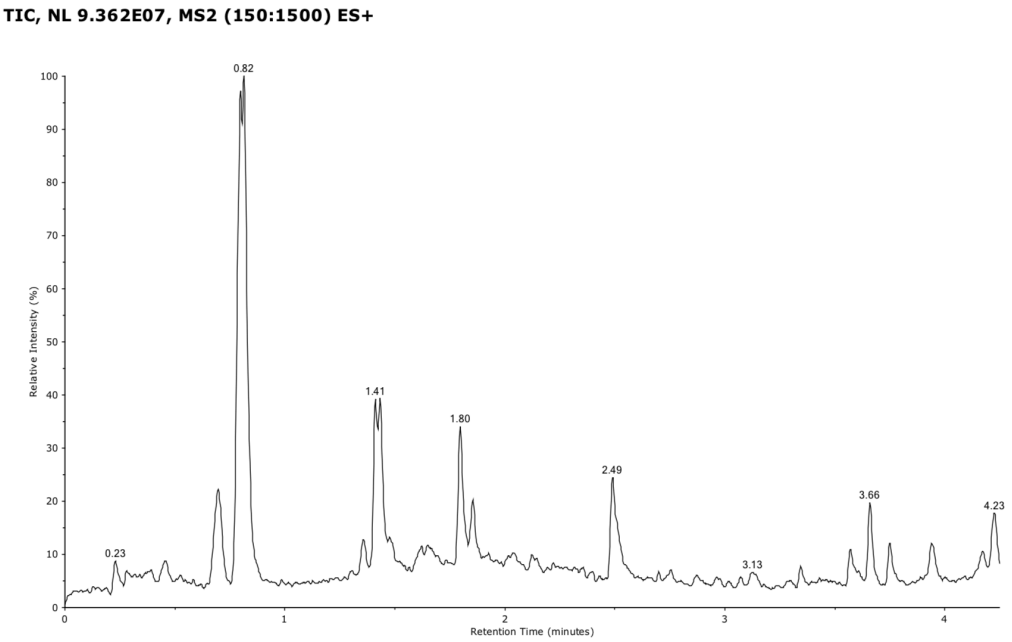


Figure 3.4: LC data of the reaction between MCAPFP and generation 1 lysine dendrimer.

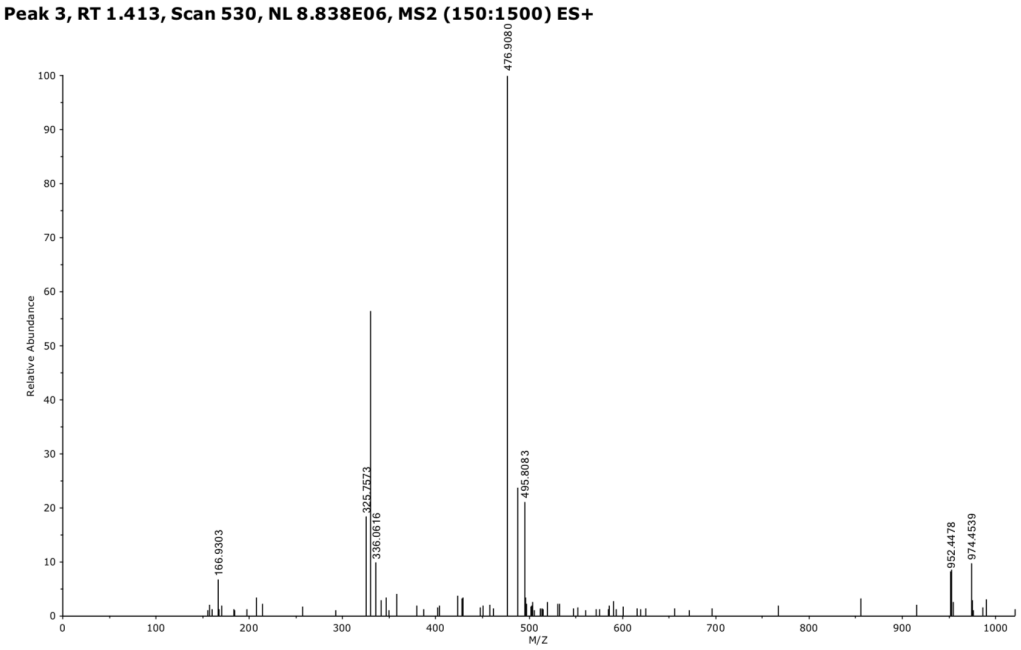


Figure 3.5: MS data for peak with rt= 1.413 min

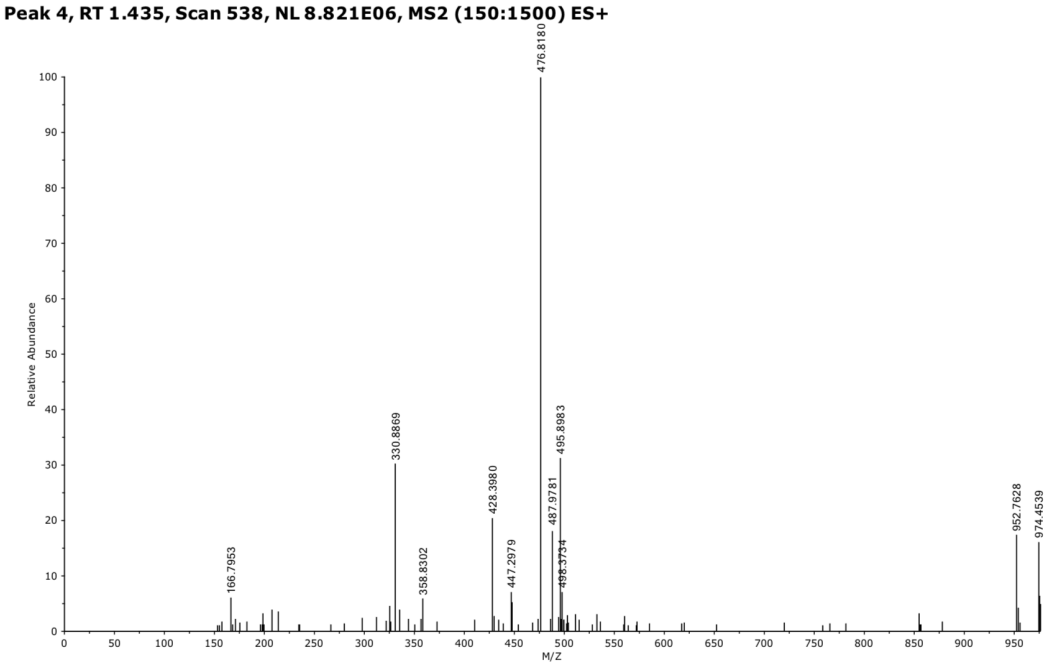


Figure 3.6: MS data for peak with rt= 1.435 min

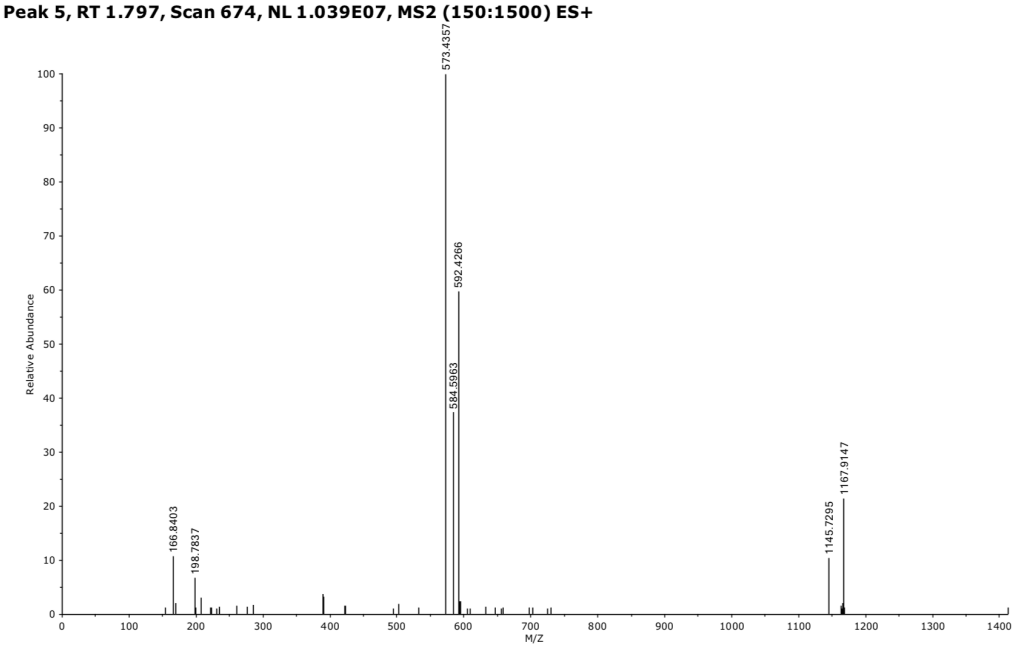


Figure 3.7: MS data for peak with rt= 1.797 min

3.2.2 Solid Phase Dendrimers – Convergent Approach

Convergent synthesis, as discussed in section 1.6.1.1, is another technique to synthesise multimeric oligonucleotides. In contrast to the post-synthetic approach described in section 3.2.1, convergent synthesis takes place on the solid support and requires a bis-phosphoramidite reagent.

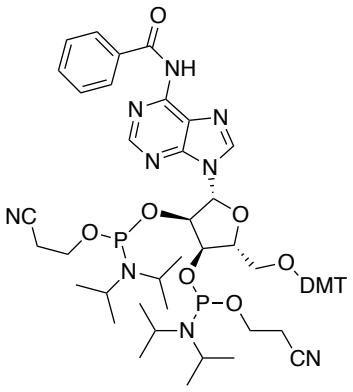


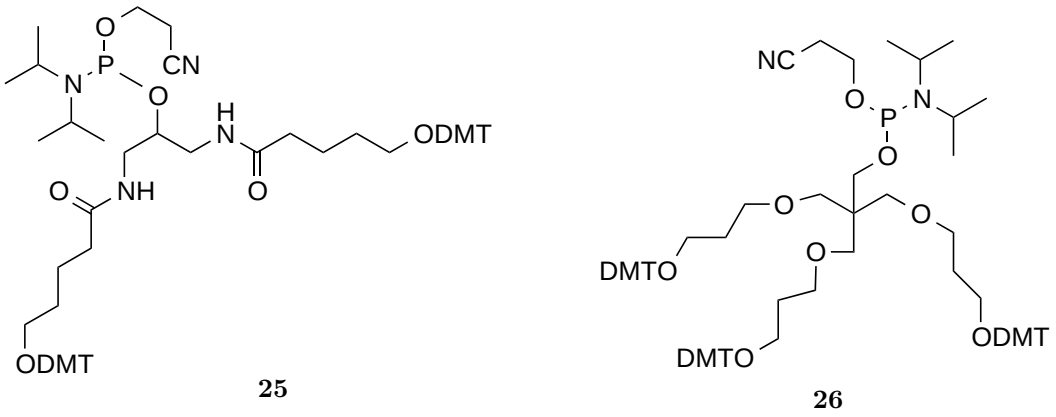
Figure 3.8: Bisphosphoramidite used in Damha's original synthesis of convergent dendrimers

While this method of synthesis was attempted, it was abandoned the work as there was evidence of poor couplings in the initial attempts which hampered yields even when just preparing a poly thymidine oligonucleotide. There was also concern that the presence of an additional ribose residue (Fig. 3.8) at oligonucleotide junctures might perturb future silencing experiments. While it is easy to conceptualise abasic bisphosphroamidite reagents it was determined that it would be a wiser use of time to focus on the better-established divergent approach to dendrimer synthesis (section 3.2.3).

3.2.3 Solid Phase Dendrimers – Divergent Approach

Shchepinov has detailed the synthesis of several useful phosphoramidites that have been used to synthesise solid-phase oligonucleotide dendrimers in a divergent manner.[156–158] Shchepinov's approach requires that a *doubler* (**25**) or *trebler* (**26**) phosphoramidite be reacted with the solid support first, in as many iterations as are required to give a

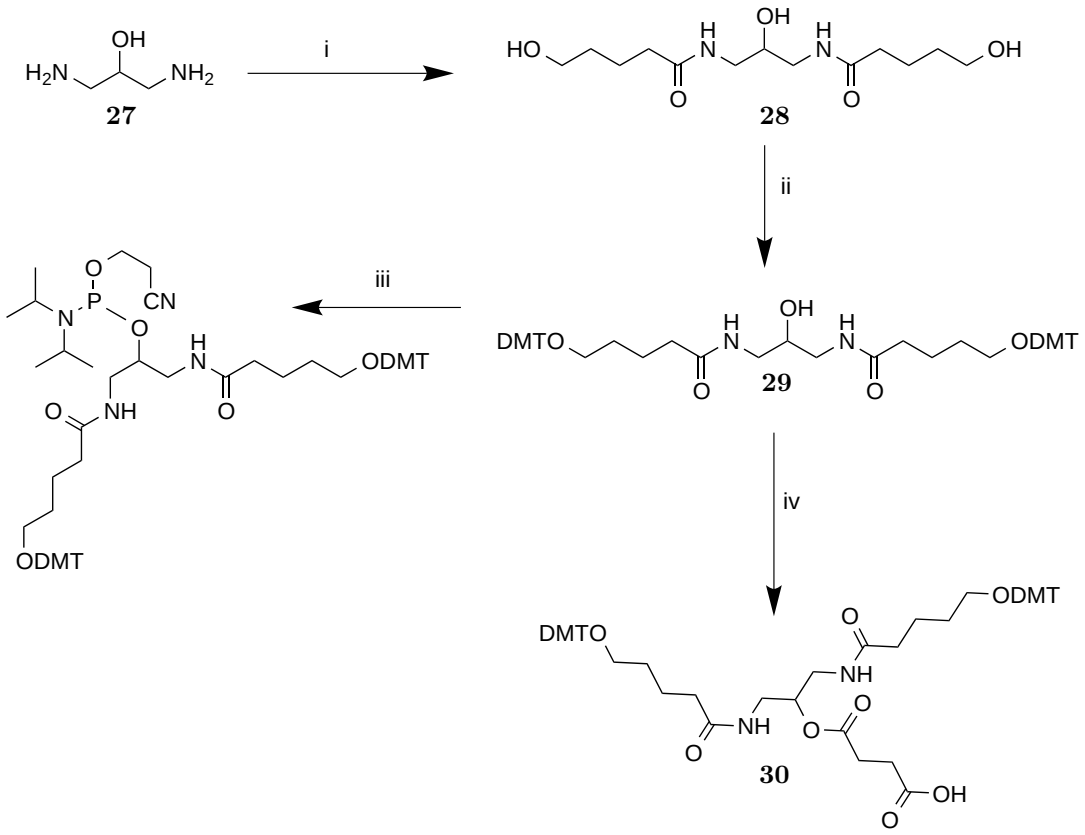
dendrimer with the desired number of arms. The desired sequence of nucleobases is then added using modified oligonucleotide synthesis conditions.



25 **26**

Scheme 3.2: Shchepinov's doubler (**25**) and trebler (**26**) phosphoramidites.

Shchepinov's phosphoramidites are commercially available, however they are expensive and the synthesis of **25** is straightforward (Scheme 3.3).[158] **27** is readily converted to **28**, however the bis-protection to give **29** occurred in a surprisingly poor yield. It may simply be an issue of steric hinderance, though the two reactive centres appear sufficiently remote from one another to mitigate these effects. Phosphitylation to give **25** was facile under standard conditions and succinylation occurred in excellent yield to give the modifier **30**.



Scheme 3.3: Synthesis of Shchepinov's doubler phosphoramidite and succinylated modifier i) δ-Valerolactone, DMAP, MeOH, overnight RT, **99%** ii) DMT-Cl, pyridine, overnight RT, **57%** iii) 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite, DIPEA, THF, **72%** iv) Succinic anhydride, DMAP, pyridine, 3 d RT, **95%**

In addition to the phosphoramdite, a succinylated version of the doubler (**30**) was prepared so that the doubler could be attached directly to CPG; this is beneficial because it allows for one fewer couplings in the synthesis cycle. Having a pre-attached doubler unit also precludes the use of Unylinker (Fig. 3.9). While Unylinker allows for the synthesis of oligonucleotides with atypical 3′ bases, its removal requires extended reaction times with hot ammonia and it is often preferable to avoid its use when possible.

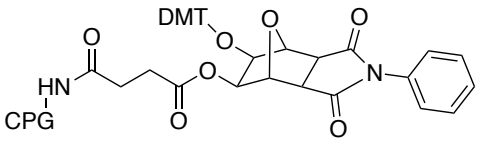


Figure 3.9: Unylinker modifier that allows for tag-free synthesis of oligonucleotides.

The preparation of **26** is more involved and was not pursued for this work as it was considered that having the 4, 8 and potentially 16-arm dendrimers available to us would be sufficient for this stage of the investigation.

It is worthy of note that Shchepinov recommends the attachment of a pentaethyleneglycol spacer before the coupling of subsequent doubler or trebler modifiers This is presumably to mitigate the deleterious effects of steric crowding that would invariably result from the growth of the multimeric oligonucleotide.[156]

3.2.4 Solid Phase Synthesis Experiments

In working to improve the synthesis of these structures, some consideration needed to be given to the conditions of their solid phase synthesis. In the published work, there are very limited details regarding the specific conditions of solid-phase synthesis. The majority of the work details the use of the trebler phosphoramidite and gives little information about the doubler phosphoramidite.[174] One might expect that an increased phosphoramidite coupling time could be required for sufficient yield owing to the ever increasing steric demands of the oligonucleotide dendrimer as it grows, and indeed this is alluded to in the literature.[156–158,174]

While a 2 min coupling time is recommended for use with the trebler phosphoramidite by Shchepinov, Glen Research suggests that this be extended to 15 min. Shchepinov recommends that the doubler phosphoramidite be used with 'extended coupling times',

again the vendor suggests a 15 min coupling time.[156] Shchepinov details that dendrimer growth is possible on 500 Å CPG, with high yields and further couplings possible on 1000Å CPG, while the vendor suggests only using 1000Å CPG.[158] With such disparate information available, a logical starting point was chosen expecting to have to fine-tune the conditions for synthesis of the molecules of interest.

1000 Å lcaa-CPG was modified with a low loading (15 μmol/g as opposed to a typical 40- 60 μmol/g) of **30** to give a solid support that should have diminished steric effects compared to typical CPG. This step was not reported in the literature, but seemed a logical starting point. Between each insertion of the doubler synthon was added a hexaethylene glycol (HEG) phosphoramidite spacer, again to decrease the steric effects. Both the doubler and HEG phosphoramidites were prepared at 0.1 M concentration in anhydrous ACN and were given extended coupling times of 10 min. It was decided to begin with making dendrimers with 2, 4 and 8 branches, requiring 1, 2 and 3 insertions of the doubler phosphoramidite respectively. The 2 arm dendrimer was prepared using 0.5 μmol of CPG, the 4 arm with 0.25 μmol of CPG and the 8 arm with 0.125 μmol of CPG. All were then subject to a 1 μmol synthesis cycle starting with serial couplings of the branching phosphoramidite.

Instead of using an active sequence for these initial trials, a poly-T 5-mer sequence was used instead to reduce any confounding base-dependent variables. The dendrimers were cleaved and phosphates deprotected using AMA at 65°C for 10 min, concentrated and re-suspended in water before desalting on a NAP-10 sephadex column and quantification using UV spectrophotometry. The yields were poor (c. 5%) and the analytical HPLC showed that what was isolated was a mixture of different molecules without a characteristic main product.

Predictably, adjustments were required to the process of synthesising dendrimeric oligonucleotides. There was choice and flexibility in the type of synthesiser, chemistry of the oligonucleotide, choice of doubling reagent, choice of solid support and choice of spacer inclusion.

3.2.4.1 Synthesiser

Not all oligonucleotide synthesisers function in the same way. While the same reactions occur in the same sequence, different models of synthesiser handle the integral coupling step in different ways. When the initial experiments were performed to synthesise dendrimers, the group was limited to using solely an ABI394 – an older synthesiser that pushes a set volume of activated phosphoramidite through the synthesis column and waits for a predetermined period of time for the coupling reaction to occur. This is a relatively poor method for ensuring phosphoramidite coupling because during the coupling step, the CPG sits in a volume of activated phosphoramidite solution much smaller than the total delivered volume and hence is exposed to fewer equivalents of the activated reagent.

When the group moved to the new laboratory in the USA, the group was able to purchase and use some different synthesisers. The first was a Dr. Oligo 48 open-bed synthesiser that handles the coupling step by dropping a pre-determined volume of activated phosphoramidite solution over CPG under an atmosphere of argon. It was felt that the yield would likely suffer from a similar issue to the ABI394 in that the coupling step is static (non-dynamic), thus no attempts were made to make dendrimers using this synthesiser.

An AKTA Oligopilot synthesiser was also available for use. This uses a unique recirculating protocol during the coupling step that keeps activated phosphoramidite solution flowing over the solid support during the entirety of the coupling step allowing for constant exposure to fresh reagent and diminished phosphoramidite equivalents. It was felt that this would be particularly useful in ensuring that every coupling step was as efficient as possible, especially so once the branching phosphoramdites were in place. Hence all further synthetic protocols were developed using this instrument. The additional significant benefit to the AKTA Oligopilot is its ability to synthesise at a much larger scale than the other synthesisers (up to 400 µmol). With the additional

synthesis capacity a diminished yield would still allow sufficient oligonucleotide to purified to complete the desired *in vivo* experiments.

3.2.4.2 Modification Chemistry

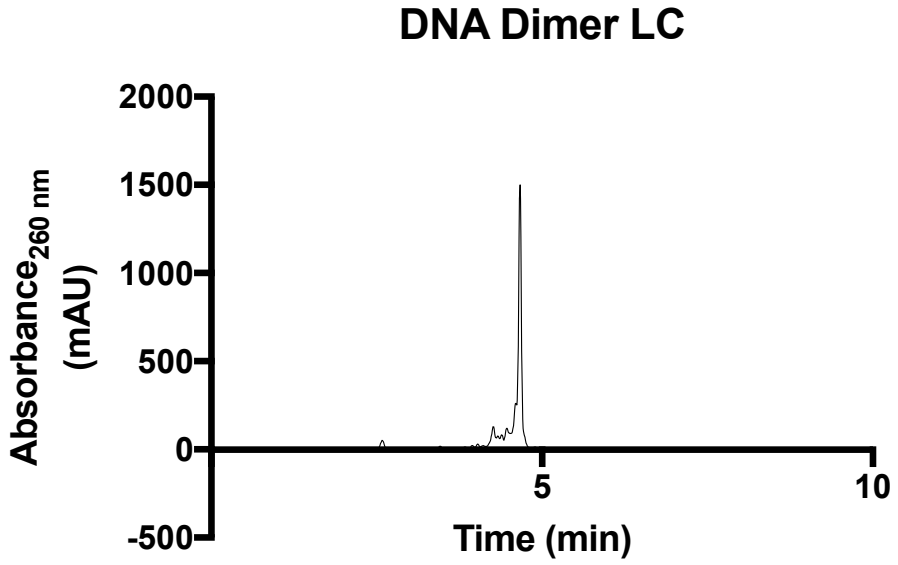


Figure 3.10: LC trace showing the dimeric DNA dendrimer.

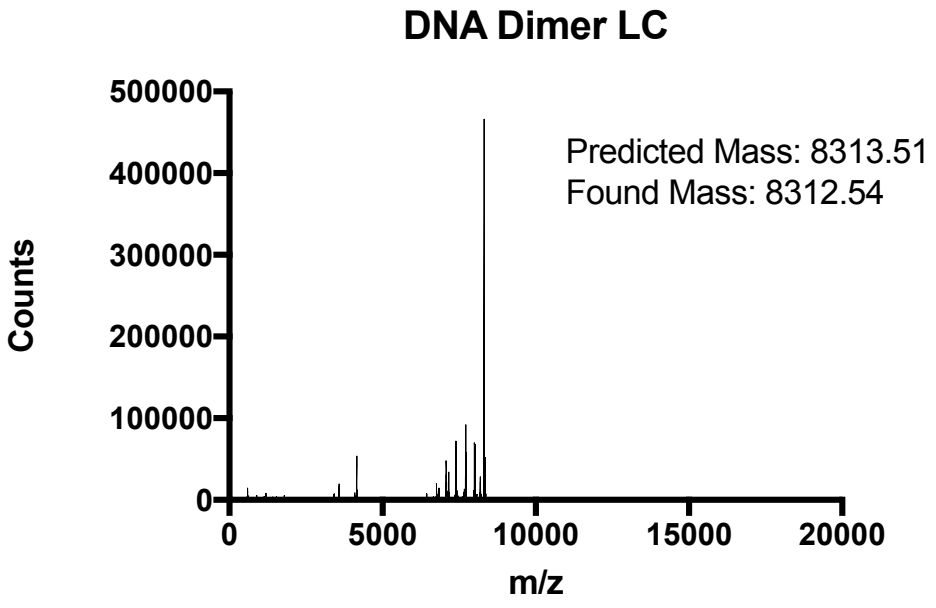


Figure 3.11: MS trace showing the dimeric DNA dendrimer.

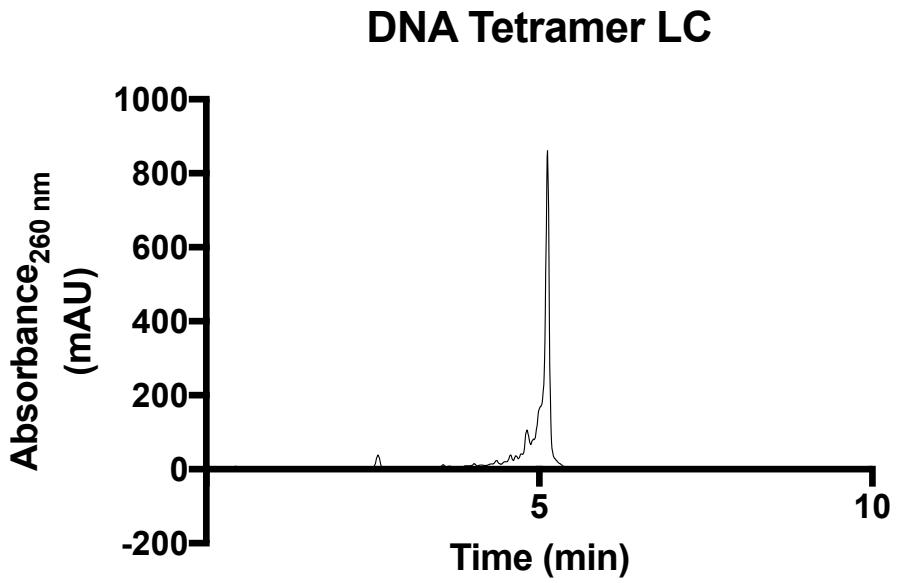


Figure 3.12: LC trace showing the tetrameric DNA dendrimer.

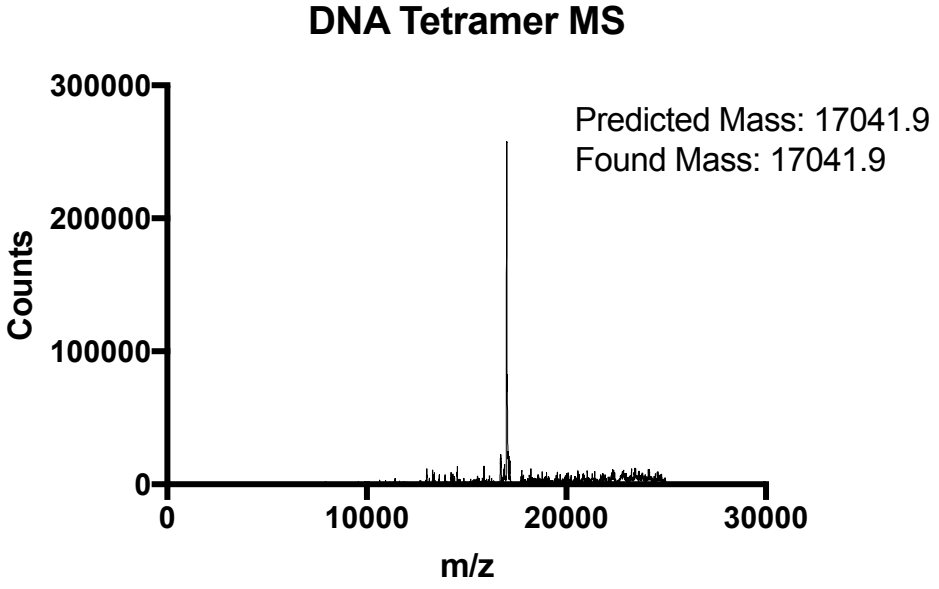


Figure 3.13: MS trace showing the tetrameric DNA dendrimer.

DNA-only oligonucleotide dendrimers were sucessfully synthesised using the 10 µmol synthesis scale on the ABI394 (Fig. 3.10, 3.11, 3.12, 3.13) however, the synthesis cycle wasteful, and the results were not translatable to the more therapeutically relevant

LNA modification. LNA is a desirable modification in oligonucleotide therapeutics because of its enhanced binding properties and nuclease resistance, but the corresponding phosphoramidites couple with somewhat lower efficiency,[259] a property that makes synthesis of dendrimers containing LNA challenging. This issue is compounded by the fact that the phosphoramidites remain under patent protection and hence are either prohibitively expensive ($1000+/g from Exiqon) or require in-house synthesis from the 5′-DMT, 3′-OH nucleoside, the option chosen. Because these phosphoramidites were synthesised in-house and at relatively small scale unfortunately meant that some impurities resulting from synthesis (solvent, water, H-phosphonate) were likely present, also lowering the coupling yields.

After successfully preparing DNA-only dendrimers, synthesis of a series of LNA-containing dendrimers was attempted. While pure dendrimers were able to be isolated from the crude mixture after repeated HPLC purification steps (Fig. **??** and **??**), the yields were very poor (<1%) and in the instance of the tetramer, the isolated material was still impure with traces of n-x products in the mass spectrum.

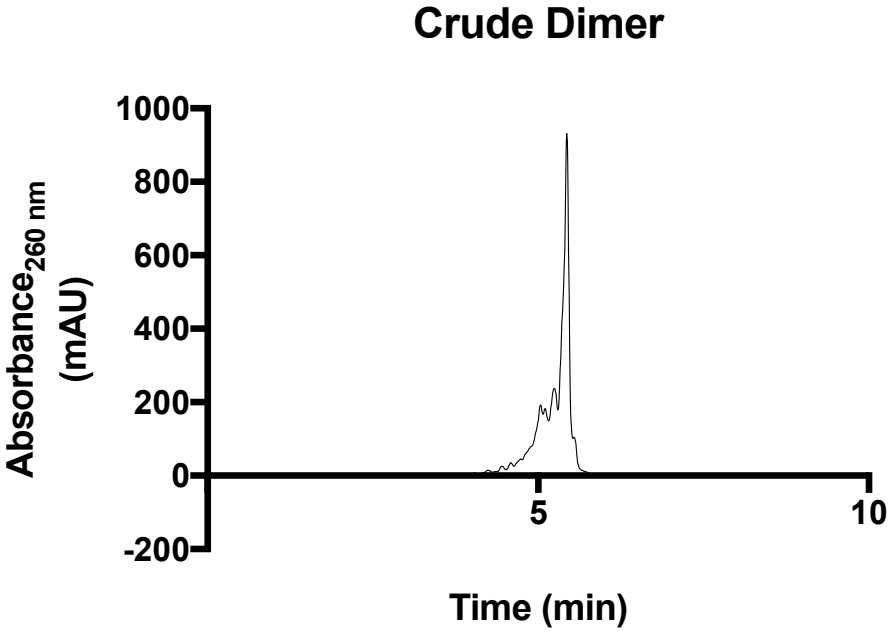


Figure 3.14: LC trace showing the crude dimeric LNA dendrimer.

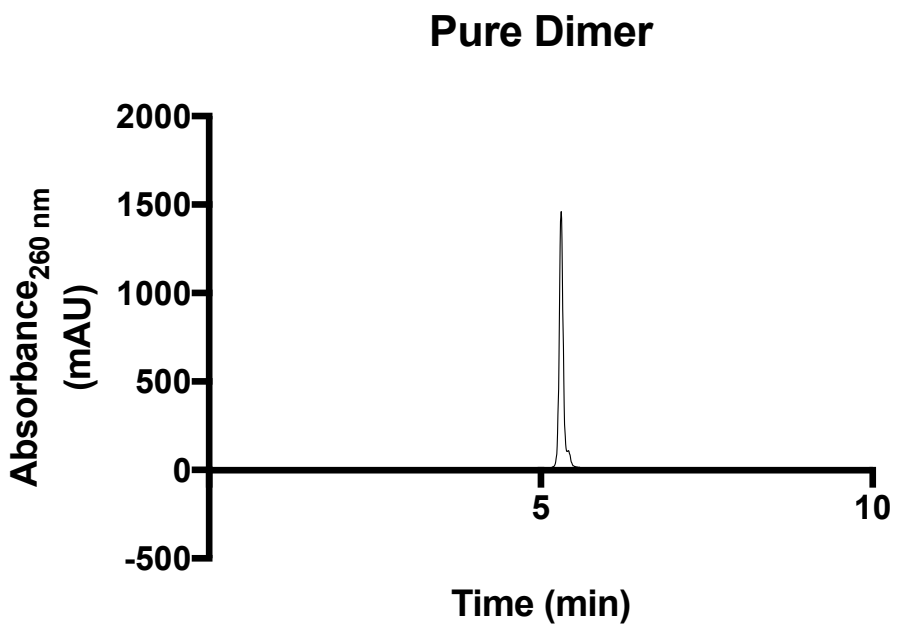


Figure 3.15: LC trace showing the pure dimeric LNA dendrimer.

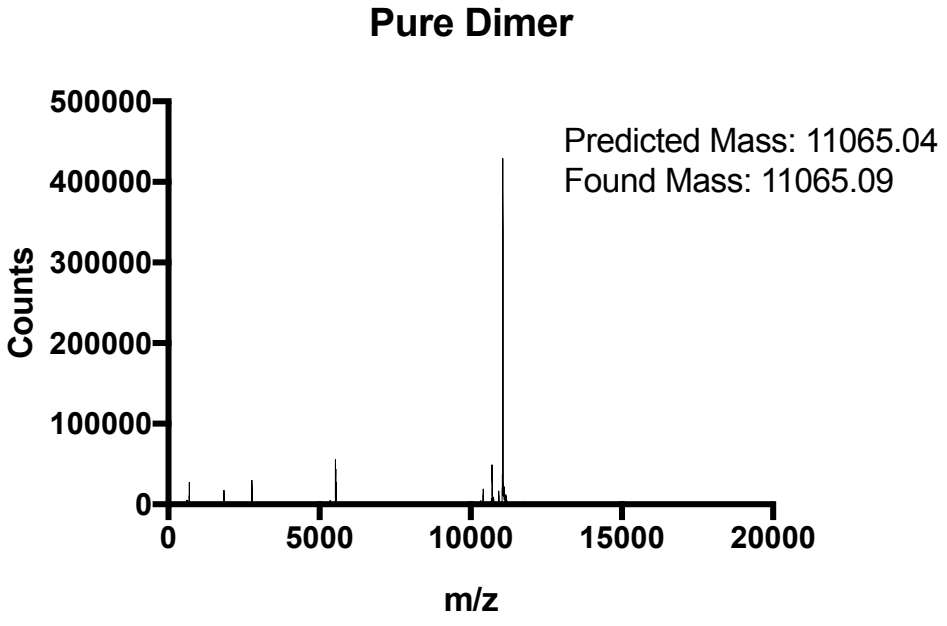


Figure 3.16: MS trace showing the pure dimeric LNA dendrimer.

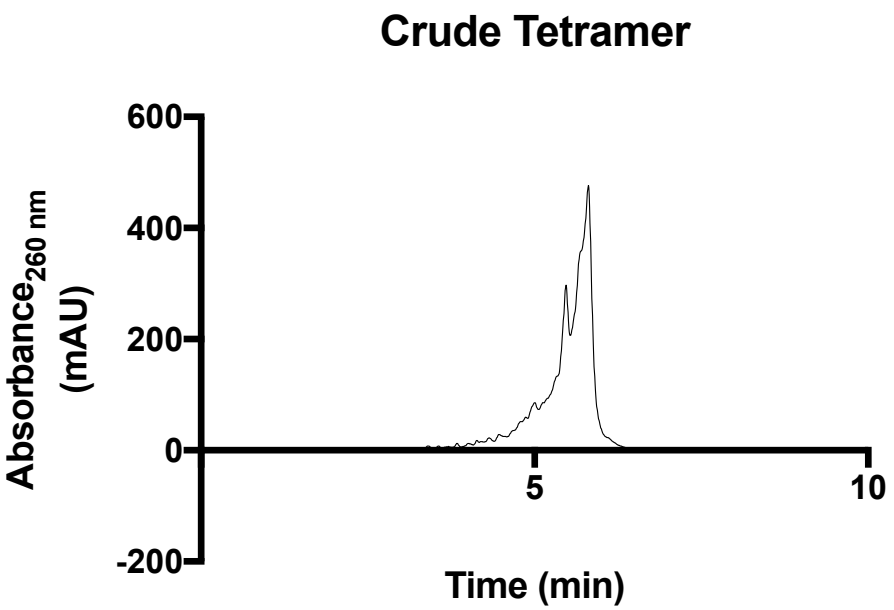


Figure 3.17: LC trace showing the crude tetrameric LNA dendrimer.

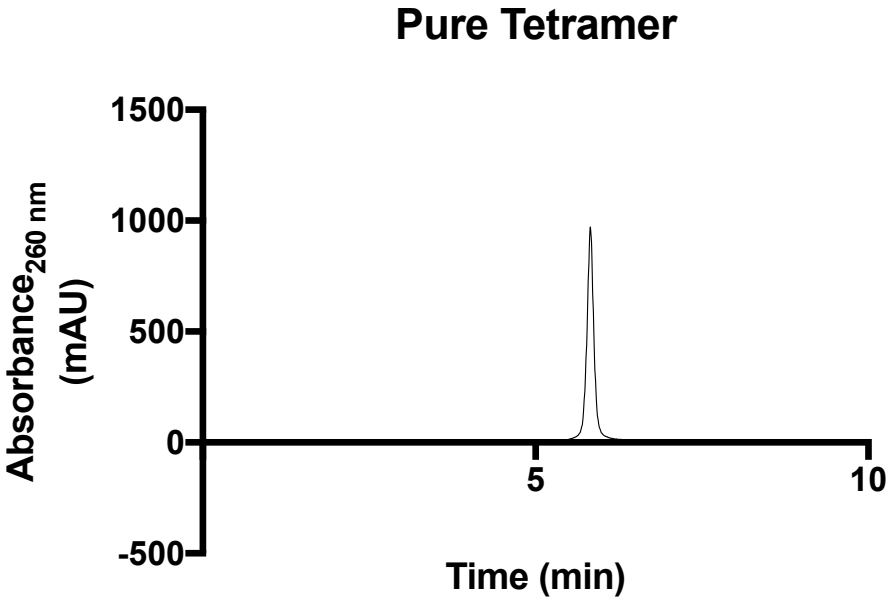


Figure 3.18: LC trace showing the pure tetrameric LNA dendrimer.

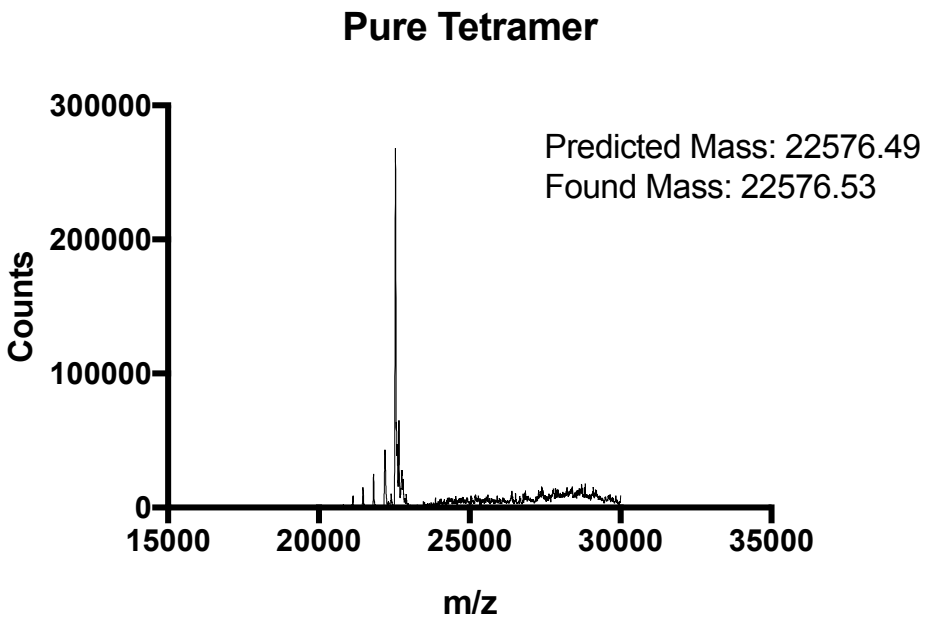


Figure 3.19: MS trace showing the pure tetrameric LNA dendrimer.

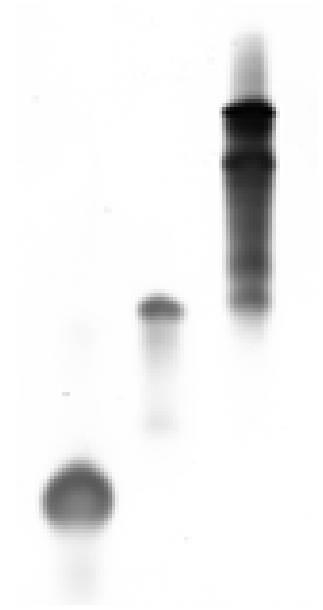


Figure 3.20: Analytical PAGE showing (left to right) purified monomer, dimer and tetramer LNA-containing oligonucleotides stained using SYBR Gold

MOE phosphoramidites came off-patent in early 2017 and hence were purchased and incorporated into the synthetic methodology as a high-affinity RNA analogue.

3.2.4.3 Choice of Doubling Reagent

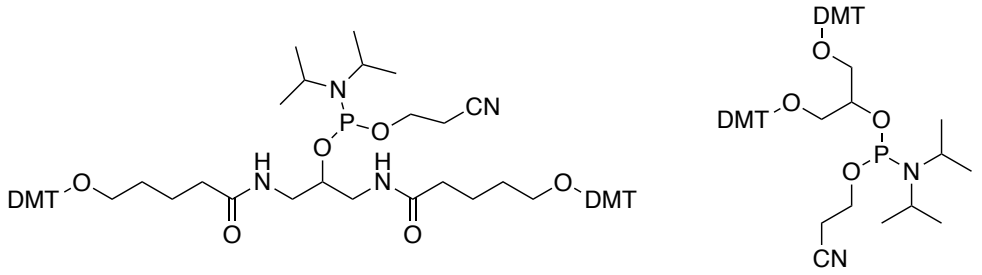


Figure 3.21: A comparison between the Shchepinov doubler synthon (left) and the glycerol doubler synthon (right)

It was considered that the Shchepinov doubling phosphoramidite was likely a more effective choice than the glycerol analogue because of the decreased steric hinderance provided by the C5 arms. ChemGenes however advised that their customers generally had better results with the glycerol modifier than the Shchepinov modifier. Hence it was decided to use the glycerol based synthon on the basis of this recommendation as well the fact that the Shchepinov modifier would have needed to be synthesised in-house because of the high commercial cost.

3.2.4.4 Solid Support

It was necessary to select the optimal solid support to use for these syntheses. Typically, this solid support is controlled-pore glass; a fine glass powder (ca. 100 μm/particle) with pores of a specified size on the surface of each particle. As a general rule, 500 Å pore CPG is suitable for synthesis of a 20-40 mer, after which the yield will decrease owing to steric congestion of the pore. 1000 Å CPG can be used for oligonucleotides up to 100 bases in length while larger pore sizes can accommodate even larger oligonucleotides. To synthesise something quite as large and branched as a dendrimer, it was considered likely beneficial to try and make the synthesis environment as similar as possible to a solution-phase

reaction. Nittophase, a crosslinked polystyrene resin that swells in acetonitrile, provides a more solution-like environment for coupling. The significant downside to using nittophase is that it is typically loaded heavily ($200\,\mu mol\,g^{-1}$) which could create steric congestion between strands and negatively compensate for the gain obtained by swelling.[260]

There is also a combined product called hybrid-CPG that comprises standard controlled-pore glass coated in a thin film of polystyrene to afford the best of both CPG and resin solid supports.[259] hybCPG was chosen for use in our initial experiments, though it was subsequently found that Nittophase performs similarly.

3.2.4.5 Spacing

The final synthetic consideration was whether or not to introduce oligoethylene glycol spacers at points in the synthetic cycle. This was indicated the original work by Shchepinov to be beneficial in forming large structures.[157] Despite the inevitable loss in yield by introducing additional coupling steps, it was considered to be a logical decision to include spacers in order to allow greater space between the growing oligonucleotides.

3.2.5 Synthesis of MALAT1 Dendrimers

It was important to target a gene that was expressed ubiquitously so that it would be possible to assay silencing *in vitro* and *in vivo* throughout a variety of tissues. While targeting a housekeeping gene was considered, these are not always expressed at useful levels throughout an organism, and occasionally they vary with immunological stimulation- something that was potentially possible with these dendrimers.[261,262] It was decided to make dendrimers targeting the ubiquitously expressed lncRNA MALAT1, using a known ASO sequence.[263]

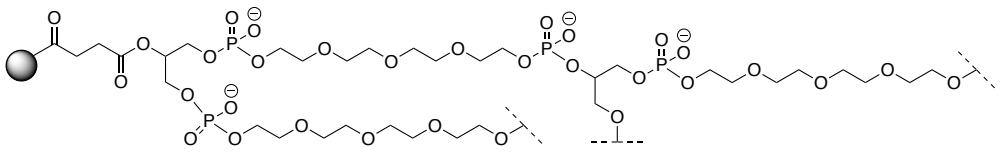


Figure 3.22: Schematic of the final dendrimer design on solid support.

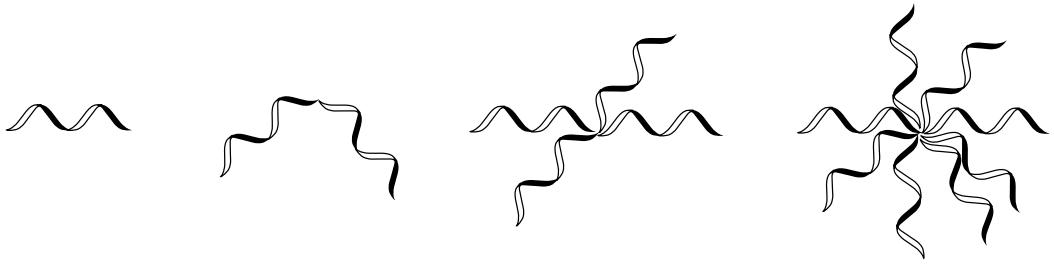


Figure 3.23: A schematic representation of the monomeric, dimeric, tetrameric and octameric structures synthesised.

For the subsequent studies, dendrimers were synthesised using hybCPG, with the glycerol modifier already attached, using a tetraethylene glycol spacing phosphoramidite between each coupling of the doubling phosphoramidite and between the final doubling moiety and the start of the oligonucleotide (Fig. 3.22). The AKTA Oligopilot synthesiser was employed, using MOE and DNA chemistry. In this manner a monomeric control, a dimer, tetramer and octamer all targeting MALAT1 were synthesised (Fig. 3.23). Post-synthesis, dendrimers were deprotected in the usual manner and purified using ion-exchange HPLC (details in section 3.8.2).

The synthesised dendrimers were analysed by RP-HPLC and RP-LCMS as opposed to IEX-HPLC owing to the improved resolution that this affords for larger oligonucleotides. The method in use on the LCMS was insufficient to adequately resolve the larger dendrimers (Fig. 3.24) and so a more stringent analytical method was developed using analytical HPLC (Fig. 3.25). A denaturing TBU PAGE gel (Fig. 3.26)

was run to help determine approximate purity. It was somewhat surprising to see the smeared bands in the gel, given the purity indicated by HPLC, however it is not inconceivable that the 3-dimensional structure of these dendrimers altered the electrophoretic mobility causing some distortion of the bands. It is however equally possible that the n-x impurities were chromatographically similar enough to the parent compound that they were hidden beneath the main peak.

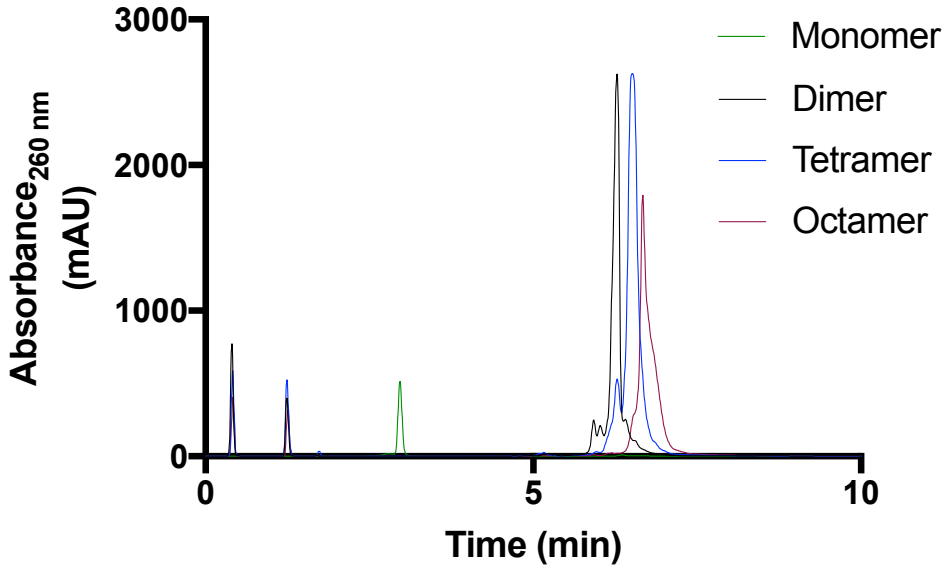


Figure 3.24: Overlayed RP-HPLC traces of monomeric, dimeric, tetrameric and octameric oligonucleotides demonstrating the challenge with HPLC purification.

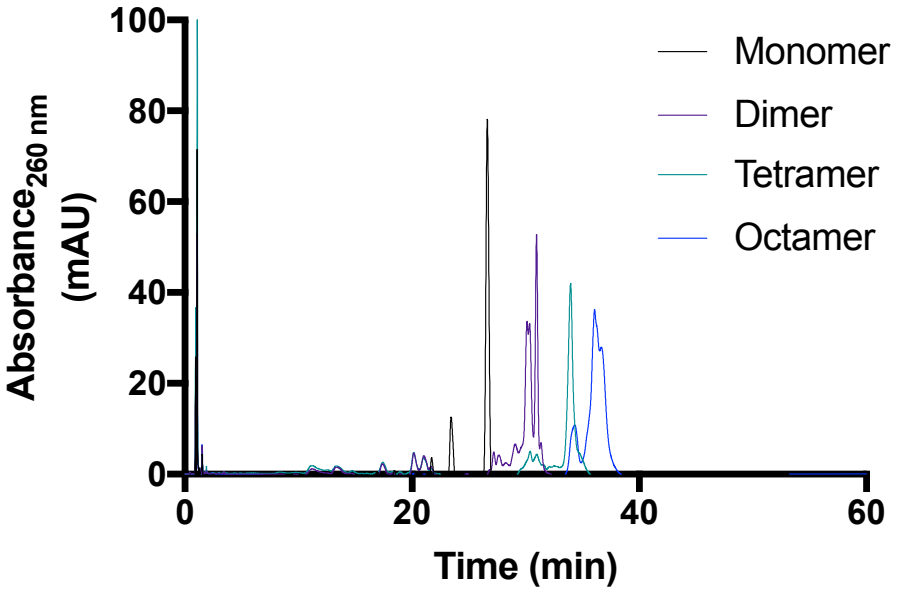


Figure 3.25: Analytical RP-HPLC of each synthesised oligonucleotide dendrimer, using a longer method, showing residual impurities after purification.

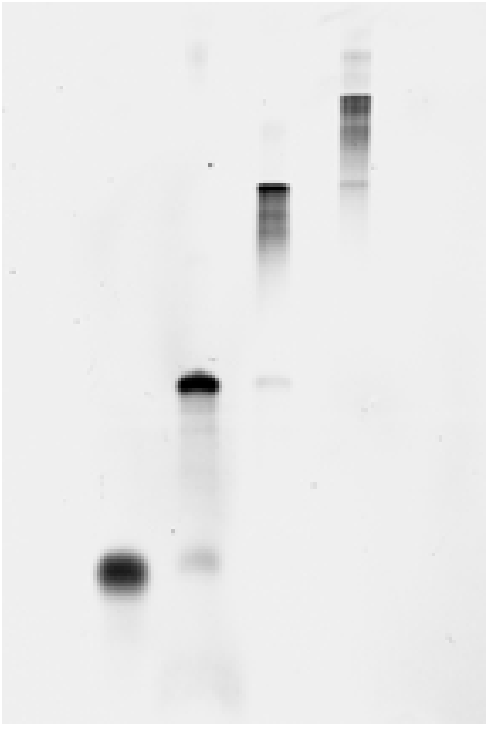


Figure 3.26: Analytical PAGE showing (left to right) monomer, dimer, tetramer and octamer stained using SYBR gold.

ESI mass spectrometry provided further evidence that the compounds of interest had been obtained, with the exception of the octameric species, for which the mass spectrum was unable to be deconvoluted (Fig. **??**). Thus, while the monomeric, dimeric and tetrameric structures have accurate mass spectra, corresponding to their predicted mass – the octameric species does not. Nevertheless, the PAGE analysis indicates synthesis of a substantially larger species than for the tetramer, dimer or monomer. The degree of structural uncertainty should be borne in mind when considering the results of the downstream biological assays using the octameric species.

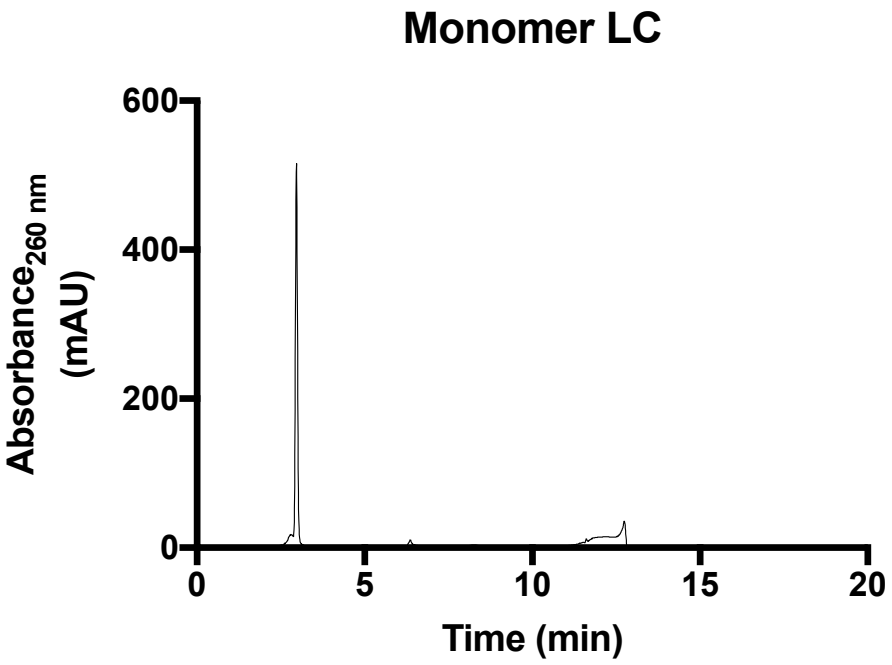


Figure 3.27: LC trace showing the monomeric MOE oligonucleotide.

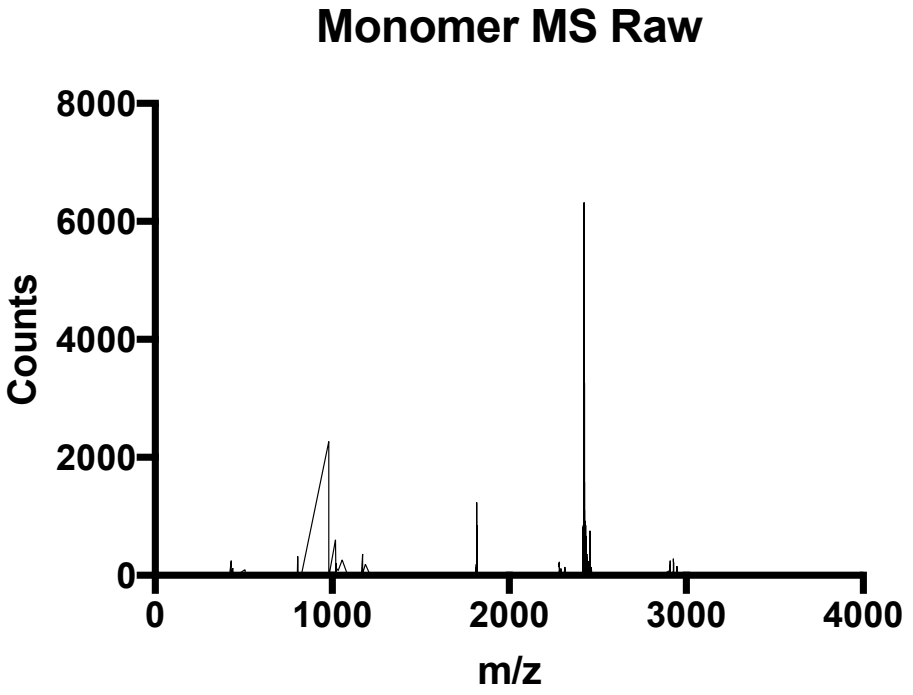


Figure 3.28: Raw MS trace showing the monomeric MOE oligonucleotide.

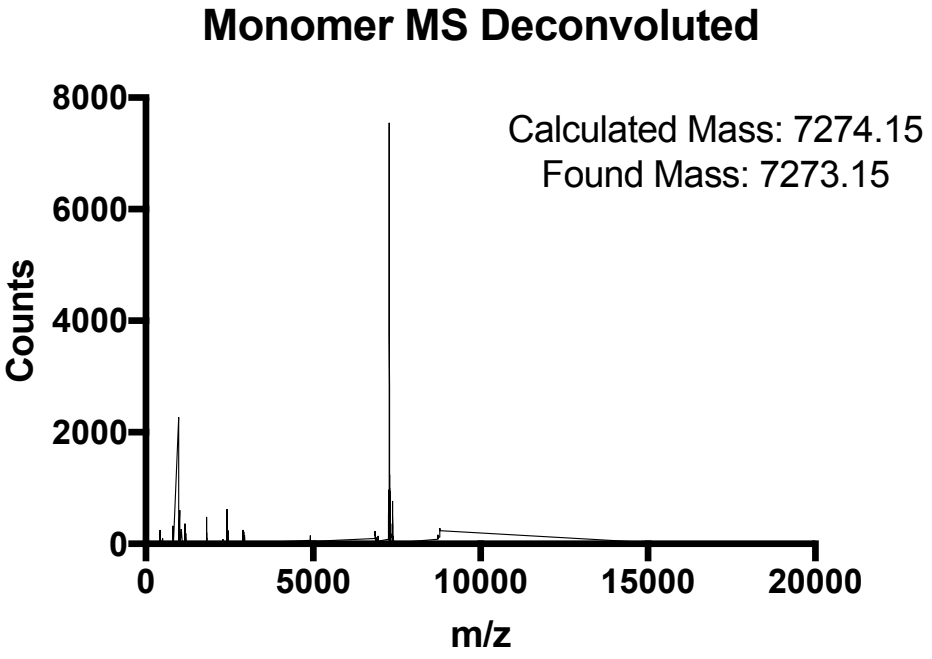


Figure 3.29: MS trace showing the pure tetrameric LNA dendrimer.

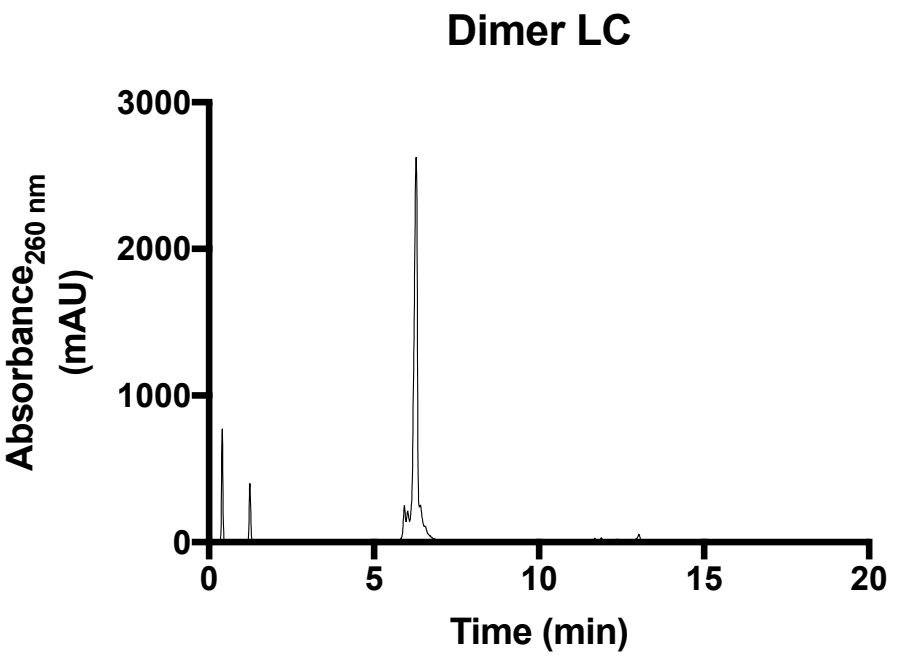


Figure 3.30: LC trace showing the dimeric MOE dendrimer.

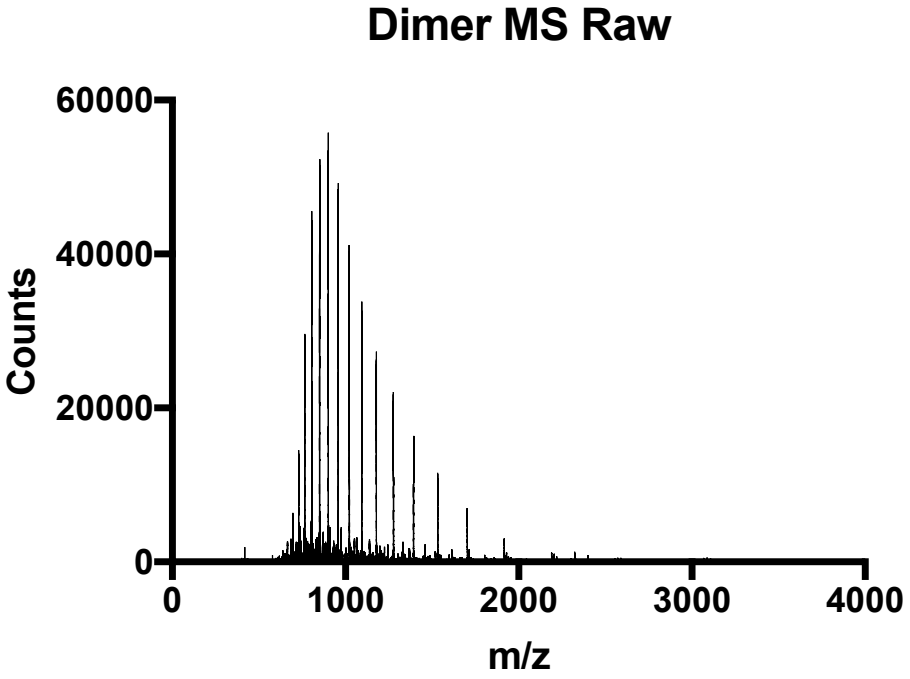


Figure 3.31: Raw MS trace showing the pure dimeric MOE dendrimer.

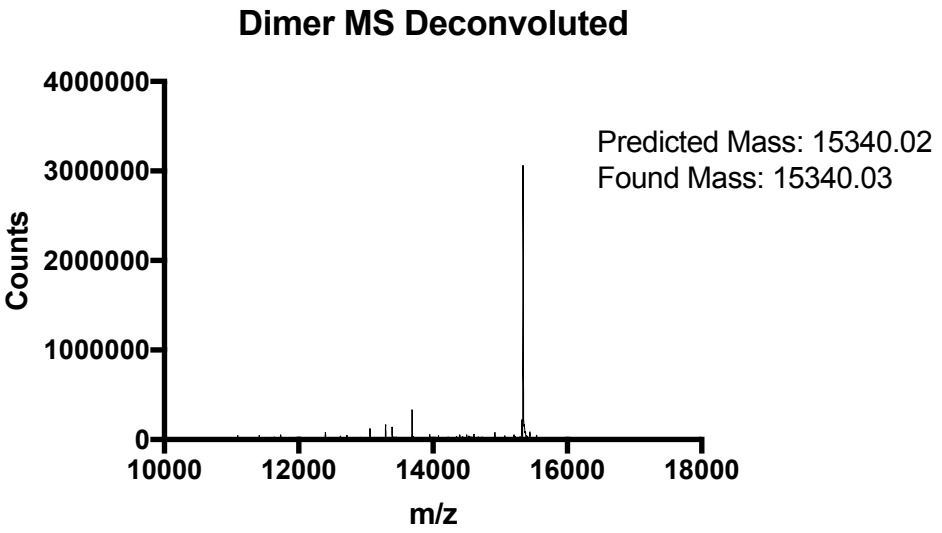


Figure 3.32: Deconvoluted MS trace showing the pure dimeric MOE dendrimer.

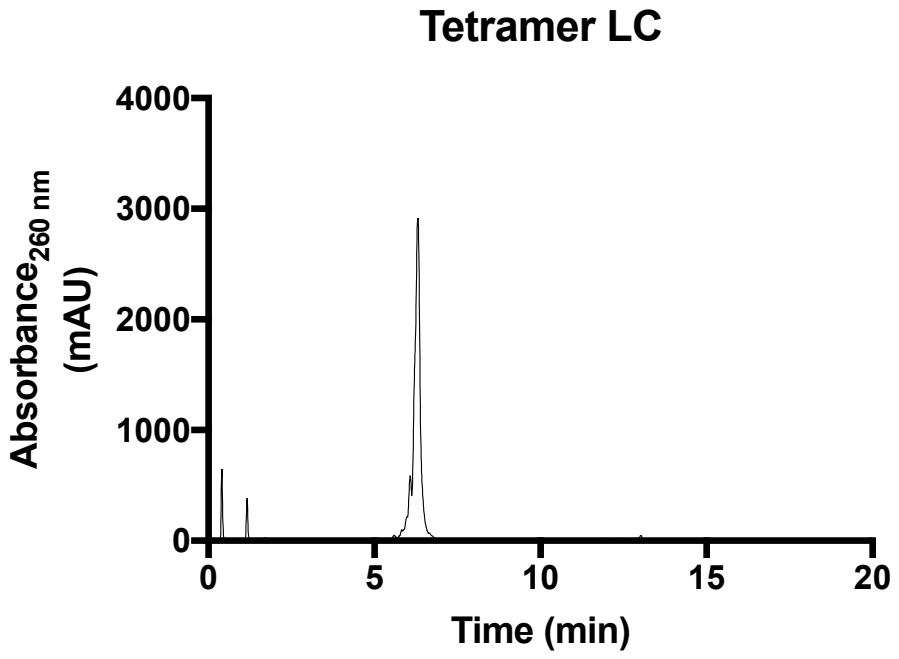


Figure 3.33: LC trace showing the tetrameric LNA dendrimer.

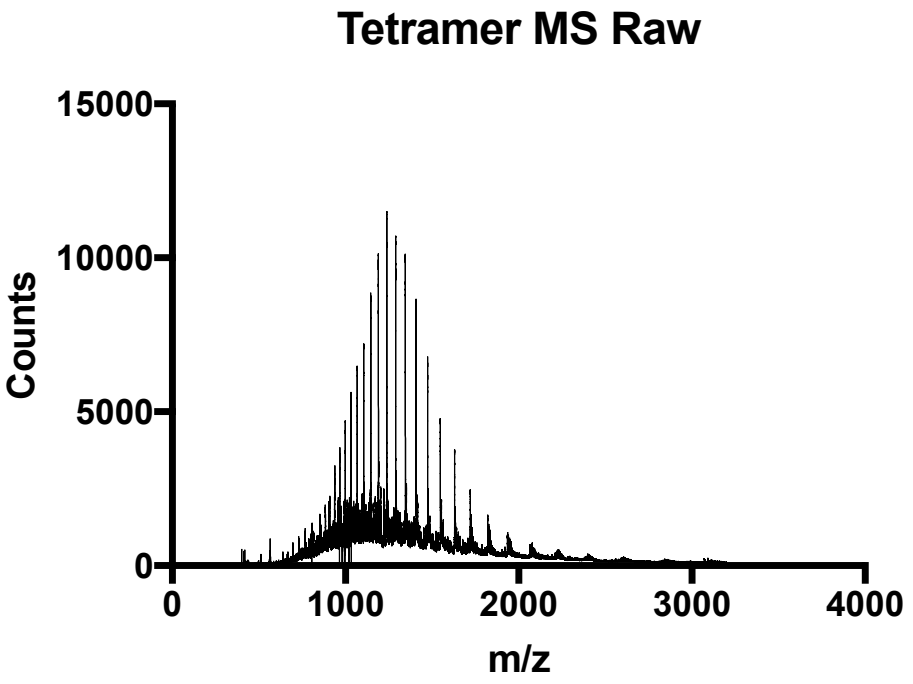


Figure 3.34: Raw MS trace showing the tetrameric MOE dendrimer.

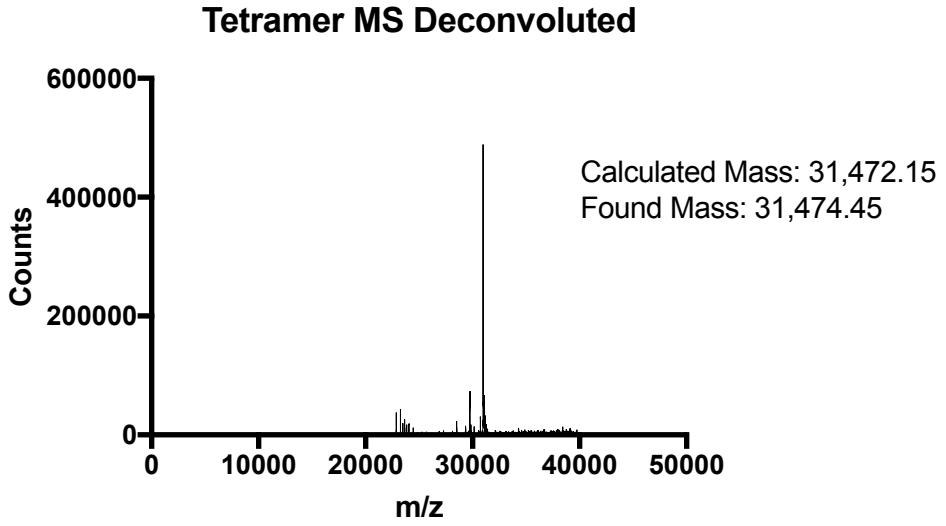


Figure 3.35: Deconvoluted MS trace showing the pure tetrameric MOE dendrimer.

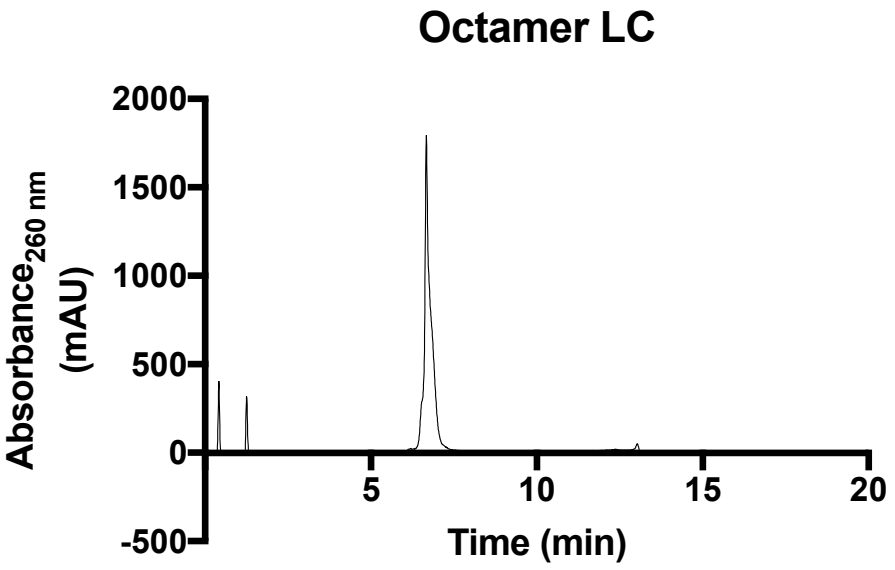


Figure 3.36: LC trace showing the octameric MOE dendrimer.

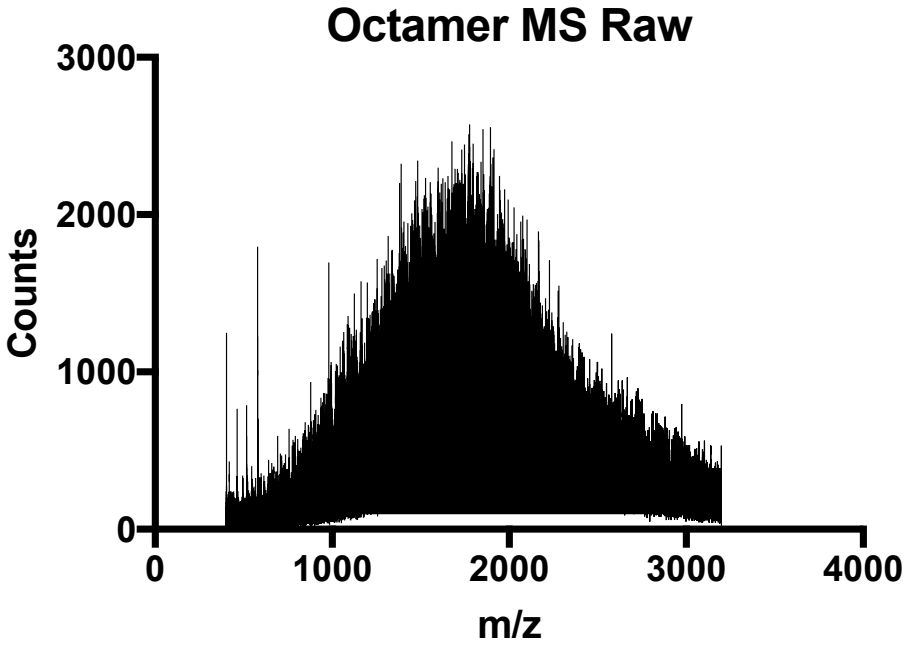


Figure 3.37: Raw MS trace showing the octameric MOE dendrimer.

3.3 Cellular Uptake of Dendrimers

One of the first things that was examined after successfully having synthesised dendrimeric oligonucleotides was how they would be taken up in cultured cells. In order to assay this, mouse embryonic fibroblast (MEF) cells were grown on coverslips and treated with naked oligonucleotide dendrimers (no transfection reagents were used). To keep the comparison the same, it was important to ensure the same absolute quantity of oligonucleotide between dendrimers, that is to say, if a 20 μM concentration of each test oligonucleotide was used, there would be 2 fold as much material for a doubler relative to a monomer, a tetramer relative to a doubler and an octamer relative to a tetramer. Clearly this is not an even comparison and so the concentration was halved as dendrimer size increased, termed herein as 'single strand equivalence'. It is also appropriate to consider this as 'moles of arms'; 5 µmol of a dimer comprises 10 µmol of the corresponding monomer oligonucleotide.

To assay uptake, the antibody generated as described in Chapter 2 was used to detect uptaken oligonucleotide in an immunofluorescent fashion. Uptake was assessed at different timepoints in order to determine whether there were any qualitative differences in rate of uptake between dendrimeric structures. Hence a slide was prepared for each dendrimer every 24 hours for 4 days, stained and imaged (Fig. 3.38). It is important to note that each timepoint is normalised in fluorescence intensity to the controls. Cells were treated with 1 μM single strand equivalent of each oligonucleotide.

Figure 3.38 shows both a time and a size-mediated trend of the cellular uptake of these dendrimers. Larger dendrimers are taken up more quickly and to a greater extent than smaller dendrimers. For example, at 24 h there is a significant uptake of the tetramer and octamer, some of the doubler but very little of the monomer. The overall gross uptake scales with dendrimer size.

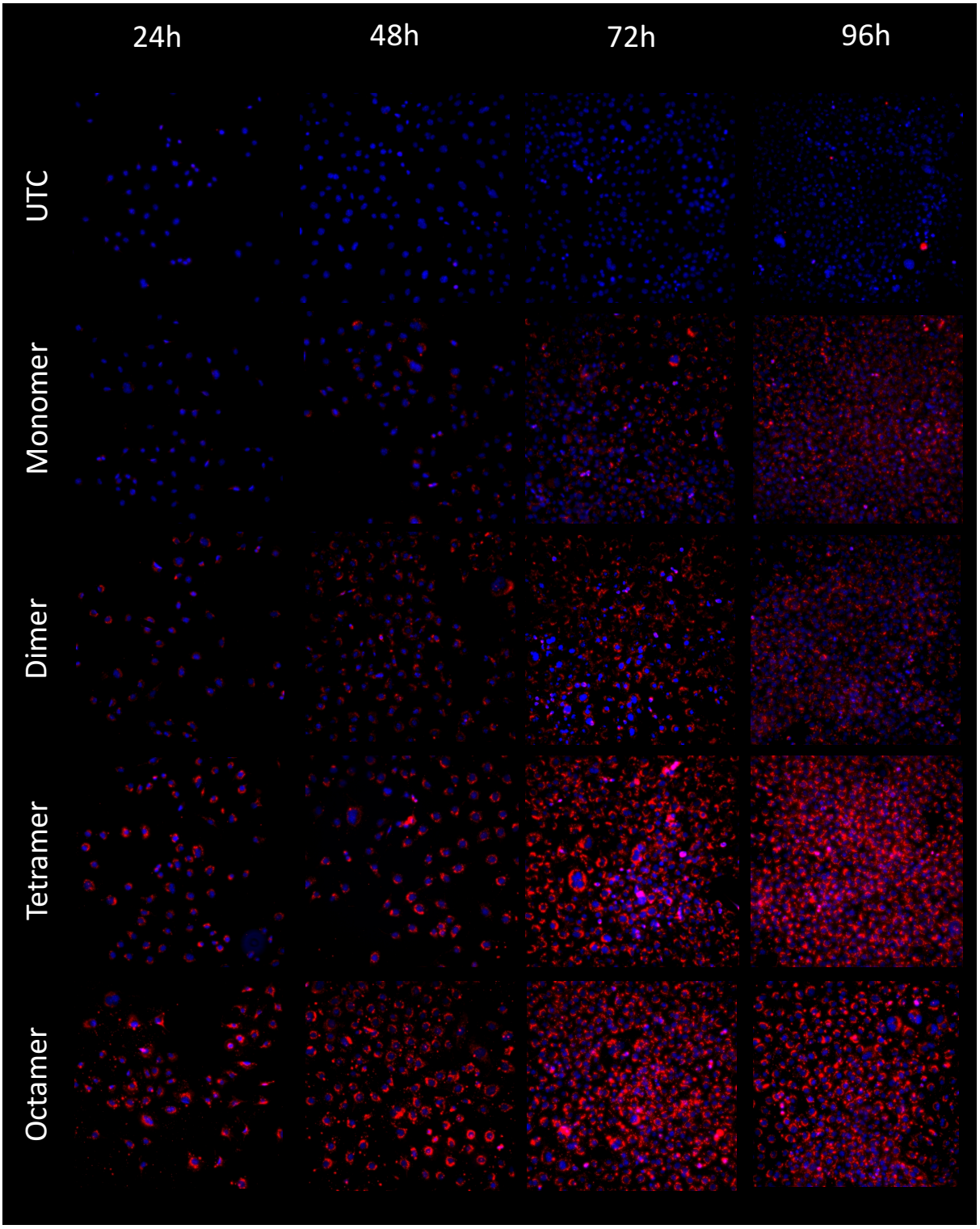
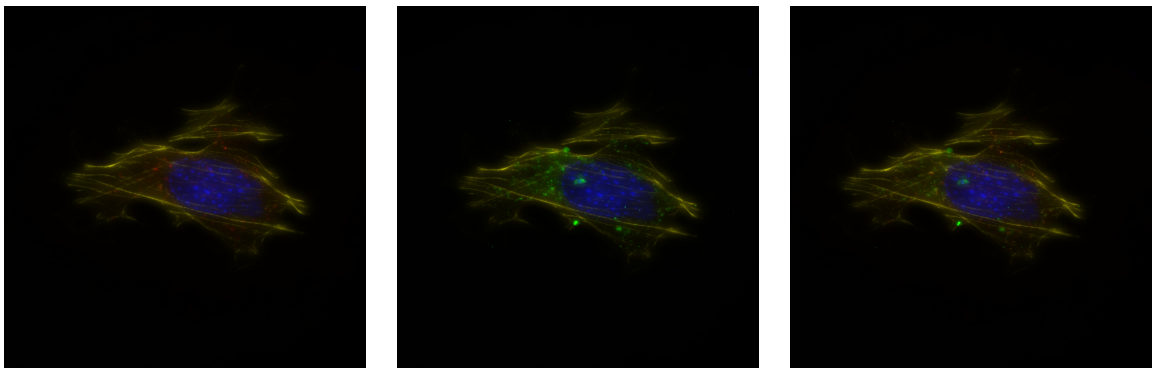


Figure 3.38: Figure showing the time-dependent uptake of multimeric oligonucleotides in MEF cells.
Blue: DAPI, Red: Anti-ASO pAb

3.3.1 Colocalisation with the Lysosome

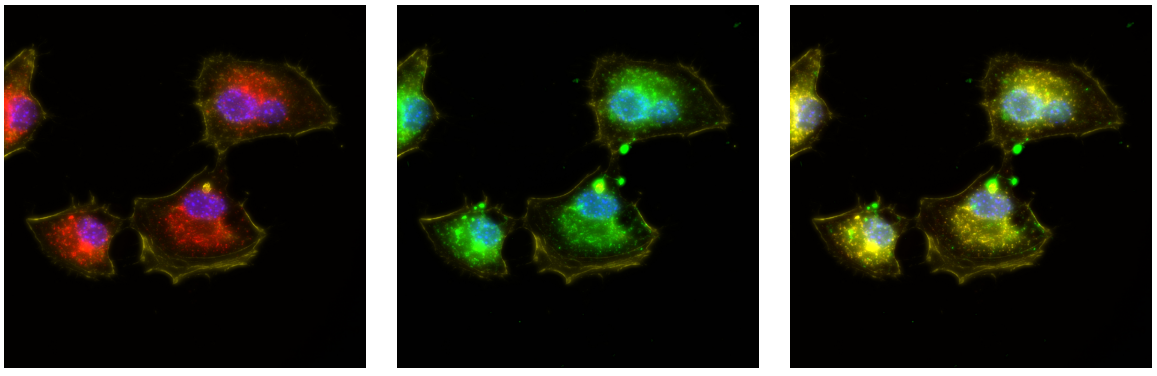
It was observed in Figure 3.38 that the anti-ASO staining occurred predominantly in perinuclear foci prompting the question as to whether these were endosomes or lysosomes. To determine how the dendrimeric oligonucleotides would interact with endosomes and lysosomes, the commercial product 'Lysotracker', a weakly basic, amine containing BODIPY dye that selectively labels acidic organelles, such as lysosomes in cells, was used. Lysotracker was in combination with the antibody previously generated (Chapter 2) to examine colocalisation of dendrimers with the lysosome. The nucleus (using DAPI), the cytoskeleton (using Phalloidin) as well as the lysosome and oligonucleotide were imaged simultaneously using the 4 separate fluorescent channels of the microscope. Images were taken 24 hours after treatment with 1μM single strand equivalent of each oligonucleotide, while lysotracker was added 30 min before fixation and staining. All images were taken under the same conditions and corrected identically.

Figure 3.39 shows untreated cells stained identically to the treated cells. This experiment served to provide background for the pAb staining. It can be seen that there is some promiscuous nuclear staining evident likely owing to the conditions of permeabilisation (Triton X100). Figure 3.40 shows the uptake of the tetrameric ASO. While much of it seems to be correlated with lysotracker, there does appear to be some extra-lysosomal distribution.



(a) UTC Lysotracker **(b)** UTC anti-ASO pAb **(c)** UTC, merge

Figure 3.39: Untreated control (UTC) cells stained with lysotracker and anti-ASO pAb. DAPI is shown in blue, the cytoskeleton (AF488- Phalloidin) in yellow, lysotracker in red and the oligonucleotide in green. Colocalisation of the oligonucleotide and lysotracker appears as yellow.



(a) Tetramer treated, Lysotracker **(b)** Tetramer treated, anti-ASO pAb **(c)** Tetramer treated, merge

Figure 3.40: Tetramer-treated cells stained with lysotracker and anti-ASO pAb. DAPI is shown in blue, the cytoskeleton (AF488- Phalloidin) in yellow, lysotracker in red and the oligonucleotide in green. Colocalisation of the oligonucleotide and lysotracker appears as yellow.

The microscopy conducted has demonstrated a significant difference in the uptake profiles of the different valency dendrimers, it seems to be the case in all tested systems that oligonucleotides with more arms appear to internalise more rapidly and extensively than oligonucleotides with fewer arms. What is not clear from this experiment however, is the mechanism of internalisation and most importantly whether the oligonucleotides are being productively internalised; that is, whether they are able to effect gene silencing once taken in to the cell. There is a strong lysosomal association in all instances of oligonucleotide uptake.

3.4 Efficacy of Dendrimers *in vitro*

To further characterise the silencing ability of these multimeric compounds, they were screened in mouse embryonic fibroblast (MEF) cells by lipid transfection (see 3.9.1 for details). The use of lipid to deliver the dendrimers allowed for a more direct comparison of their inherent silencing ability, as opposed to their ability to enter the cell. Silencing data is shown in figure 3.41.

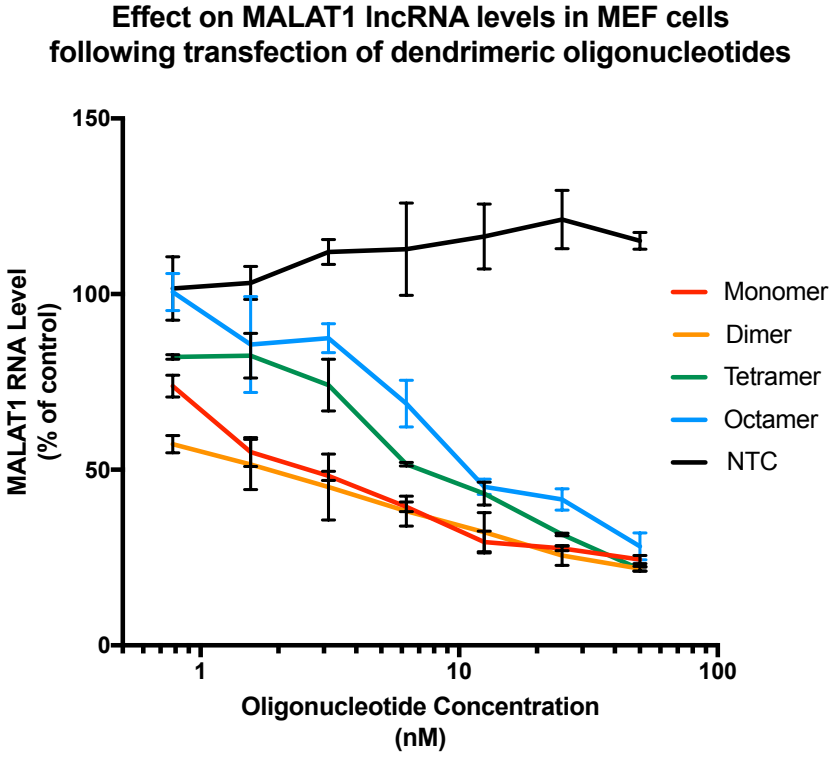


Figure 3.41: Dose-dependent silencing resulting in MEF cells after 17 hour transfection with different oligonucleotide dendrimers.
RNA level was quantitated using Affymetrix Quantigene 2.0 and normalised to HPRT (Section 3.9.1)

Interestingly, while all compounds are able to induce significant gene silencing, the dimeric oligonucleotide appears to be marginally more efficacious than the monomeric species, while both monomer and dimer are more effective than either the tetramer or the octamer.

Despite the clearly enhanced cellular uptake, both the tetramer and the octamer species were less efficacious.

It was considered that there were several possible reasons for this diminishing activity in the higher dendrimers. These molecules may have been taken up *via* a non-productive pathway, sequestered in lysosomes (as Fig. 3.40 may indicate). These larger molecules may also not be able to recruit RNase H as effectively as their smaller counterparts owing to significant steric congestion around the core.

3.5 Biodistribution of Dendrimers *in vivo*

There are often significant discrepancies between *in vitro* and *in vivo* biodistribution. This typically comes as a result of the vastly different environments: *in vitro* uptake involves exposure of a monolayer of cells to a static, relatively concentrated solution of oligonucleotide over a period of hours while *in vivo* uptake occurs with a circulating oligonucleotide solution of only transiently high concentration. Oligonucleotides assessed by *in vivo* biodistribution are subject to the various serum proteins and cell-surface receptors intrinsic to a living organism again, in contrast to the single cell type generally employed *in vitro*.

Saline, monomeric, dimeric, tetrameric and octameric oligonucleotides ($10\,\mathrm{mg\,kg^{-1}}$) were administered to mice by both subcutaneous and intravenous injection. Two mice were treated per route of injection, per compound. Oligonucleotides were diluted in 100 μL of sterile saline for intravenous administration and 200 μL for subcutaneous administration. 48 h after injection, animals were euthanised and perfused transcardially with PBS followed by NBF before post-fixation and embedding, details can be found in section 3.9.2.

Figures for all tissues by both routes of administration are available in the appendix. Data presented in figures 3.42 - 3.45 is particularly unexpected and so is included and discussed here in the main text.

3.5.1 Liver - Intravenous

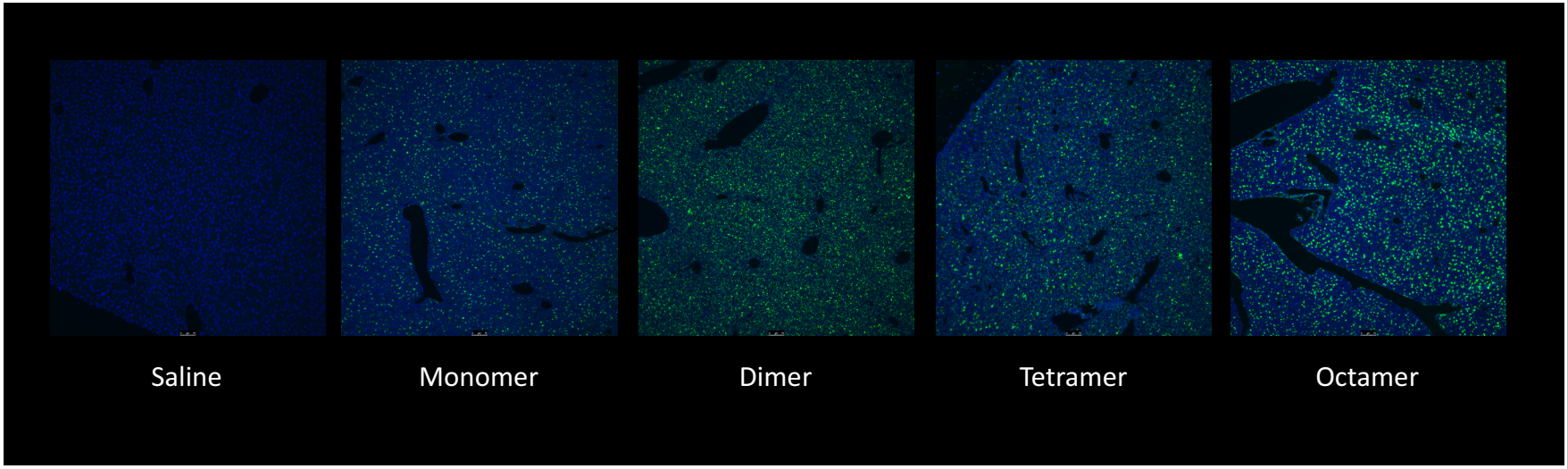


Figure 3.42: Biodistribution of oligonucleotide dendrimers following intravenous injection of $10\,\mathrm{mg\,kg^{-1}}$
Blue: DAPI, Green: Anti-ASO pAb

The liver is one of the primary tissues in which oligonucleotides bioaccumulate. It was observed that the dimeric species appeared most strongly uptaken within this tissue compared with tetramers and octamers. This qualitative measure does correlate well with the more quantitative approach taken by Subramanian *et al* in recent literature wherein dimeric oligonucleotides were shown to accumulate to a greater extent in liver than their monomeric analogues.[175,183]

3.5.2 Kidney Cortex - Intravenous

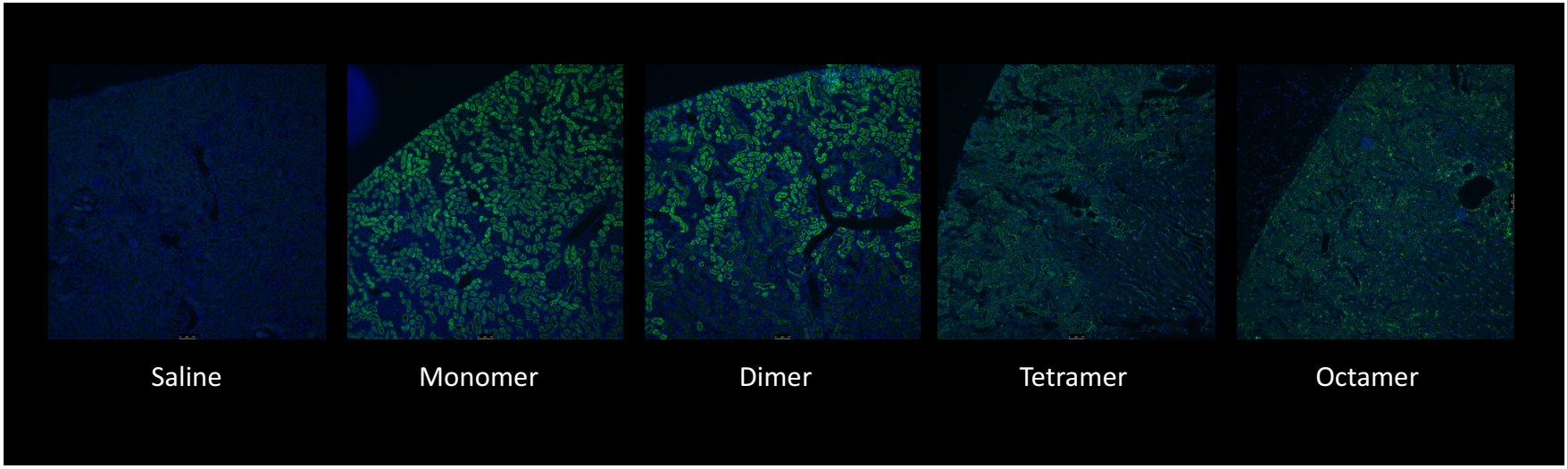


Figure 3.43: Biodistribution of oligonucleotide dendrimers following intravenous injection of $10\,\mathrm{mg\,kg^{-1}}$
Blue: DAPI, Green: Anti-ASO pAb

It is generally considered that most ASOs do not cause significant nephrotoxicity.[264] While this holds true for MOE-modified oligonucleotides (excepting one patient with pre-existing metastatic melanoma[265]), a recent clinical trial of an LNA-containing oligonucleotide showed acute tubular necrosis in an otherwise healthy volunteer.[266] Keeping this in mind, it was hoped that with multimerisation, oligonucleotide may be redirected away from the kidney to potentially preclude such nephrotoxic events. Indeed, it appears as though as one looks from monomer to octamer there is a decrease in accumulation in the renal cortex.

3.5.3 Kidney Medulla

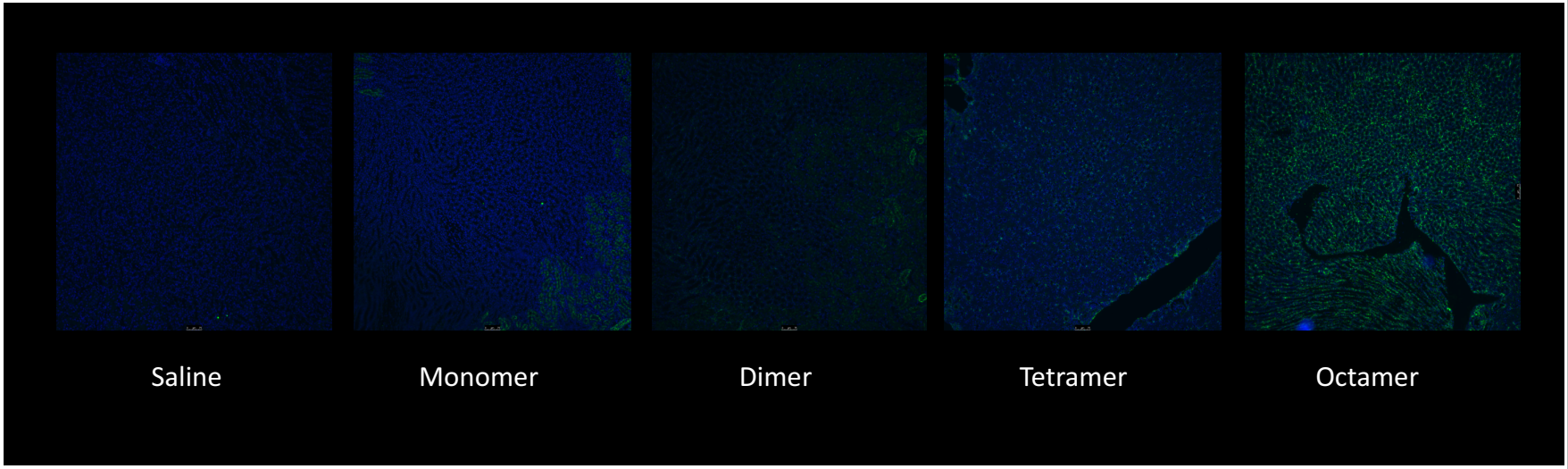


Figure 3.44: Biodistribution of oligonucleotide dendrimers following intravenous injection of $10\,\mathrm{mg\,kg^{-1}}$
Blue: DAPI, Green: Anti-ASO pAb

Something utterly unexpected was observed in the renal medulla. This is typically an area of the body that antisense oligonucleotides do not accumulate in, and indeed this appears to hold true for monomeric, dimeric and tetrameric species. The octamer, however showed profound distribution throughout the renal medulla. A disease such as medullary cystic kidney disease, an autosomal dominant disease caused by mutations in the genes encoding uromodulin (UMOD), hepatocyte nuclear factor-1β (HNF1B), renin (REN), and mucin-1 (MUC1) in the renal medulla,[267] could be amenable to treatment using an octameric oligonucleotide that reaches that part of the tissue.[268]

3.5.4 Lung

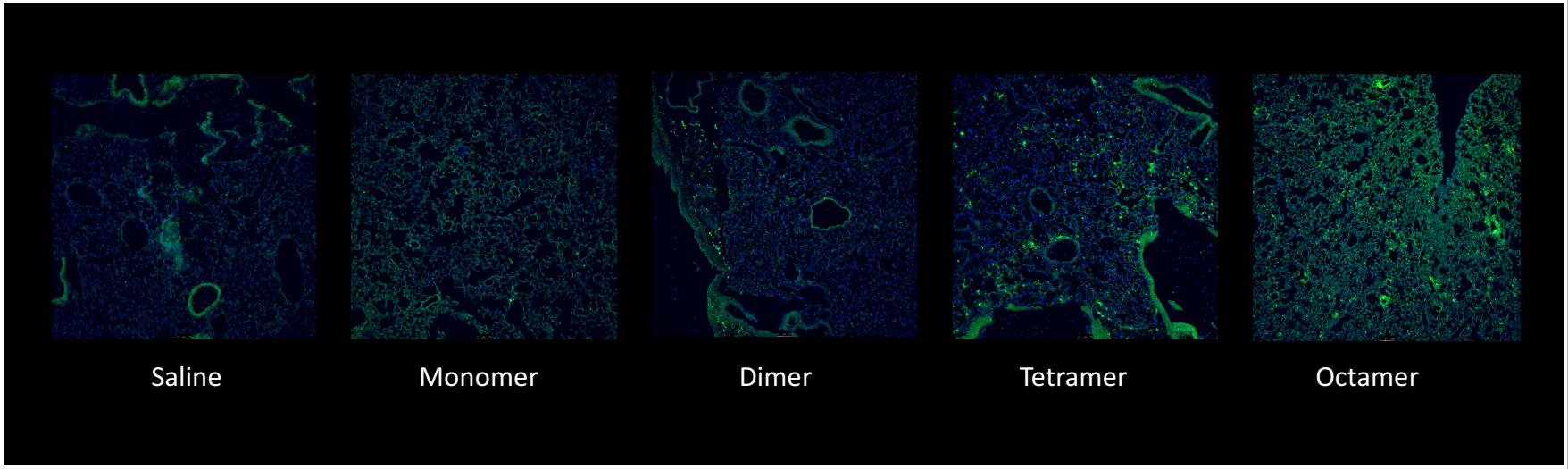


Figure 3.45: Biodistribution of oligonucleotide dendrimers following intravenous injection of $10\,\mathrm{mg\,kg^{-1}}$
Blue: DAPI, Green: Anti-ASO pAb

The lungs presented some interesting data. In the first instance, there was significant background fluorescence. This may be due to the open nature of the tissue: the thin walls of the alveoli may not have adhered properly to the slide, allowing both primary and secondary antibodies to become trapped between these thin tissue edges and the slide, explaining the 'pattern' of non-specific staining in the UTC. Accounting for this high background, the octameric structure appeared to accumulate most significantly in the lungs. This is expected to occur because following intravenous injection, pulmonary capillaries are the first capillaries encountered and perhaps this environment induced precipitation or aggregation of the dendrimer within the vascular endothelium.

3.6 Efficacy of Dendrimers *in vivo*

Following on from the *in vivo* biodistribution data, it was necessary to explore how these multimeric oligonucleotides affect gene expression in various tissues in mice. 8 animals per group were treated, in order to enable statistical confidence in silencing. After intravenous injection of oligonucleotide ($10\,\mathrm{mg\,kg^{-1}}$), animals were left in their home cage for 6 days before being euthanised and tissues harvested. The intravenous route was chosen after examination of the biodistribution data because of the seemingly increased tissue uptake it afforded. Tissues were taken directly from the animal and placed into RNAlater for storage to prevent nonspecific degradation. Two days after this, three separate punches were taken from the tissue (to act as technical replicates) and homogenised using Affymetrix homogenising solution with added proteinase K in a Qiagen tissuelyzer. Homogenate was incubated at 65 °C in a water bath for one hour to ensure each sample was adequately homogenised then frozen before RNA quantitation using the Quantigene 2.0 reagent system. Details can be found in section 3.9.2.

The four tissues discussed below were chosen because they are all therapeutically relevant and in the case of heart, considered to be a challenging tissue to target with systemic oligonucleotide therapy.

Statistics for figures 3.46 and 3.49 were calculated using a one way ANOVA with Tukey post-hoc multiple comparison test, after confirming normality of data with the D'Agostino and Pearson test. Statistics for figures 3.47 and 3.48 were calculated using the Kruskal-Wallis test with Dunn's correction as a result of at least one treatment group failing the normality test. p values are only shown between saline and treatment groups when a significant result occurs. Bars represent mean and standard deviation.

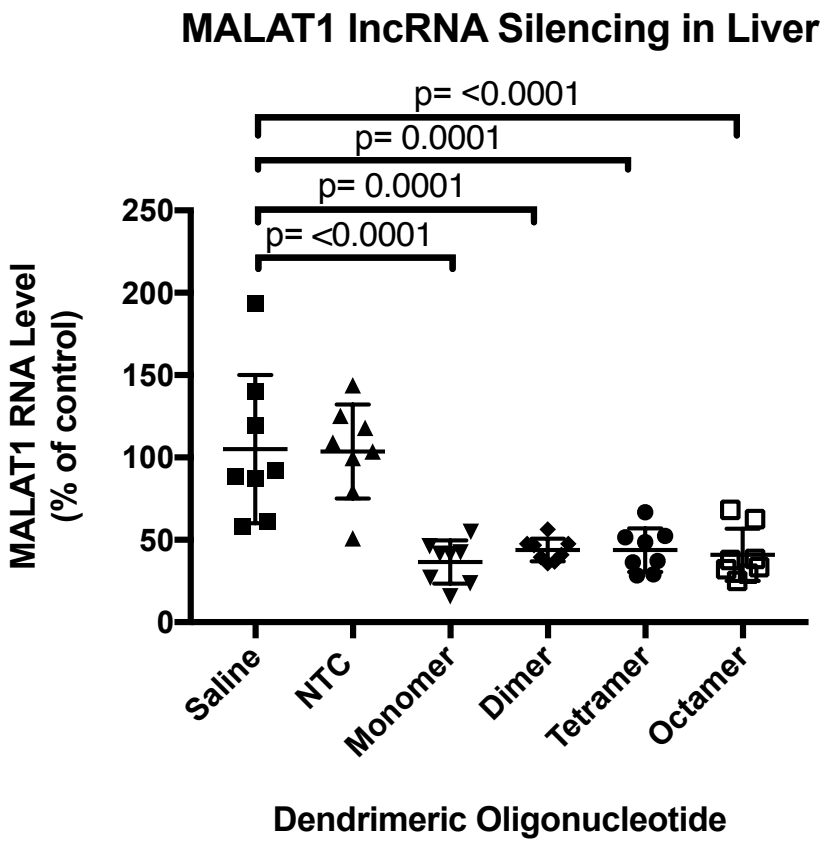


Figure 3.46: MALAT1 silencing for different dendrimers in the liver.
RNA level was quantitated using Affymetrix Quantigene 2.0 and normalised to HPRT (Section 3.9.2.3).

The first tissue examined was the liver. The liver has traditionally been the main tissue targeted by antisense oligonucleotides because large amounts of the injected dose accumulate in this tissue. Interestingly, despite the relative differences in biodistribution from monomer to octamer, the degree of silencing of all four oligonucleotide designs was consistent. This raises some interesting questions. Given that all compounds were injected on a mg kg^{-1} basis (that is to say, the absolute quantity of nucleic acid delivered remains the same) there are clearly fewer molecules of dendrimer than there are of monomer. Looking at the microscopy there is qualitatively less oligonucleotide in the liver for say, octamer than for monomer, yet the silencing remains comparable.

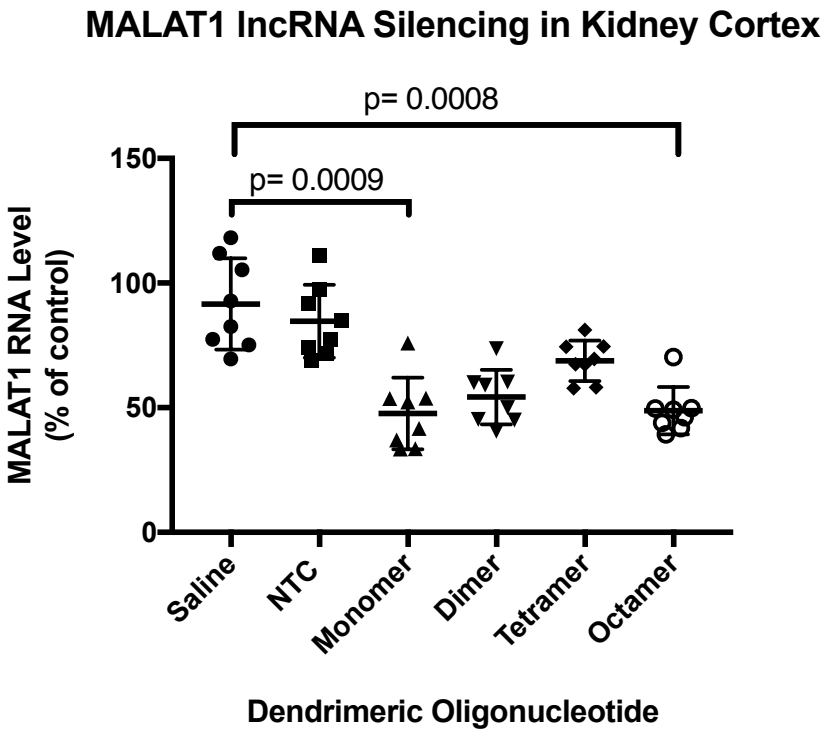


Figure 3.47: MALAT1 silencing for different dendrimers in the kidney cortex.
RNA level was quantitated using Affymetrix Quantigene 2.0 and normalised to HPRT (Section 3.9.2.3).

Silencing in the kidney cortex diminished from monomer to tetramer, but the octamer seemed to silence a little more effectively than the tetramer again. All data was non-significant between groups, which was unexpected given that the biodistribution data indicated decreasing accumulation as dendrimer size increases. The partial increase in silencing at the octamer level could arise from the apparently greater distribution throughout the whole kidney.

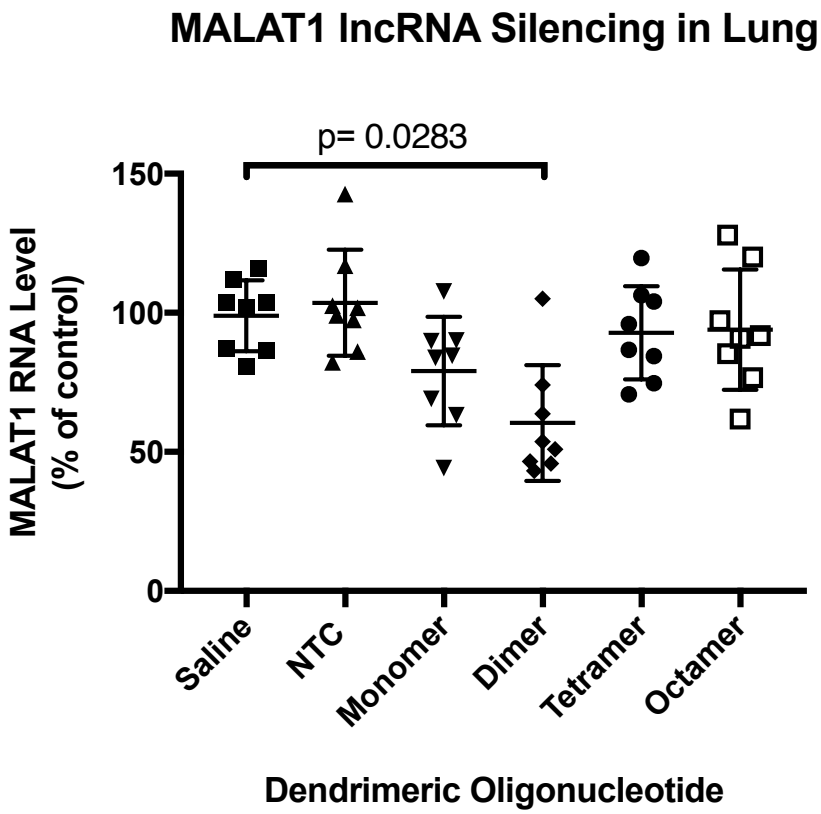


Figure 3.48: MALAT1 silencing for different dendrimers in the lung.
RNA level was quantitated using Affymetrix Quantigene 2.0 and normalised to HPRT (Section 3.9.2.3).

Silencing in the lung followed an unpredictable pattern; increasing from monomer to dimer and dropping off for tetramer and octamer. While the monomer and dimer groups are not statistically significant from one another, the dimer group is the only group that shows significance from the NTC with 40% silencing of the target lncRNA. To achieve 40% silencing with $10\,\mathrm{mg\,kg^{-1}}$ of a systemically-administered MOE gapmer is an impressive feat, made more remarkable by the fact that specifically the dimeric structure espouses it. This result is particularly encouraging because of ongoing work in the group concerning asthma.[269] With the lead-compound already identified, dimerisation and systemic screening *in vivo* should shed some light on the potential therapeutic implications of the dimeric structure.

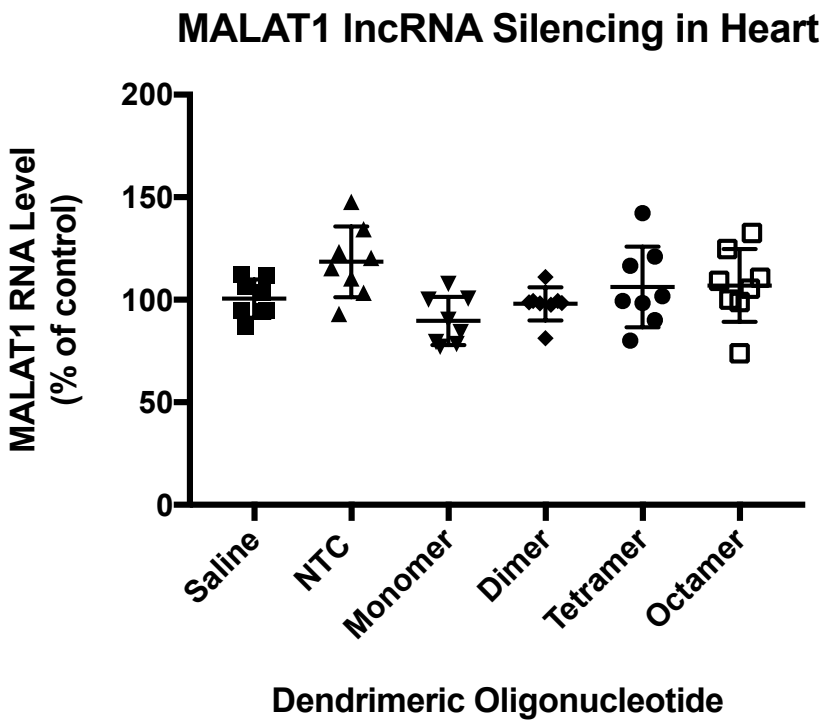


Figure 3.49: MALAT1 silencing for different dendrimers in the heart.
RNA level was quantitated using Affymetrix Quantigene 2.0 and normalised to HPRT (Section 3.9.2.3).

Unsurprisingly, in the heart there was no significant efficacy across the spectrum of multimeric oligonucleotides. As has been described in the literature, cardiac muscle is a profoundly challenging target to effectively silence genes in. There is a small, non-significant amount of MALAT1 silencing with monomeric oligonucleotides, however. It is not unreasonable to suggest that perhaps by using more potent oligonucleotide chemistry there may be some potentially significant targets that could be reached in this tissue.

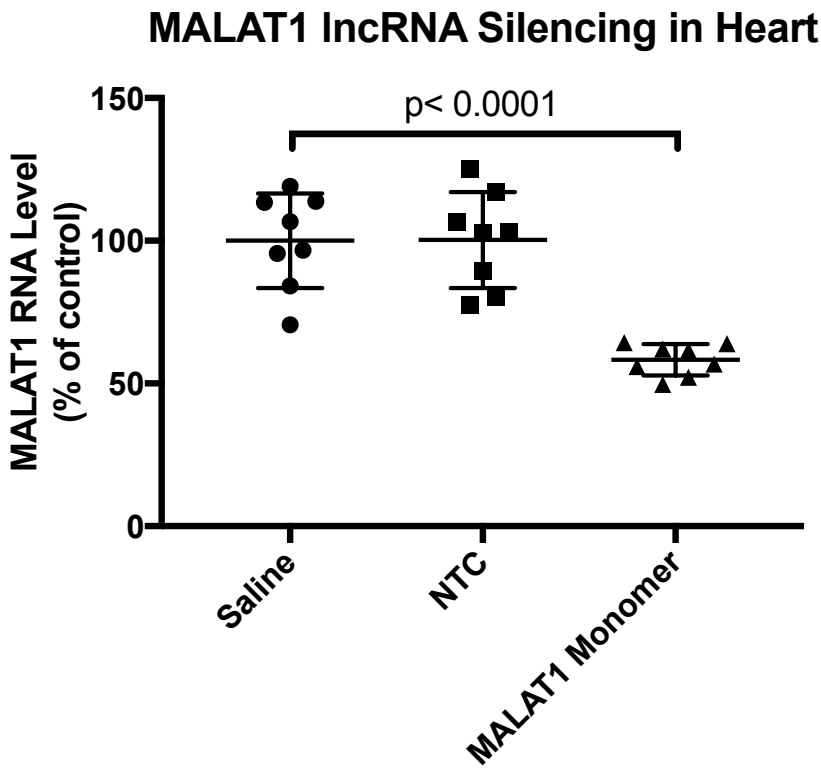


Figure 3.50: MALAT1 silencing for an LNA gapmer in the heart
RNA level was quantitated using Affymetrix Quantigene 2.0 and normalised to HPRT (Section 3.9.2.3).

56% silencing had been obtained in the heart with an LNA-winged monomeric ASO targeting MALAT1 at a $5\,\mathrm{mg\,kg^{-1}}$ dose, administered intravenously (Fig. 3.50).

3.7 Conclusions and Future Work

Conditions have been derived that allow for the solid-phase synthesis of MOE-containing oligonucleotide dendrimers targeting MALAT1. Larger dendrimers have been demonstrated to be taken up more effectively and more quickly in cultured cells, and that they exhibit different profiles of biodistribution in the mouse following intravenous and subcutaneous injection. The ability for dimeric oligonucleotides to effect silencing within the lung at moderate systemic doses, that octameric oligonucleotides are comparable to monomeric oligonucleotides in silencing in the renal cortex and that all tested oligonucleotide multimers are effective in the liver.

While the discrete dendrimers synthesised and tested in this work showed disparate and unexpected ability to penetrate cultured cells, the silencing activity *in vivo* was only significantly improved in the instance of the dimeric structure relative to the monomeric structure in the lung. Despite clearly significant changes in biodistribution following intravenous administration, silencing efficacy did not correlate. One presumptive hypothesis to explain these apparently discordant results is that while dendrimers seem to be well taken up into cells, that the uptake is less productive, that is that the oligonucleotides are less-able to engage their targets, either owing to their chemical nature or to their sub-cellular distribution. It is important to note that the additional challenges presented with the synthesis of these dendrimers are disproportionate to any gain seen in silencing ability. In almost all assays, the monomeric oligonucleotide was the most potent and effected silencing to the greatest extent. One potential solution to this is to make the dendrimers biocleavable, such that when they enter in to cells, the individual oligonucleotide arms separate from the core and are then free to be trafficked as a normal antisense oligonucleotide. The use of biocleavable chemistry has been described in previous oligonucleotide dendrimer work and remains a potentially very interesting use of this multimer technology.[183] It would be beneficial to understand the mechanism of cellular uptake utilised by these dendrimers and indeed to examine whether it differs between dendrimer sizes.

3.8 Experimental methods

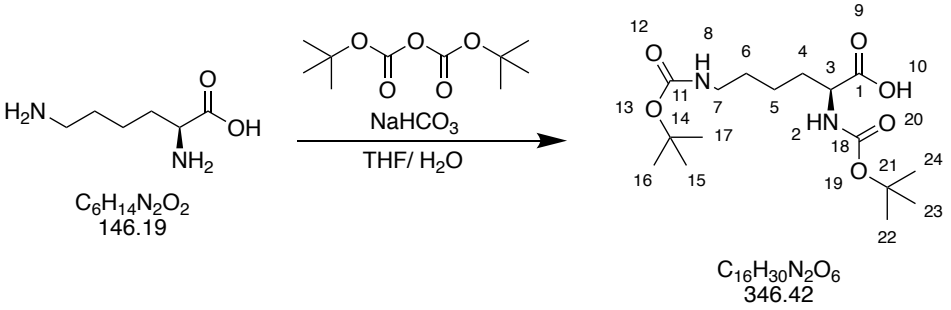
3.8.1 Chemical Methods

All reagents were purchased from Aldrich, Fisher, Alfa or Fluorochem and used without further purification unless otherwise stated. TLC was conducted on fluorescent silica plates from Merck and visualised with both U.V. and $KMnO_4$ or vanillin stains. Column Chromatography was conducted using Silica purchased from Merck or on a Biotage Isolera 4 automated column chromatography instrument using either SNAP or SNAP ULTRA pre-packed cartridges with either a linear or step gradient.

NMR spectra were recorded on Bruker AVII400, Bruker AVIIIHD400 or Bruker AVIIIHD500 FT-NMR spectrometers in the indicated solvent at 298 K. Chemical shifts for proton and carbon spectra are reported on the delta scale in ppm.

Samples were analysed using a Waters (Manchester, UK) TQD mass spectrometer equipped with a triple quadrupole analyser. Samples were introduced to the mass spectrometer via an Acquity H-Class quaternary solvent manager (with TUV detector at 254nm, sample and column manager). Ultra-performance liquid chromatography was undertaken via a Waters BEH C18 column (50 mm x 2.1mm 1.7µm). Low resolution mass spectra were recorded using positive ion electrospray ionisation, using a gradient of 20% acetonitrile (0.2% formic acid) to 100% acetonitrile (0.2% formic acid) in 5 min at a flow rate of 0.6 mL/min. Alternatively, low-resolution mass spectra were recorded using negative ion electrospray ionisation using a gradient of 20% acetonitrile (0.2% ammonium hydroxide) to 100% acetonitrile (0.2% ammonium hydroxide) in 5 min at a flow rate of 0.6 mL/min.

3.8.1.1 (*S*)-2,6-Bis((tert-butoxycarbonyl)amino)hexanoic acid



L-lysine HCl (5.0 g, 27.3 mmol) was dissolved in H_2O (50 mL) with $NaHCO_3$ (6.9 g, 82.1 mmol). Boc_2O (11.25 mL, 98.25 mmol) was added as a solution in THF (50 mL) producing a cloudy solution which was allowed to stir at RT for 20 h. The THF was removed from the reaction *in vacuo* and the remaining aqueous phase washed (Et_2O) and acidified to pH 3-4 (citric acid). Finally, the aqueous phase was extracted (DCM), the organics pooled and dried ($MgSO_4$) and the solvent removed *in vacuo* forming a white, brittle foam under high-vacuum.

Yield: 6.3 g (18.3 mmol, 67%)

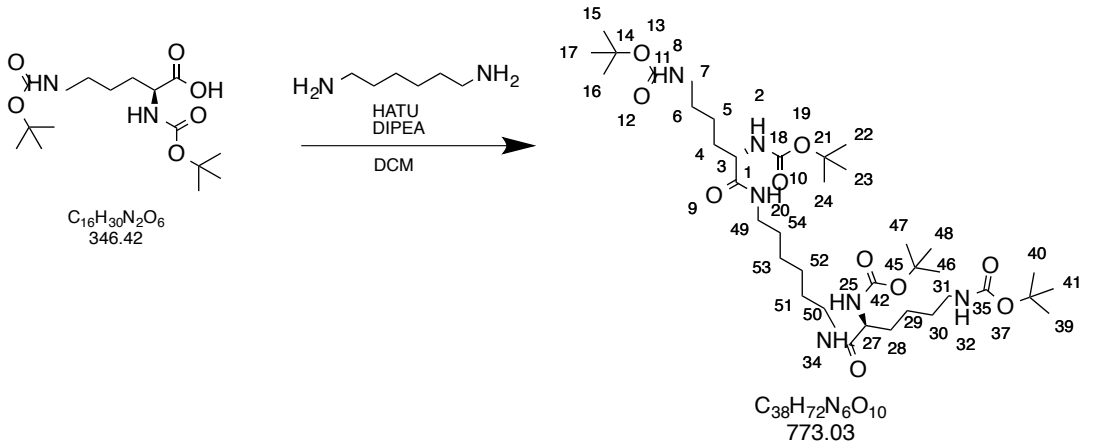
^{1}**H NMR** (400 MHz, d-6-DMSO) δ 6.99 (1H, br d, J = 7.9 Hz, H-10), 6.76 (1H, br t, J = 5.2 Hz, H-2), 3.77 - 3.89 (1H, m, H-3), 2.82 - 2.95 (2H, m, H-7, 8), 2.51 (1H, dt, J = 3.5 Hz, J = 1.7 Hz, H-7), 1.47 - 1.71 (2H, m, H-4), 1.38 (21H, d, J = 2.9 Hz, H-17, 16, 15, 22, 24, 23, 6, 5)

^{13}**C NMR** (100 MHz, d-6-DMSO) δ 174.7 (C-1), 156.0 (C-18, 11), 78.4, 77.8, 53.9 (C-3), 31.8 (C-7), 30.9 (C-6), 29.5 (C-4), 28.7 (C-22, 24, 23, 17, 16, 15), 23.4 (C-5)

LRMS (ESI) m/z= 347.0 $[M+H]^+$

The above analytical data are in agreement with the literature[270]

3.8.1.2 Tetra-tert-butyl ((5*S*,5'*S*)-(hexane-1,6-diylbis(azanediyl))bis(6-oxohexane-6,5,1-triyl))tetracarbamate



HATU (1.14 g, 3 mmol), DCM (10 mL) and DIPEA (1.04 mL, 5 mmol) were added to a flask followed by (S)-2,6-bis((tert-butoxycarbonyl)amino)hexanoic acid (1.00 g, 2.88 mmol) and all was allowed to stir at RT for 15 min. 1,6-diaminohexane (140 mg, 1.2 mmol) was added as a solution in DCM (5 mL) producing a yellow solution. All was left to stir at RT for 20 h, after which the solvents were removed *in vacuo*. The residue was taken up into water and extracted with DCM, which was subsequently washed with water and brine and dried ($MgSO_4$), the produced solid was purified by column chromatography (10% MeOH in DCM) to yield a white powder.

Yield: 1.11 g (1.42 mmol, wet)

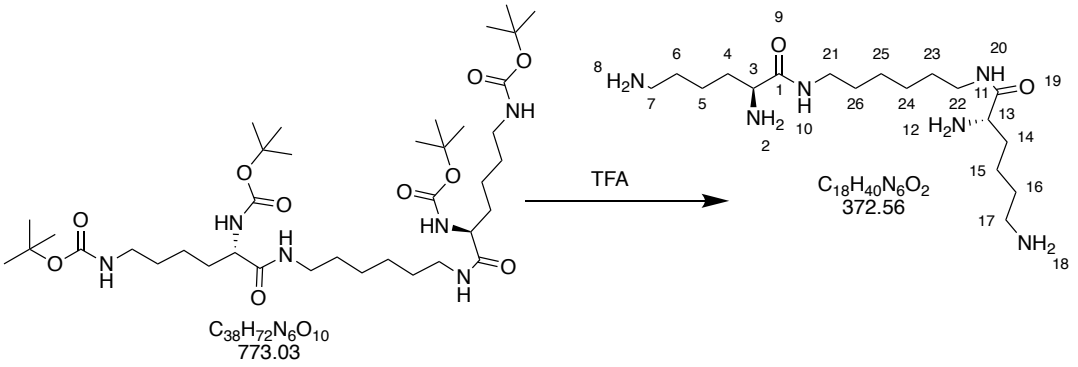
^{1}**H NMR** (400 MHz, d-6-DMSO) δ 6.81 (1H, br s), 5.47 - 5.63 (2H, m), 4.74 (2H, br s,), 4.09 (2H, br s, H-9, 34), 2.97 - 3.23 (7H, m), 2.06 (4H, s), 1.21 - 1.93 (49H, m)

^{13}**C NMR** (100 MHz, d-6-DMSO) δ 174.8, 172.7 (C-19, 32), 156.0 (C-4, 15, 45, 36), 46.3, 32.1, 29.5, 28.4, 28.3, 25.4, 22.7, 20.9, 8.6

LRMS (ESI) m/z= 773.5 $[M+H]^+$

The above analytical data are in agreement with the literature[271]

3.8.1.3 (2*S*,2'*S*)-*N*,*N*'-(hexane-1,6-diyl)bis(2,6-diaminohexanamide)



Tetra-tert-butyl ((5S,5′S)-(hexane-1,6-diylbis(azanediyl))bis(6-oxohexane-6,5,1-triyl))tetracarbamate (1.1060 g, wet from previous reaction) was dissolved in TFA (10 mL) and all allowed to stir at RT for 2 h. The solution was precipitated into Et_2O (400 mL) and the formed white solid collected by filtration. It was allowed to remain damp with Et_2O and dried under high vacuum to give a fine white powder.

Yield: 530.3 mg (1.35 mmol, wet)

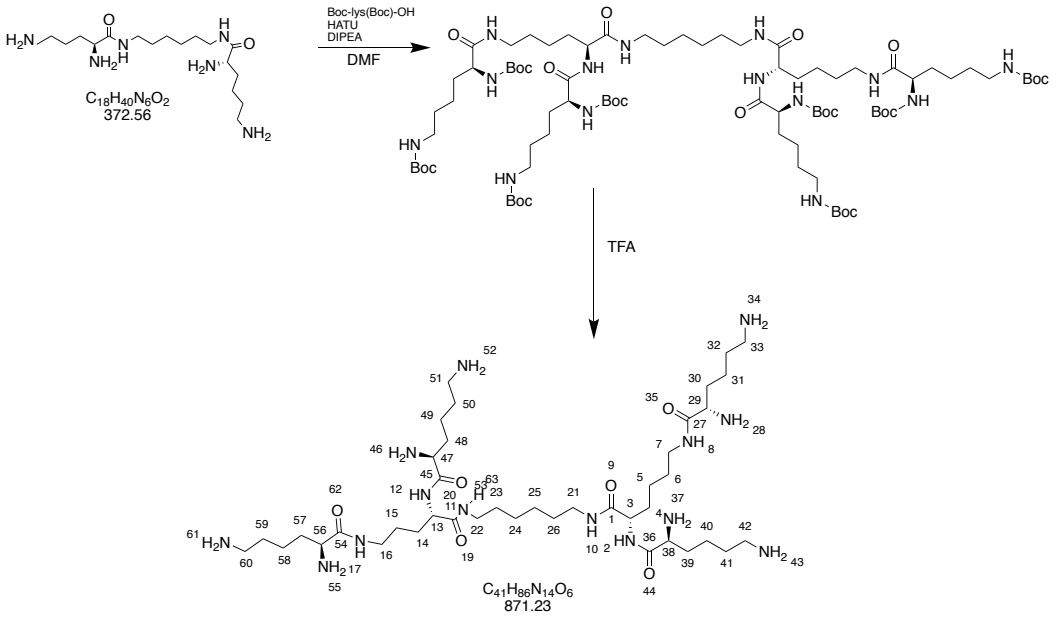
^{1}**H NMR** (400 MHz, D_2O) δ 3.85 (2H, t, J = 6.7 Hz, H-13, 3), 3.09 - 3.22 (4H, m, H-21, 26), 2.92 (4H, t, J = 8.4 Hz, H-7, 17), 1.75 - 1.89 (4H, m, H-14, 4), 1.63 (4H, quin, J = 7.8 Hz, H-16, 6), 1.44 (21H, dq, J = 7.1 Hz, J = 6.2 Hz, H-25, 22), 1.36 (4H, tt, J = 8.8 Hz, J = 7.5 Hz, H-5, 15), 1.20 - 1.30 (4H, m, H-24, 23)

^{13}**C NMR** (100 MHz, D_2O) δ 169.3 (C-1, 11), 117.8, 114.9, 66.0, 53.1 (C-13, 3), 39.5 (C-21, 26), 39.0 (C-7, 17), 30.3 (C-14, 4), 28.1, (C-25, 22), 26.3 (C-16, 6), 25.6 (C-24, 23), 21.3 (C-5, 15)

LRMS (ESI) m/z= 373.2 $[M+H]^+$

Novel compound, no data available

3.8.1.4 OctaAmine dendrimer



Boc-lys(Boc)-OH (866 mg, 2.5 mmol) was added to a solution of HATU (114 mg, 3 mmol) in DMF (10 mL). DIPEA (1.5 mL, 5 mmol) was added, producing a light yellow colour. All was allowed to stir at RT for 10 min under argon. (2S,2'S)-N,N'-(hexane-1,6-diyl)bis(2,6- diaminohexanamide) (186.3 mg, 0.5 mmol) in DMF (10 mL) was added, darkening the solution. The reaction was left to stir overnight and then poured into H_2O (150 mL) and extracted with DCM (3 x 50 mL). The organic phase was washed with 10% citric $acid_{aq}$, $NaHCO_3$ and brine. The solvent was removed *in vacuo* and the residue immediately dissolved in TFA and left to stir at RT for 90 min. The solution was precipitated into Et_2O and the residue filtered.

Yield: 157.2 mg (35%)

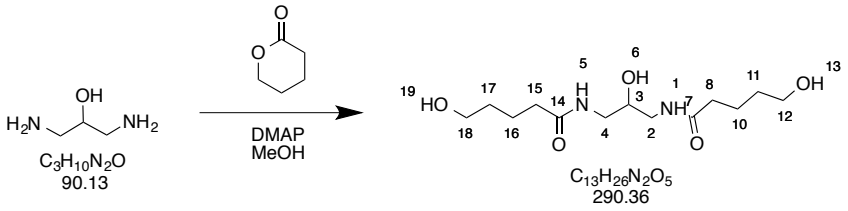
^{1}**H NMR** (400 MHz, D_2O) δ 3.91 (4H, t, J = 6.6 Hz, H-47), 3.25 - 3.42 (8H, m), 2.95 (8H, t, J = 7.7 Hz, H-51), 1.76 - 1.94 (8H, m, H-48), 1.66 (8H, quin, J = 7.7 Hz, H-50), 1.33 - 1.45 (8H, m, H-49), 1.25 - 1.31 (4H, m)

^{13}**C NMR** (100 MHz, D_2O) δ 169.8 (C-45), 53.1 (C-47), 38.9 (C-51), 38.5, 30.3 (C-48), 26.3 (C-50), 21.3 (C-49), 17.7, 16.2

LRMS (ESI, MeOH) m/z= 885.7 $[M+H]^+$

Novel compound, no data available

3.8.1.5 Doubler Alcohol



1,3-diamino-2-propanol (1 eq., 1.80 g, 20 mmol) was dissolved in MeOH (30 mL) and δ-valerolactone (2.2 eq., 4.40 g, 4 mL, 43.9 mmol) was added with DMAP (1 %, 24.4 mg, 0.2 mmol). The mix was bought to reflux overnight. The following morning, excess solvent was removed *in vacuo* and the resulting material dried *in vacuo*, the excess solvent evaporated leaving behind a thick oil that solidified on standing. The mass was broken up and boiled in DCM (50 mL) then filtered to give the final compound as a white solid.

Yield: 5.75 g (19.8 mmol. 99%)

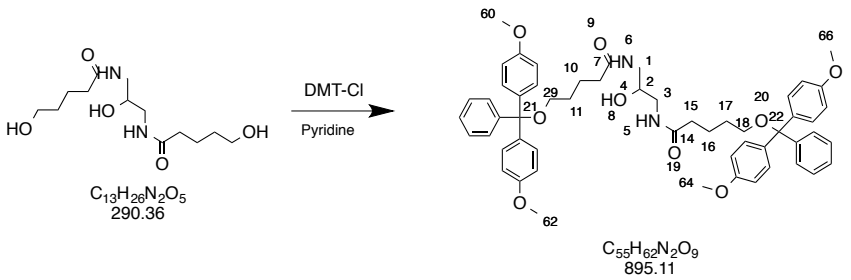
^{1}H NMR (400 MHz, d-6 DMSO) δ 7.76 (2H, br t, J = 5.7 Hz, H-1, 5), 4.96 (1H, br s, M09), 4.36 (2H, br s, H-19, 13), 3.49 (1H, t, J = 5.5 Hz, H-3), 3.37 (3H, br s, H-12, 18), 3.02 (4H, tdd, J = 19.5 Hz, J = 13.4 Hz, J = 5.8 Hz, H-2, 4), 2.08 (3H, t, J = 7.4 Hz, H-8, 15), 1.45 - 1.58 (4H, m, H-10, 16), 1.35 - 1.43 (3H, m, H-11, 17)

^{13}C NMR (100 MHz, d-6 DMSO) δ 173.0 (C-7, 14), 69.2 (C-3), 60.9 (C-12, 18), 43.3 (C-2, 4), 35.7 (C-8, 15), 32.5 (C-11, 17), 22.4 (C-10, 16)

LRMS (ESI) m/z= 291 $[M+H]^+$, 313 $[M+Na]^+$

The above analytical data are in agreement with the literature[158]

3.8.1.6 DMT Doubler alcohol



DMT-Cl (6.777 g, 20 mmol) was dissolved in pyridine (30 mL) under argon. The doubler alcohol (2.612 g, 9 mmol) was slurried into pyridine (20 mL) and added to the DMT-Cl solution. The mixture was allowed to react overnight at room temperature. The following morning, the solvents were removed *in vacuo* and the residue dissolved in DCM. The organic phase was washed with water and then brine before being dried (Na_2SO_4) and the solvent was removed *in vacuo*. The residue was purified using column chromatography (5% MeOH in DCM) to give the product as a light yellow foam.

Yield: 4.167 g (4.66 mmol, 52%)

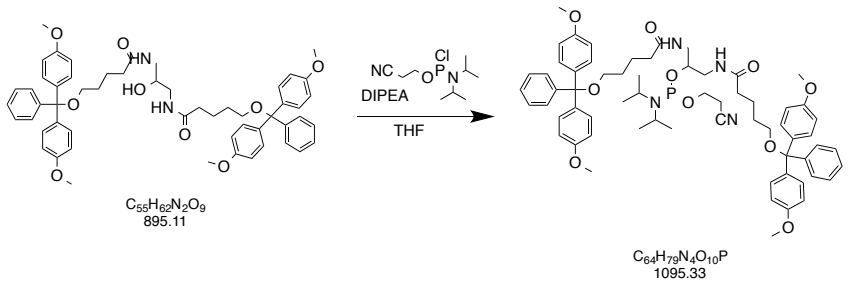
^{1}**H NMR** (400 MHz, DMSO-d_6) δ p7.73 (2H, t, J = 5.8 Hz, H-6, 5), 7.27 - 7.42 (11H, m), 7.23 (8H, d, J = 8.9 Hz), 6.88 (8H, d, J = 8.8 Hz), 4.92 (1H, d, J = 4.9 Hz, H-4), 3.73 (12H, s, O-C$\mathbf{CH_3}$), 3.44 - 3.54 (1H, m, H-2), 2.96 - 3.10 (4H, m, H-3, 1), 2.89 - 2.96 (3H, m, H-18, 29), 1.90 - 2.24 (4H, m, H-15, 7), 1.53 (9H, br s, H-17, 11, 16, 10)

^{13}**C NMR** (100 MHz, DMSO-d_6) δ 172.7 (C-14, 9), 158.4 (C-59, 65, 43, 30), 145.7 (C-68, 46), 136.5 (C-62, 56, 33, 40), 130.0, 128.1, 124.4, 113.6, 85.6 (C-21, 22), 69.1 (C-2), 63.1 (C-18, 29), 55.5, 43.2 (C-3, 1), 35.7 (C-15, 7), 29.7 (C-17, 11), 22.8 (C-16, 10)

LRMS (ESI) m/z= 918 $[M+Na]^+$

The above analytical data are in agreement with the literature[158]

3.8.1.7 DMT Doubler amidite



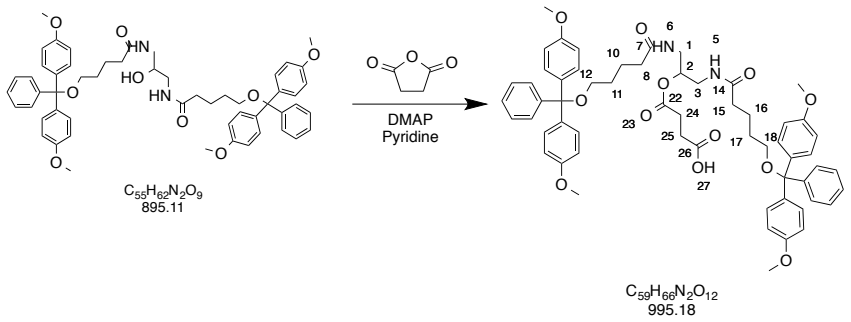
The DMT doubler (2.596 g, 2.9 mmol) was dissolved in THF (20 mL) under argon. DIPEA (1.28 mL, 7.5 mmol) was added followed by 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (1.000 g, 4.48 mmol) and the mixture allowed to stir at room temperature for 2 h. Neutralised EtOAc (10 mL) was added and the mixture washed with degassed KCl $_{(sat)(aq)}$ (3 x 20 mL) before drying (Na_2SO_4). The solvent was removed *in vacuo* to give the product as a white, collapsable foam. The foam was dissolved in DCM (5 mL) and dropped into hexane (200 mL) to give an oily material which was re-dissolved in DCM and re-concentrated *in vacuo* to provide the product as a crisp white foam.

Yield: 2.305 g (2.1 mmol, 72%)

^{31}**P NMR**(135 MHz, CD_3CN) δ 149.09

The above analytical data are in agreement with the literature[158]

3.8.1.8 DMT Doubler Succinic acid monoester



5-[bis(4-methoxyphenyl)(phenyl)methoxy]-N-(3-5-[bis(4-methoxyphenyl)(phenyl)methoxy]pentanamido-2-hydroxypropyl)pentanamide (1 eq., 895 mg, 1 mmol) and DMAP (10 mol%, 12.2 mg, 0.1 mmol) were dissolved in pyridine (10 mL) and succinic anhydride (2.1 eq., 210 mg, 2.1 mmol) was added. The mixture was allowed to react at RT. TLC was taken at 45 min. Some product was noticed, but significant starting material remained. The reaction was allowed to proceed overnight. The following morning TLC indicated that starting material was still present. Succinic anhydride (300 mg, 3 eq) was added and the reaction allowed to proceed for 2 further days. Excess pyridine was removed *in vacuo* and the residue taken into EtOAc (50 mL). The aqueous phase was washed with water (3 x 50 mL), sat. aq. $NaHCO_3$ (50 mL) and brine (50 mL) before being dried (Na_2SO_4) and concentrated *in vacuo*. The product was isolated as an off white foam.

Yield: 943 mg (948 umol, 95%)

^{1}**H NMR** (400 MHz, $CDCl_3$) δ 8.53 - 8.65 (2H, m, H-5, 6), 7.38 - 7.48 (5H, m), 7.17 - 7.38 (25H, m), 6.78 - 6.87 (11H, m), 4.75 - 4.88 (1H, m, H-2), 3.78 (12H, s), 3.47 - 3.58 (2H, m), 3.18 - 3.31 (2H, m), 3.01 - 3.10 (5H, m, H-18, 12), 2.98 (3H, s), 2.58 -2.68 (2H, m, H-25), 2.48 - 2.58 (2H, m, H-24), 2.11 - 2.21 (4H, m, H-15, 8), 1.51 - 1.78 (9H, m, H-17, 16, 11, 10)

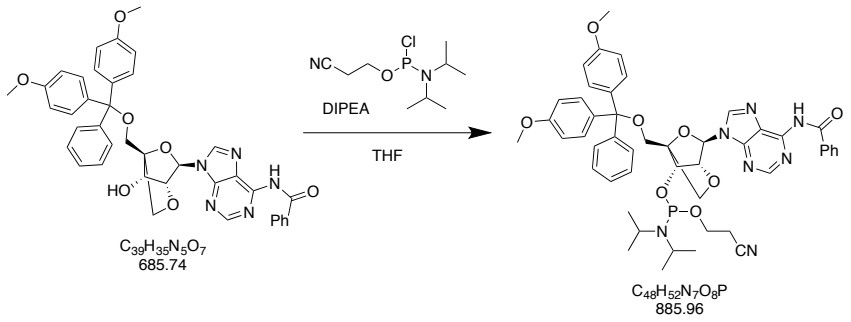
^{13}**C NMR** (100 MHz, $CDCl_3$) δ 175.6 (C-26), 174.1, 171.9 (C-22), 170.5, 158.6, 158.3, 149.0, 145.3, 139.5, 136.7, 136.6, 130.1, 130.0, 129.1, 128.2, 127.8, 127.8, 127.7, 127.1,

126.6, 124.0, 113.2, 113.0, 85.8, 81.4, 71.4 (C-2), 62.9 (C-18, 12), 55.3, 55.2, 38.5, 36.4 (C-15, 8), 29.8, 29.8 (C-25), 29.6 (C-17, 16, 11, 10, 24), 28.4, 22.6

LRMS (ESI) m/z= 1018 $[M+Na]^+$

Novel compound, no data available

3.8.1.9 LNA A



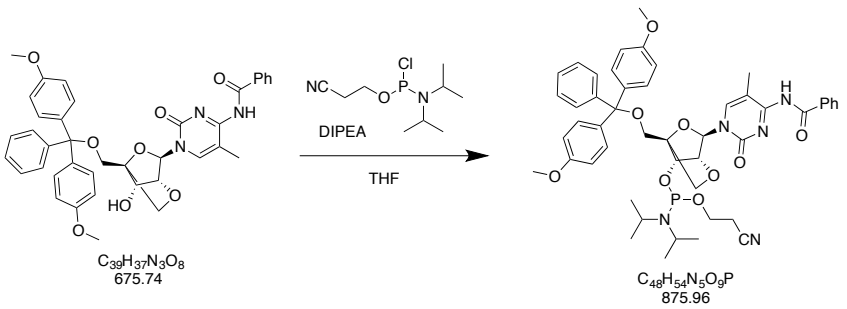
LNA-A-OH (1 eq., 1.36 g, 1.99 mmol) was dried *in vacuo* overnight and then dissolved in THF (14.1 mL) under argon. DIPEA (3 eq., 0.77 g, 0.985 mL, 5.96 mmol) was added followed by 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (1.2 eq., 0.564 g, 0.532 mL, 2.38 mmol). The clear solution was allowed to react at RT. After 3 h, additional 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (75 μL) was added. The reaction mixture was transferred to an argon purged separatory funnel and diluted with neutralised EtOAc (25 mL). The organics were washed with sat $NaHCO_3$ (2 x 20 mL) and sat KCl (3 x 20 mL), the solvents were dried (Na_2SO_4) and concentrated in vacuo to give a crisp foam which was purified by column chromatography.

Yield: 857 mg (968 μmol, 49%)

^{31}**P NMR**(135 MHz, CD_3CN) δ 150.47, 150.0

No data available

3.8.1.10 LNA C



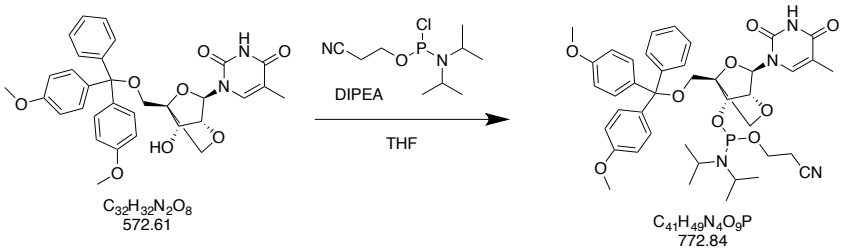
LNA-C-OH (1 eq., 1.9 g, 2.82 mmol) was dissolved in THF (25 mL) and DIPEA (3 eq., 1.09 g, 1.4 mL, 8.45 mmol) was added. 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (1.5 eq., 1 g, 0.943 mL, 4.23 mmol) was added slowly under argon and all left to stir at RT under argon. The reaction mixture was transferred to an argon purged separatory funnel and diluted with neutralised EtOAc (25 mL). The organics were washed with sat $NaHCO_3$ (2 x 20 mL) and sat KCl (3 x 20 mL), the solvents were dried (Na_2SO_4) and concentrated in vacuo to give a crisp light yellow foam.

Yield: 2.269 g (2.59 mmol, 92%)

^{31}**P NMR**(135 MHz, CD_3CN) δ 150.54, 149.55

No data available

3.8.1.11 LNA T



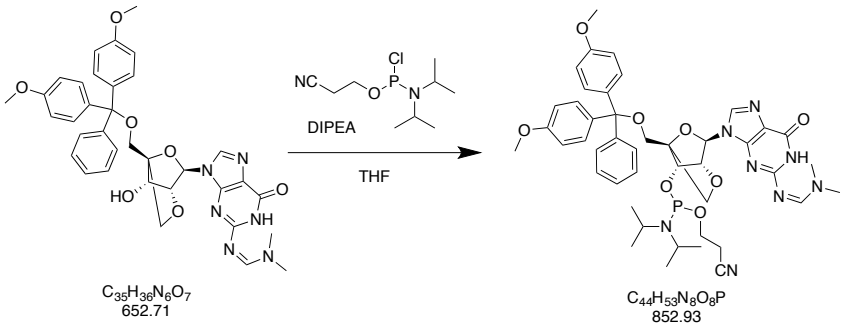
LNA-T-OH (1 eq., 2.02 g, 3.52 mmol) was dried under hi vac overnight and then dissolved in THF (25 mL) under argon forming a slightly cloudy solution. DIPEA (3 eq., 1.37 g, 1.75 mL, 10.6 mmol) was added followed by 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (1.2 eq., 1 g, 0.943 mL, 4.23 mmol) and all allowed to react at RT. At 3 h, additional 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (150 μL) was added. The reaction mixture was transferred to an argon purged separatory funnel and diluted with neutralised EtOAc (25 mL). The organics were washed with sat $NaHCO_3$ (2 x 20 mL) and sat KCl (3 x 20 mL), the solvents were dried (Na_2SO_4) and concentrated in vacuo to give a crisp foam.

Yield: 2.486 g (3.22 mmol, 91%)

^{31}**P NMR**(135 MHz, CD_3CN) δ 150.22, 149.64

No data available

3.8.1.12 LNA G



LNA-G-OH (1 eq., 1.84 g, 2.82 mmol) was dissolved in a mixture of THF (25 mL) and DMF (8 mL) and DIPEA (3 eq., 1.09 g, 1.4 mL, 8.45 mmol) was added. 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (1.5 eq., 1 g, 0.943 mL, 4.23 mmol) was added dropwise and the mixture left to stir at RT under argon. The reaction mixture was transferred to an argon purged separatory funnel and diluted with neutralised EtOAc (25 mL). The organics were washed with sat $NaHCO_3$ (2 x 20 mL) and sat KCl (3 x 20 mL), the solvents were dried (Na_2SO_4) and concentrated in vacuo to give a crisp white foam.

Yield: 2.302 g (2.699 mmol, 96%)

^{31}P NMR(135 MHz, CD_3CN) δ 149.83 (presumably overlapped diastereoisomers)

No data available

3.8.2 Oligonucleotide Synthesis

LNA phosphoramidites were synthesised in house. MOE, DNA and other non-nucleoside phosphoramidites were purchased (ChemGenes). Modified nucleoside phosphoramidites (LNA or MOE) were coupled for 10 min and standard DNA phosphoramidites for 1 min.

Intermediate scale (1 µmol) oligonucleotides were synthesised on an ABI394 oligonucleotide synthesiser on specified CPG (ChemGenes, packed into twist top columns). 0.1M DDTT (ChemGenes) was used as the sulfurising reagent and 0.25M BTT (AIC) as the activator. Oligonucleotides were deprotected using concentrated ammonia at 55 °C for 18 h and purified using ion-exchange chromatography (eluting with 30% acetonitrile in water containing increasing gradients of $NaClO_4$). Oligonucleotides has their identity confirmed by LCMS and were purified by diafiltration in an Amicon spin filter.

In vivo scale oligonucleotides were synthesised either at 10 µmol using an AKTA Oligopilot 10 (GE Life Sciences) or 100 µmol using an AKTA Oligopilot 100 (GE Life Sciences). 0.1M DDTT (ChemGenes) was used as the sulfurising reagent and 0.25 M BTT (AIC) as activator. The oligonucleotide was deprotected using concentrated ammonia at 55 °C for 18 h and purified using ion-exchange chromatography (eluting with 30% acetonitrile in water containing increasing gradients of $NaClO_4$). The purified oligonucleotide was dialysed and concentrated using Amicon spin filters.

3.9 Biological Experimental

3.9.1 *In Vitro* Screening

Mouse embryonic fibroblast (MEF) WT cells were purchased from ATCC and cultured in regular DMEM supplemented with 10 % FBS. Cells were maintained in 5% CO_2 humidified incubator.

For lipid transfection, MEF cells were plated in 96 well plates at ca. 8000 cells/well at least 16 h prior to transfection. Cells were transfected with oligonucleotides using Lipofectamine RNAiMax reagent, then harvested 16 hours post transfection. MALAT1 lncRNA levels were quantified using the Quantigene 2.0 assay kit (Affymetrix, QS0011).

3.9.2 Mouse Work

Female FVB/NCr mice were obtained from Charles River Laboratories at 8 weeks of age and were maintained on standard rodent chow and acidified water *ad libitum*. Sodium pentobarbital (>800 mg/kg as Fatal Plus) was administered intraperitoneally as a dilution in saline for terminal anaesthesia. Oligonucleotides were administered intravenously using the lateral tail vein, or subcutaneously over the flank as dilutions in saline.

3.9.2.1 Euthanasia and Tissue Harvest

Sodium pentobarbital (>800 mg/kg as Fatal Plus administered intraperitoneally as a dilution in saline) or an isoflurane overdose was used for terminal anaesthesia. Animals were immediately perfused transcardially (chest fur was wetted with 70% EtOH, thoracic incision made and diaphragm cut to expose the heart. The right atrium was snipped and perfusate introduced through a needle in the left ventricle).

3.9.2.2 FFPE-IF

Following PBS perfusion, 10% neutral buffered formalin (Sigma) was perfused until fixation was complete. Tissues of interest were excised and post-fixed in NBF at 4 °C for at least 24 h. Once the tissue was removed from NBF, it was placed in a cassette, paraffin embedded and sectioned to 4 µm before mounting to charged slide and dewaxing at 60 °C for 1 h. Slides were then deparaffinized and rehydrated (two washes in xylene, two washes in 100% ethanol, two washes in 95% ethanol, one wash in water).

Slider were treated with 3% H_2O_2 in methanol for 10 min followed by two washes in TBST. Sections were treated with Proteinase K (Dako) for 10 min (Dako) then washed twice with TBST. Sections were treated with Cyto-Q Background Buster (Innovex Biosciences) then washed twice. The diluted antisera was applied (1:500 in 2% BSA 5% normal goat serum in TBST). The slides were incubated with antisera overnight at 4 °C, washed 3 times for 5 min each with TBST, AF488/555/647 goat anti-rabbit IgG (CST) at 1:2000 in 2% BSA 5% normal goat serum in TBST For 1h at RT. The slides were then washed 3 times for 5 min each with TBST. Sections were covered with SignalStain Boost with DAPI and coverslipped with Leica Surgipath glass slides.

3.9.2.3 RNA Quantitation

Oligonucleotide treated animals were euthanised and tissues immediately excised and placed in RNALater. After 24 hours, tissue punches were taken and homogenised (Affymetrix homogenising solution with additional Proteinase K), after homogenisation the lysate was frozen at -80°C. Before RNA quantitation, the lysate was warmed in an oven at 50°C until completely melted. RNA levels were quantitated using the Quantigene 2.0 single-plex bDNA assay from Affymetrix.

3.9.2.4 Microscopy

Fluorescent images were obtained with a Leica DMi8 inverted fluorescence microscope equipped with a DAPI, GFP, Y3 and Y5 filter cube and HC PL FLUOTAR 5x/0.15, HC PL FL 10x/0.3 PH1, HC PLAN APO 20x/0.7 PH2, HC PL FL 20x/0.4 CORR PH1, HCX PL FL L 40x/0.6 CORR PH2, HCX PL APO 40x/1.3 OIL, HCX PL APO 63x/1.4-0.6 OIL objectives.

Chapter 4

Oligonucleotides for *C9ORF72* Repeat-Expanded ALS

4.1 Declaration of Work Done

I synthesised all of the oligonucleotides. *In vitro* experiments for *C9ORF72* work were completed by Helene Tran and all data pertaining to those experiments were generated by her. Animal work was completed by Courtney Pinto and I. Silencing data from these animals was generated by Helene Tran. I generated all of the immunofluorescent immunohistochemical images presented. Toxicological data was obtained from the UMMS Department of Animal Medicine.

4.2 Introduction

Oligonucleotide therapeutics are emerging as a potential tool for treating several neurological diseases of genetic origin. Antisense oligonucleotides have showed consistently that they are able to correct aberrant genetic causes of disease from preclinical animal models though to early clinical trials.

The CNS is the primary extra-hepatic target for Ionis Pharmaceuticals, with disease targets such as Huntington's disease, frontotemporal dementia and Alzheimer's disease among others represented in their pipeline. Ionis' recent and remarkable success with nusinersen, a splice-switching oligonucleotide that allows for production of the correct SMN2 protein in patients with spinal muscular atrophy has served to encourage further research in the field.[105]

A significant number of neurological diseases have a genetic element to them as evidenced by the diversity of pathologies in the pipeline. With the availability of different oligonucleotide chemistries, enabling upregulation, downregulation and splice-correction of aberrant RNA, oligonucleotides present a very flexible platform for therapeutic development in the CNS.

4.2.1 Routes of Administration for Neurotherapeutic Oligonucleotides

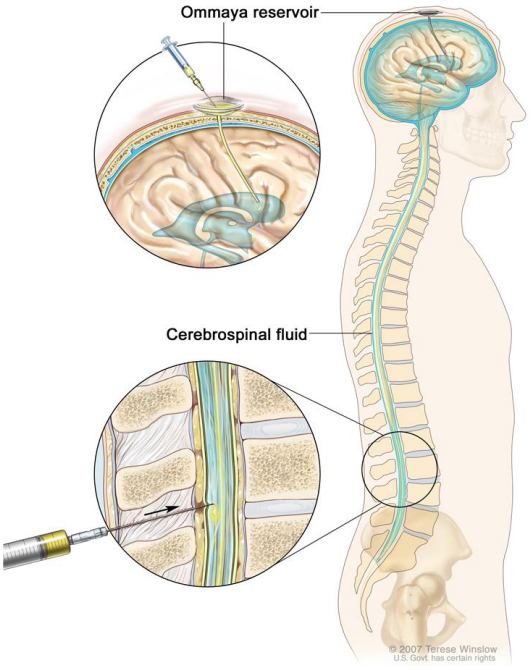


Figure 4.1: Routes of Administration for drug injection into the CSF. For the National Cancer Institute © 2007 Terese Winslow, U.S. Govt. has certain rights. Permission Granted.

One of the major challenges in developing oligonucleotide drugs for neurological applications is that they do not cross the blood-brain-barrier (BBB). The BBB exists as a variety of cells, primarily endothelia, that form tight-knit junctions, separating the brain from circulating blood and extracellular fluid.[272] The main function of the BBB is to prevent the crossing of toxins, pathogens and other unwanted substances from the blood into the brain and thus ensure that CNS homeostasis is maintained, ensuring the correct functioning of neurones.[273] As a result of this, oligonucleotides administered into the blood supply do not generally show any significant uptake or effect within the CNS, except in rare instances and when they are administered in exceptionally high doses.[9] Thus, in order to adequately deliver these molecules to the neurological tissues of interest requires central administration (directly into the cerebrospinal fluid of the patient or experimental animal, see Fig: 4.1[274]). Central administration of drugs is typically achieved using an intrathecal injection, or by implantation of an Ommaya reservoir into the lateral ventricle of the brain (Fig. 4.1). In experimental mice however, the technical challenges associated with an intrathecal injection typically result in the practise of bolus injection into the lateral ventricle (intracerebroventricular, or ICV injection).[275] There are clear anatomical differences between a lumbar intrathecal injection in a human, and an injection into one

of the lateral ventricles in a mouse. Indeed, safety and tolerability studies in non-human primates tend to rely on intrathecal injections to avoid this discrepancy.

4.3 Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis is a progressive neurodegerative disease that results in the death of motor neurones. Patients are usually 50-60 years of age at presentation and diagnosis requires exclusion of other, more common neurological pathologies. While initial symptoms are usually mild (muscle twitching and muscle stiffness), they progress rapidly to include muscle atrophy, dysarthria, dysphagia and dyspnea. Death typically follows 2-4 years after diagnosis as a result of complete respiratory failure. The disease remains relatively rare, with an instance of approximately 2 per 100,000 people in the USA and through Europe. It is nevertheless the most commonly occurring motor neurone disease.

Most cases of ALS are of unknown cause, though some can be traced through familial inheritance, genetic roots for this disease have been identified for small percentages of the patient population in both familial and sporadic ALS. The first, superoxide dismutase 1 (SOD1) was reported in 1993 by Rosen *et al.*,[276] with several others shown to be implicated in small percentages of ALS cases, namely TARDBP[277] and FUS.[278,279]

A significant step in the field of ALS was the discovery of a hexanucleotide 'GGGGCC' repeat expansion in a non-coding region of *C9ORF72*.[135] Neurotypical persons typically have 20-30 repeats of this hexanucleotide, while affected patients can have copy numbers from hundreds to thousands.[280] This mutation is the highest presently known causative genetic origin of ALS, representing approximately 34% of familial ALS and approximately 5% of sporadic ALS.[281]

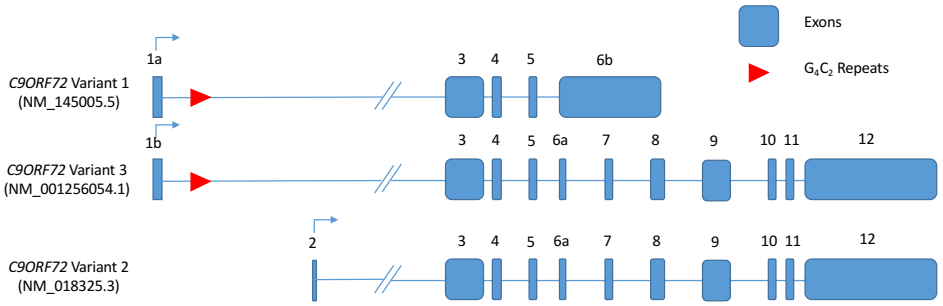


Figure 4.2: A figure showing the three variants of *C9ORF72*.

C9ORF72 is expressed as three different splice variants, V1, V2 and V3. Variants V1 and V3 contain promoters before the repeat expansion and so express the repeat. The repeat-expanded RNAs can accumulate as RNA foci (inducing sequestration of RNA binding proteins), or be translated to produce toxic polydipeptides. The V2 variant has its promotor region after the hexanucleotide repeat and hence is not considered to have a role in ALS pathology.[282,283] The V2 variant is expressed more highly than either V1 or V3.[284]

4.4 Objective

A paper by Ionis recently described antisense oligonucleotide-mediated inhibition of *C9ORF72* in a mouse model of C9-dependent ALS, albeit at high doses and with a single type of chemical modification.[275] Given the promise of antisense technology in the CNS, this work set out to explore whether changes to the chemistry of these oligonucleotides could provide an improvement in therapeutic properties. It was considered that it may be possible to apply design insights learned in the context of *C9ORF72* to work on other neurodegenerative diseases in the future.

For this study, a collaboration was formed with Robert H. Brown Jr., a clinical and academic neurologist at UMMS who specialises in ALS. Over the course of his career, he has discovered several causative genes for ALS[276,278] and other neurodegenerative

diseases.[285–288] He continues to treat ALS patients, and has two patients beginning to show symptoms of *C9ORF72*-ALS. The patients were eager to participate in and fund experimental treatment options. Our collaborator has previously received compassionate use approval[1] from the FDA for a different therapeutic modality, and our efforts were focussed on generating a compound with appropriate potency and therapeutic index to attempt to treat these two patients.

4.5 Screening ASOs

The initial experiments attempted were to screen Ionis' most promising molecules injected alongside LNA – containing analogues of the same. Each of the oligonucletides used in the original Ionis work was termed a 'MOE-Gapmer' with no further information provided. With just this information, it was presumed that the structure was that of a fully phosphorothioate-modified oligomer with 5 MOE nucleotides in each 'wing'.

ASO Name	**Sequence (5′ – 3′)**
Ionis 1	CCCGGCCCCTAGCGCGCGAC
Ionis 2	GCCTTACTCTAGGACCAAGA
Ionis 3	TGGTTAATCTTTATCAGGTC
Ionis 4	ACAGGCTGCGGTTGTTTCCC
Ionis 5	GCCCCTAGCGCGCGACTC
Ionis NTC	CCTATAGGACTATCCAGGAA

Table 4.1: Sequences described by Jiang *et al.* to target the repeat-expanded *C9ORF72* RNA. No modification or backbone pattern were described.

Oligomers **Ionis 1-5** were reported as active sequences, while **Ionis NTC** was their non-targeting control.[275] Our experimental efforts were focussed on sequences based on **Ionis 1** and **5**, by synthesising the following oligonucleotides;

[1] Expanded access, sometimes called 'compassionate use,' is the use outside of a clinical trial of an investigational medical product (i.e., one that has not been approved by FDA).

ASO Name	Sequence (5′ – 3′)
ASO 23	+C*+C*+C*G*G*C*C*C*C*T*A*G*+C*+G*+C
ASO 24	+G*+G*+C*C*C*C*T*A*G*C*G*C*+G*+C*+G
ASO 25	+C*+C*+C*T*A*G*C*G*C*G*C*G*C*G*+A*+C*+T
ASO 30	&G*&C*&C*&C*&C*T*A*G*C*G*C*G*C*G*C*G*+A*+C*+T
ASO 31	&C*&C*&C*&G*&G*C*C*C*C*T*A*G*C*G*C*&G*&C*&G*&A*&C
ASO 32	&G*&C*&C*&C*&C*T*A*G*C*G*C*G*C*&G*&A*&C*&T*&C

Table 4.2: Sequences synthesised in order to test modifications.
'+' indicates an LNA nucleotide, '*' indicates a phosphorothioate linkage and '&' indicates a MOE nucleotide.

It was important that the oligonucleotides designed incorporated clinically relevant modifications such as MOE and LNA. Because no modification pattern was described in the otiginal work, it was decided several analogues of each sequence using both MOE and LNA chemistry should be made. It was presumed that the main modification utilised was MOE owing to the clinically approved drugs from Ionis using this chemistry, but it was also important to explore whether incorporation of the higher binding affinity modification would be well tolerated and potentially more efficacious. Wherein, **ASO 23** is a 3′-truncated form of **Ionis 1**, converted to a more typical 3-9-3 gapmer structure for LNA-containing therapeutic oligonucleotides. **ASO 24** is also a truncated form of **Ionis 1**, but the truncation is from the 5′ end, as opposed to the 3′ end. **ASO 25** is an LNA analogue of **Ionis 5**, while **ASO 30** is a mixed LNA/MOE derivative of **Ionis 5**. **ASO 31** and **ASO 32** correspond identically to the sequences **Ionis 1** and **Ionis 5**, synthesised as MOE gapmers.

4.6 *In Vitro* Silencing

In order to assay the ASOs in a higher throughput setting, as well as to save on animals, initial screening was performed in patient-derived fibroblast cells (Fig. 4.3).

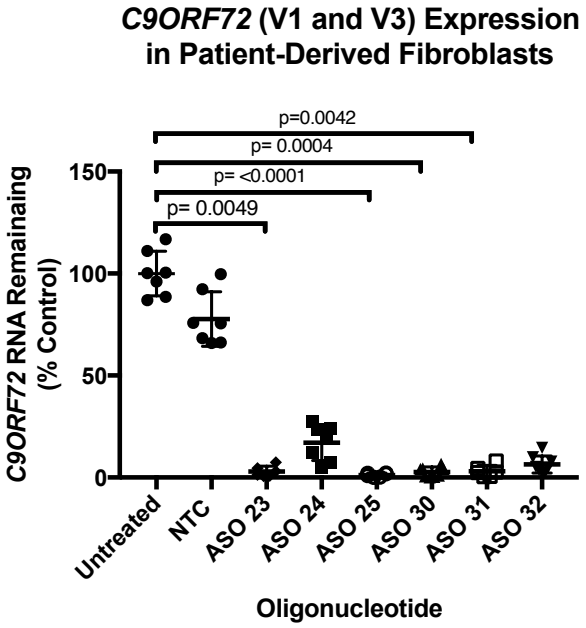


Figure 4.3: *In vitro* silencing data for ASOs 23, 24, 25, 30, 31 and 32 in cultured patient-derived fibroblasts.
Oligonucleotides were used at 100 nM. RNA level was quantitated using qRT-PCR and normalised to GAPDH.

All of the oligonucleotides that were designed were potent in silencing the V1 and V3 transcripts, with four of the six silencing significantly. In our first *in vivo* study (section 4.8.2), each of these oligonucleotides were examined.

4.7 Biodistribution of Oligonucleotides in Mouse CNS

Using the antibody generated as described in Chapter 2, the biodistribution of the oligonucleotides synthesised following intracerebroventricular injection was assessed. Two wild type C57BL/6J mice were injected intracerebroventricularly with a fully phosphorothioate-modified 5-10-5 MOE gapmer. After 48 hours the animals were euthanised and tissue was processed as described in section 4.14.2.4.

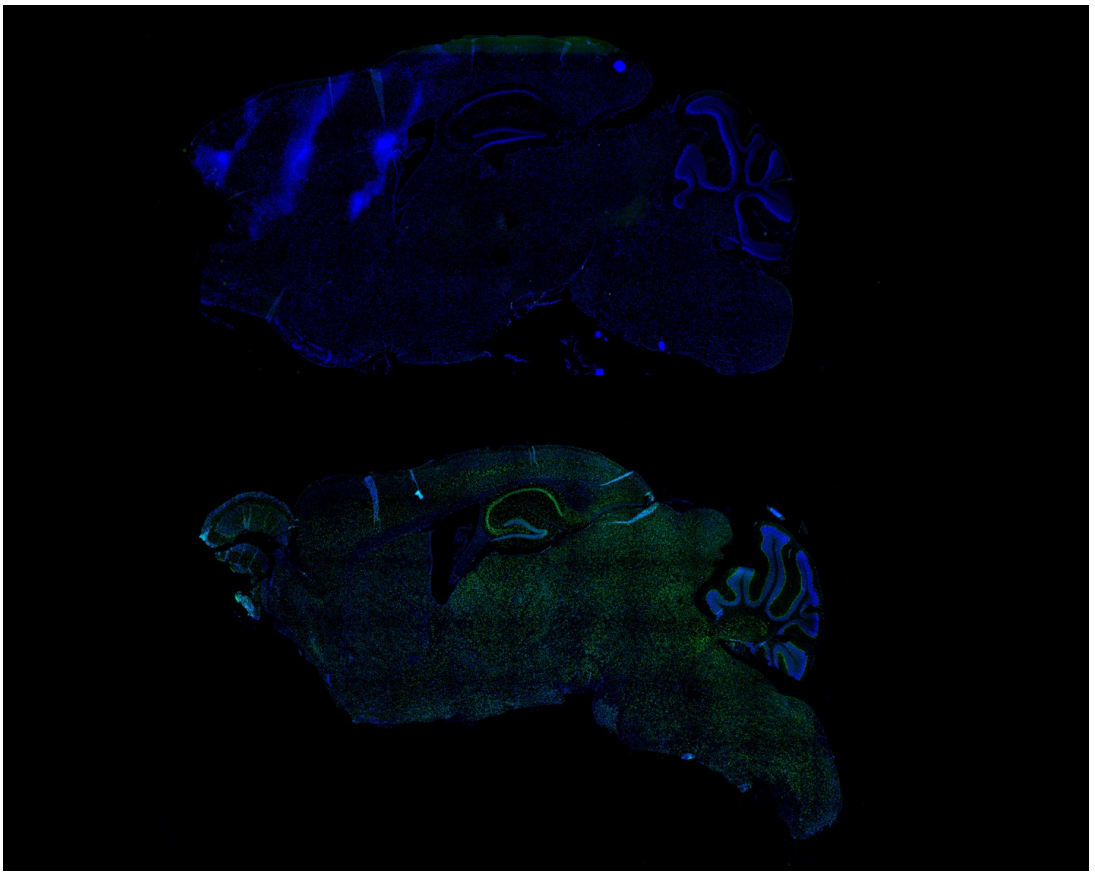


Figure 4.4: Biodistribution of ASO through the mouse brain, saggital section. Top is a PBS treated animal, bottom is ASO-treated.
DAPI is shown in blue and ASO imaged with anti-ASO pAb is shown in green.

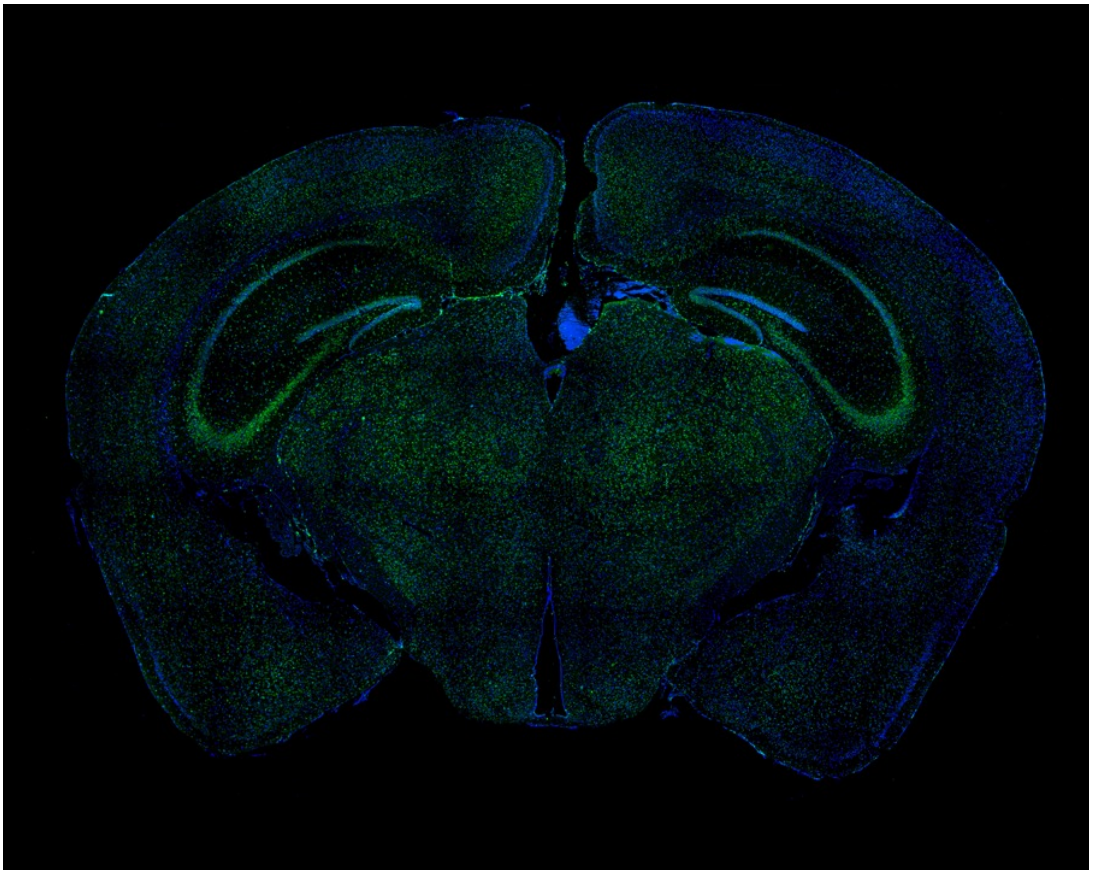


Figure 4.5: Biodistribution of ASO through the mouse brain, coronal section.
DAPI is shown in blue and ASO imaged with anti-ASO pAb is shown in green.

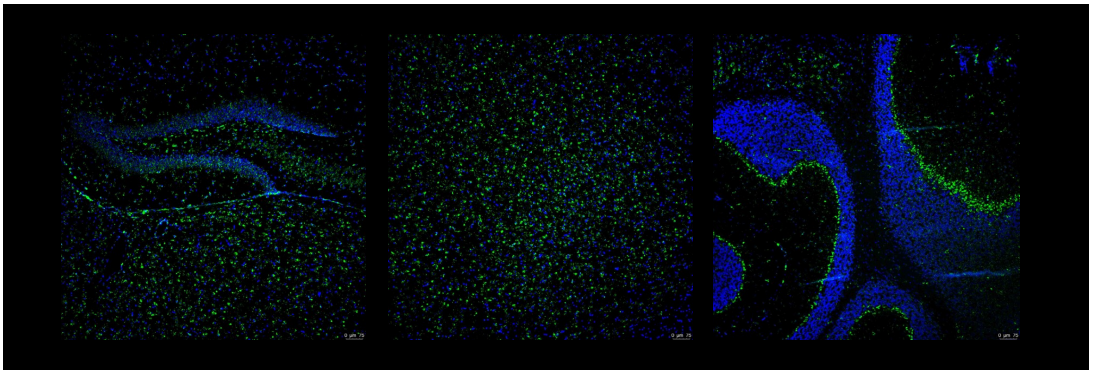


Figure 4.6: Biodistribution of ASO through the mouse brain, coronal section, enhanced to show L to R: Hippocampus and dentate gyrus, thalamus and cerebellum.
DAPI is shown in blue and ASO imaged with anti-ASO pAb is shown in green.

All of the above patterns of biodistribution are consistent with those presented in the literature.[124,230–232]

4.8 *In Vivo* Silencing

4.8.1 *C9ORF72* Transgenic Animals

In order to adequately assess the *in vivo* potency of the oligonucleotides that had been synthesised, a mouse model of the disease was required. The Brown lab had generated a line of mice that express exons 1-6 human *C9ORF72* with approximately 500 GGGGCC repeats.[289] While these animals are not known to exhibit any physical phenotype of ALS/FTD, they recapitulate the molecular features of the disease, such as presence of RNA foci and associated dipeptides.[289] These animals were purpose-bred for these experiments from the original colony of animals generated in the Brown lab.

4.8.2 Some ASOs are Unexpectedly Toxic When Centrally Administered

Jiang *et al.* described that 350 µg of oligonucleotide was injected ICV, as a solution in 10 µl pH 7.4 PBS,[275] and so the above oligonucleotides were diluted to the same concentration and injected ICV into the *C9ORF72* transgenic mice. To our surprise, these animals experienced profound seizure-like activity post-injection (or indeed, during injection in the case of **ASO 30**). Uniformly, each of these animals died without recovering from seizure post-operatively.

4.9 Improving Tolerability

At this point, there were a number of factors that had to be considered in order to attempt to identify the cause of this observed toxicity. Until the issues surrounding toxicity were solbed, it was decided to work in wild type C57BL/6J animals (the genetic background for the transgenic animals) to prevent unnecessary wastage of genetically-modified animals.

4.9.0.1 Immunogenicity

The first cause considered was that 5-methyl-C was not used *in lieu* of DNA C in our oligonucleotides. Oligonucleotides with unmethylated CG dinucleotides are typically able to elicit an immune response through TLR9 activation. It was reasoned that perhaps in some instances at least, a rapid immune signalling cascade could have caused the observed effects, though the onset was more rapid than expected for such a response.

In order to address these issues, these oligonucleotides were resynthesised with 5-methyl-C nucleotides in place of the DNA C nucleotides to produce oligonucleotides that should have diminished immunogenicity. This by itself unfortunately did not improve survival, and the animals died in the same manner as they did with unmethylated C residues. It was felt, however, that the use of 5-methyl-C modifications was an appropriate change and that despite the lack of any obvious improvement in acute toxicological profile it would continue to be used in all future oligonucleotides.

4.9.0.2 LNA Content and Length

It was felt appropriate to reduce the LNA content of our oligonucleotides marginally, considering that the presently available data surrounding constrained nucleic acids indicates generally fewer inclusions per oligonucleotide than was being used in our fledgeling screen. There is also some evidence that longer oligonucleotides possess diminsihed toxicity than shorter oligonucleotides.[290] Thus, the oligonucleotides were

resynthesised from a 3-9-3 format to a 2-16-2 format, both increasing the overall length of the oligonucleotide and decreasing the total LNA content. Finally, it was deemed inappropriate to develop **ASO 30** because of its profound toxicity. This led to the next set of oligonucleotides:

ASO Name	**Sequence (5′ – 3′)**
ASO 66	+C*+C*+C* T*A*G* ^{Me}C*G*^{Me}C* G*^{Me}C*G* +A*+C*+T
ASO 67	+C*+C*^{Me}C*G*G*^{Me}C*^{Me}C*^{Me}C*^{Me}C*T*A*G*^{Me}C*G*^{Me}C*G*^{Me}C*G*+A*+C
ASO 68	+G*+C*^{Me}C*^{Me}C*^{Me}C*T*A*G*^{Me}C*G*^{Me}C*G*^{Me}C*G*A*^{Me}C*+T*+C
ASO 69	&C*&C*&C*&G*&G*^{Me}C*^{Me}C*^{Me}C*^{Me}C*T*A*G*^{Me}C*G*^{Me}C*&G*&C*&G*&A*&C
ASO 70	&G*&C*&C*&C*&C*T*A*G*^{Me}C*G*^{Me}C*G*^{Me}C*&G*&A*&C*&T*&C
ASO 71	&C*&C*&T*&A*&T*A*G*G*A*T*A*T*^{Me}C*^{Me}C*&A*&G*&G*&A*&A

Table 4.3: Sequences of oligonucleotides synthesised after the initial screen.

These new LNA oligonucleotides are direct analogues of the active MOE sequences. **ASO 66** is the same sequence as **ASO 25**, **ASO 67** is the same sequence as **ASO 69** and **ASO 68** is the same sequence as **ASO 70**. **ASO 71** is the ^{Me}C equivalent of the NTC **Ionis NTC**.

4.9.0.3 Dosage

In our initial experiments, the 350 µg dose given in the original publication[275] was being used without due consideration that changing the chemistry from MOE to LNA for **ASO 23**, **ASO 24**, **ASO 25** and **ASO 30** may have affected the toxicological profile of the oligonucleotides and thus that the dosage should be decreased commensurately. This did not, however, explain the toxicity of the MOE-only oligonucleotides **ASO 31** and **ASO 32** and so it was considered that there may be another aspect to the toxicological problem being experienced.

Doses were reduced dramatically from 350 µg across the board to a more conservative 10 nmol (ca. 50 µg – 70 µg depending on sequence) and titrated up or down from that

reference point. The LNA containing oligonucleotides remained toxic at 10 nmol, producing seizures and death in treated animals, but these effects were not seen 5 nmol or lower. **ASO 71** could be used at a 30 nmol dose before any toxic effects were observed while **ASO 70** was tolerated up to 30 nmol. **ASO 69** was toxic past 10 nmol and thus was discounted from any future experiments.

4.9.0.4 Viscosity

Anecdotally, it was observed that the LNA oligonucleotides gave significantly more viscous solutions than did their MOE containing counterparts. Indeed, at higher concentrations of MOE, it was also observed that the solutions were significantly more viscous than for lower concentrations. This was presumed to be as a result of significantly increased interstrand binding owing to the strength of LNA binding, or simply due to the greater concentration of dissolved material in the case of the MOE compounds. At this point, there was uncertainty as to what could be done to reduce the viscosity of these solutions, however this will be discussed in section 4.9.2.

4.9.0.5 Anaesthesia

ICV injections are a stereotaxic surgical intervention and must be conducted with the experimental animal under general anaesthesia. In rodents, this is typically accomplished using a cocktail of ketamine and xylazine injected intraperitoneally, however this combination of drugs has a relatively small therapeutic window and can often result in inadequate anaesthesia, resulting in a need to re-dose which often leads to death. The ketamine/xylazine cocktail also takes a significant time to induce a suitable plane of anaesthesia, requires an extensive recovery period and provides no pain control. In order to circumvent these issues, a new drug combination was used in our experimental animals consisting of fentanyl with midazolam and dexmedetomidine. This combination of an opioid, benzodiazepine and α_2-adrenergic agonist in theory

provides neuroleptoanalgesia, a detached state without pain. The major benefits are that the animal has pain controlled from the induction phase and that each component is chemically reversible (fentanyl with buprenorphine, midazolam with flumazenil and dexmedetomidine with atipamezole) allowing for quicker recovery from anaesthesia which diminishes the chance for anaesthesia-related death.

Isoflurane is a volatile inhaled anaesthetic that has an excellent safety profile. Its use in stereotaxic surgery requires adapted nose cones that allow for the passage of volatile gasses over the snout of the animal.

Our initial experiments were performed using injectable fentanyl/ midazolam/ dexmedetomidine anaesthesia and there was concern that perhaps the seizure-like activity observed was a consequence of the post-operatively administered flumazenil. In order to discount this possibility, required nose cones were purchased and the ananesthetic moved to isoflurane.

4.9.1 Silencing Data for Improved ASOs

An *in vitro* screen of the newly generated oligonucleotides (Fig. 4.7) was conducted. Applying what had been learned from our past experiments, our next *in vivo* silencing experiments were designed to compare the potential for each of the synthesised compounds. The transgenic animals used were all at approximately 6 months of age (consistent with the originally published work), anaesthetised with isoflurane and administered the compounds in Table 4.3. Two weeks after injection, the animals were euthanised with an overdose of isoflurane and the CNS harvested post-perfusion. Tissue punches were taken throughout the brain and spinal cord, total RNA extracted and analysed using RT-qPCR. In each experiment, **ASO 66** and **ASO 68** were given as a total of 5 nmol while **ASO 70** and **NTC** were given as a total of 30 nmol.

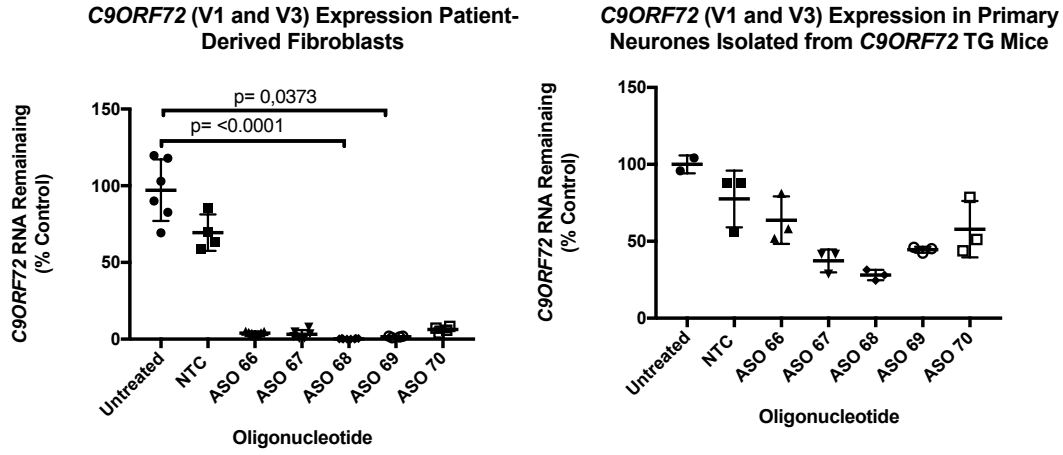


Figure 4.7: *In vitro* silencing data for ASOs 66, 67, 68, 69 and 70 in cultured patient-derived fibroblasts and primary neurones.
Oligonucleotides were used at 100 nM. RNA level was quantitated using qRT-PCR and normalised to GAPDH.

Silencing in the cortex, just above the site of injection, was encouraging with good silencing observed for both **ASO 68** and **ASO 70** (Fig. 4.8). In both the cervical and thoracic spinal cord, the same pattern of silencing was observed as for the cortex (data not shown).

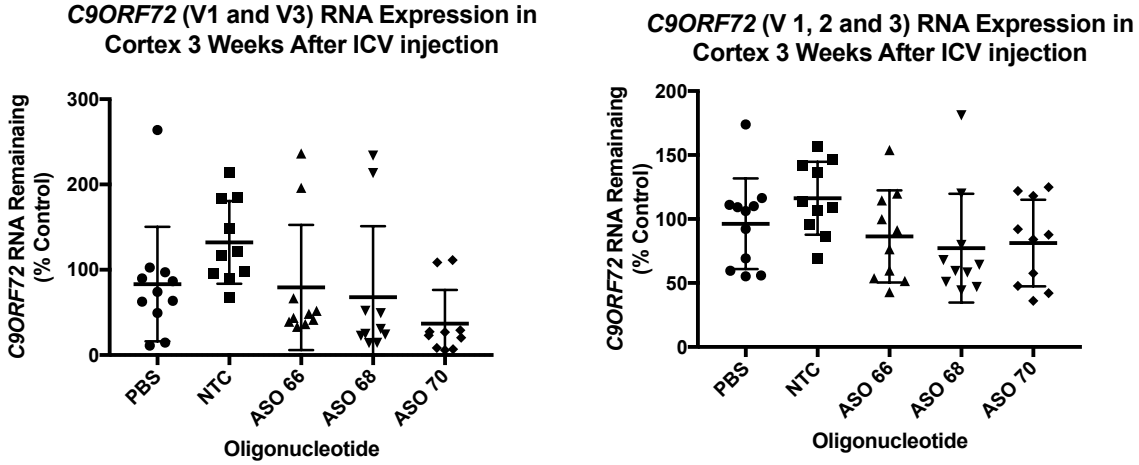


Figure 4.8: Cortical silencing data following ICV injection of oligonucleotides in transgenic *C9ORF72* mice.
RNA level was quantitated using qRT-PCR and normalised to GAPDH.

ASO 66 was less potent than **ASO 68** and on the basis of these data was excluded from further development.

4.9.2 Alzet Pumps Allow Higher Dosing Without Toxicity

Following our initial and provocative silencing results a quandry became evident; clearly these molecules are able to induce potent silencing however it was necessary to give them at doses that are simply too close to their toxic level. That is to say that these oligonucleotides have a very small therapeutic window. It was considered important to decouple whether the act of injecting the oligonucleotide was producing toxicity (i.e. that the toxic effects were acute and not the result of the total dose of oligonucleotide) or whether indeed the oligonucleotides were toxic in and of themselves past some dose threshold.

In order to accomplish this, Alzet osmotic pumps were used to deliver a constant amount of oligonucleotide over one week. Alzet pumps are small devices that implant subcutaneously. By drawing in fluid from the animal they use osmotic pressure to pump a very slow, continuous stream of their contents through an intracerebroventricularly implanted cannula for the duration of their action. These pumps have a fill volume of 200 µL and must be fully filled in order to function correctly. A dose-response study was performed wherein animals were fitted with pumps to deliver the doses in table 4.4. Three weeks after the pumps were implanted, animals were euthanised and tissue processed as detailed earlier.

ASO Name	Total Dose/week	Equivalent Dose/day
ASO 68	400 nmol, 200 nmol, 100 nmol 50 nmol	56 nmol, 28 nmol, 14 nmol, 7 nmol
ASO 70	100 nmol, 50 nmol, 25 nmol	14 nmol, 7 nmol, 3.5 nmol
ASO 71	200 nmol, 100 nmol	28 nmol, 14 nmol

Table 4.4: ASOs used for the Alzet-pump ICV silencing study.

None of the animals died at any point over the course of the surgery or the subsequent 3 weeks during and after ASO infusion. These results indicate that ASO accumulation does

not necessarily precipitate toxicity, but that the seizure-like toxicity is indeed induced by giving a bolus dose. Considering the LNA-containing oligonucleotides, bolus injections never exceeded 1 mM and the maximum concentration in the pumps was 500 μM. For MOE-containing oligonucleotides however, the maximum bolus concentration was 5 mM whereas for the pump, it was 2 mM.

Thus there is one forthcoming hypothesis that can be drawn from this, that the toxicity evident after a bolus injection comes from the concentration of the oligonucleotide in solution. This implies that the toxicity is of a biophysical nature – that a more concentrated solution has some property, likely its viscosity or osmolarity, that makes it toxic when administered centrally.

4.9.2.1 Higher Total Dose Does Not Significantly Improve Silencing

It was important to assay the silencing post-pump implantation, not just to ensure that the oligonucleotides were effective when administered in this fashion, but to make certain that pumps had indeed discharged their contents, and that the improvement in survival was not owing to non-functioning pumps.

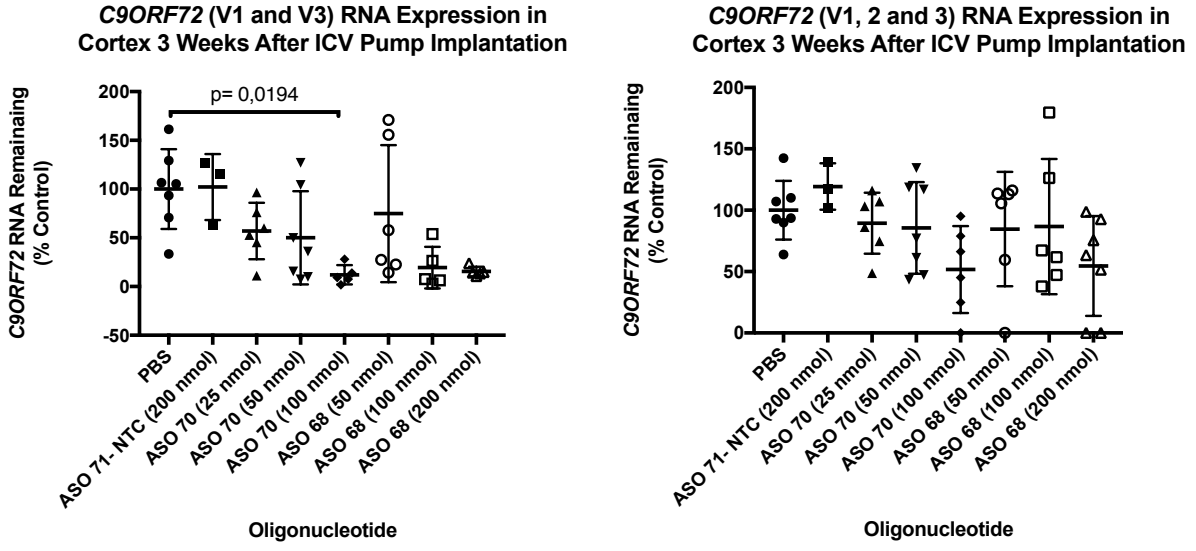


Figure 4.9: ICV Pump Silencing data in the cortex of transgenic *C9ORF72* mice. RNA level was quantitated using qRT-PCR and normalised to GAPDH.

Gratifyingly, the data show a dose-dependent response for both oligonucleotides when administered *via* pump. While it was encouraging to know that a larger dose of oligonucleotide was possible than was achieved by bolus, there was only a slight improvement in the degree of silencing obtained when doing so. Comparing the results of the LNA oligonucleotide **ASO 68** administered as a 5 nmol bolus and achieving ca. 75% silencing, 100 nmol had to be given *via* pump to achieve a similar degree of silencing. This phenomenon has been reported previously, though not with LNA-containing oligonucleotides and not against this target.[291] Clearly when considering translation to the clinic, it's preferable to be able to administer a single bolus injection (either in to an Ommaya reservoir or intrathecally) than it is to undergo a major surgical procedure to implant an intrathecal cannula for infusion, when the observed benefit from each is similar.

This data induced a state of uncertainty; it was clear that quantity of administered oligonucleotide was not toxic, however that a bolus dose is more effective than an infused dose. It was also clear that it was possible to deliver significant amounts of oligonucleotide as a bolus from the previous literature. Reevaluation of the literature, revealed that some oligonucleotides destined for use in the CNS are not fully phosphorothioate modified, though the authors are not always clear about exactly which oligonucleotides contain which modifications, nor at which positions.[292–294] Further confusing the matter, some papers do indicate that they used fully phosphorothioate-modified oligonucleotides but confound the matter of toxicity by administering lower bolus doses than reported elsewhere, or by using Alzet pumps.[295,296]

4.10 Mixed Backbone ASOs Show Improved Toxicological Profile

Considering the opaque nature of the literature surrounding backbone chemistry for neurotherapeutic oligonucleotides, it was decided to alter the backbone composition of our lead MOE-containing oligonucleotide to see whether it might be possible to improve the toxicological profile. This led to the synthesis of the oligonucleotides in Table 4.5:

ASO Name	**Sequence (5′ – 3′)**	**PS Inclusions**
ASO 70- Skip	&G*&C&C*&C&C*TA*G^{Me}CG*^{Me}C*G^{Me}C*&G&A*&C&T*&C	9 PS
ASO 70- Wings 1	&G*&C&C&C&CT*A*G*^{Me}C*G*^{Me}C*G*^{Me}C&G&A&C&T*&C	9 PS
ASO 70- Wings 2	&G*&C&C&C&C*T*A*G*^{Me}C*G*^{Me}C*G*^{Me}C*&G&A&C&T*&C	11 PS

Table 4.5: Mixed backbone ASOs used in the ICV silencing study

ASO 70- Skip has an alternating phosphorothioate/phosphodiester backbone, effectively halving the total phosphorothioate content. Both **ASO 70- Wings 1** and **ASO 70- Wings 2** are specifically modified to contain phosphodiester modifications within the wings of the gapmer. **ASO 70- Wings 2** however contains an additional phosphorothioate modification at the MOE-DNA linkage. The first step was to determine the maximum tolerated dose for these new molecules in wild-type animals. Hence these compounds were injected ICV and it was found that **ASO 70- Skip** was well-tolerated up to 50 nmol while both **ASO 70- Wings 1** and **ASO 70- Wings 2** were tolerated up to 80 nmol (and indeed perhaps higher, as 80 nmol was our maximum tested dose).

ASO Name	Total Dose	Timepoints for Each Dose
ASO 70- Skip	30 nmol, 15 nmol, 7.5 nmol	3 weeks, 6 weeks, 12 weeks
ASO 70- Wings 1	60 nmol, 30 nmol, 15 nmol	3 weeks, 6 weeks, 12 weeks
ASO 70- Wings 2	30 nmol	6 weeks, 12 weeks

Table 4.6: Mixed backbone ASOs, doses and timepoints for the followup silencing study

To establish whether the introduction of phosphodiester linkages would significantly diminish the potency of these molecules, a silencing experiment was performed in the transgenic animals. It was uncertain whether there would be an observable difference between the efficacy of **ASO 70- Wings 1** and **ASO 70- Wings 2** and because there were limited animals available, our initial efforts were focussed on **ASO 70- Wings 1** while **ASO 70- Wings 2** was only examined at a further time point. Hence, it was decided to assay the three oligonucleotides as in Table 4.6.

The **ASO 70- Wings 1** data (Fig. 4.10) was disheartening; **ASO 70- Wings 1** showed reduced potency compared with **ASO 70**. Thus while it was indeed possible to administer significantly more oligonucleotide, it didn't demonstrate significant enough potency to warrant consideration at this time point.

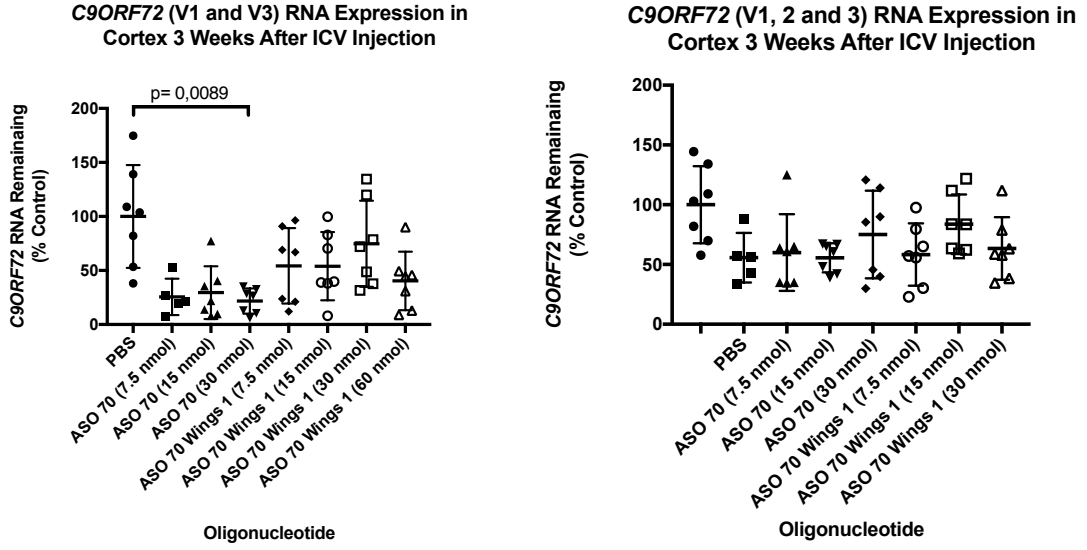


Figure 4.10: ICV Bolus injection data comparing silencing from **ASO 70** and **ASO 70- Wings 1** in the cortex by dose-response after 3 weeks.
RNA level was quantitated using qRT-PCR and normalised to GAPDH.

Data from the later timepoint (Fig. 4.11), showed a dramatic increase in silencing potential for **ASO 70- Wings 2** relative to both **ASO 70** and **ASO 70- Wings 1** at the same 30 nmol dose. This is presumably owing to the **ASO 70- Wings 1** compound being significantly more nuclease sensitive as a result of the additional phosphodiester content.

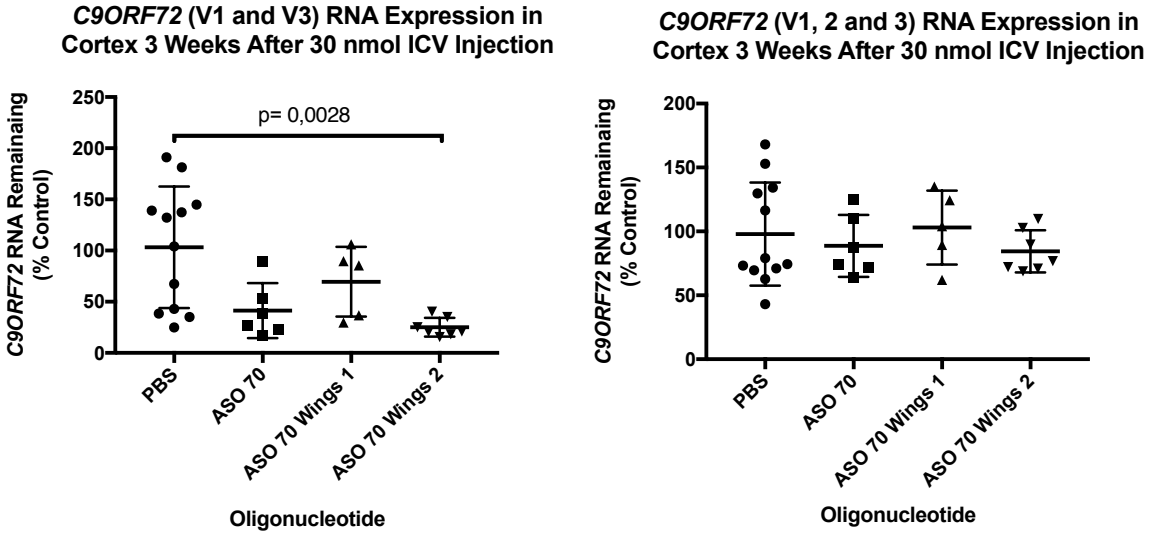


Figure 4.11: ICV Bolus injection data comparing silencing between **ASO 70**, **ASO 70-Wings 1** and **ASO 70- Wings 2** in the cortex at 30 nmol after 6 weeks. RNA level was quantitated using qRT-PCR and normalised to GAPDH.

4.11 Toxicological Data

Once administered to the CSF, ASOs do enter the bloodstream and hence bioaccumulate in tissues such as the liver and kidney.[227] Hence at point of euthanasia, the animals in the 3 week arm of the study were bled *via* the facial vein and the blood analysed using Abaxis blood chemistry rotors.

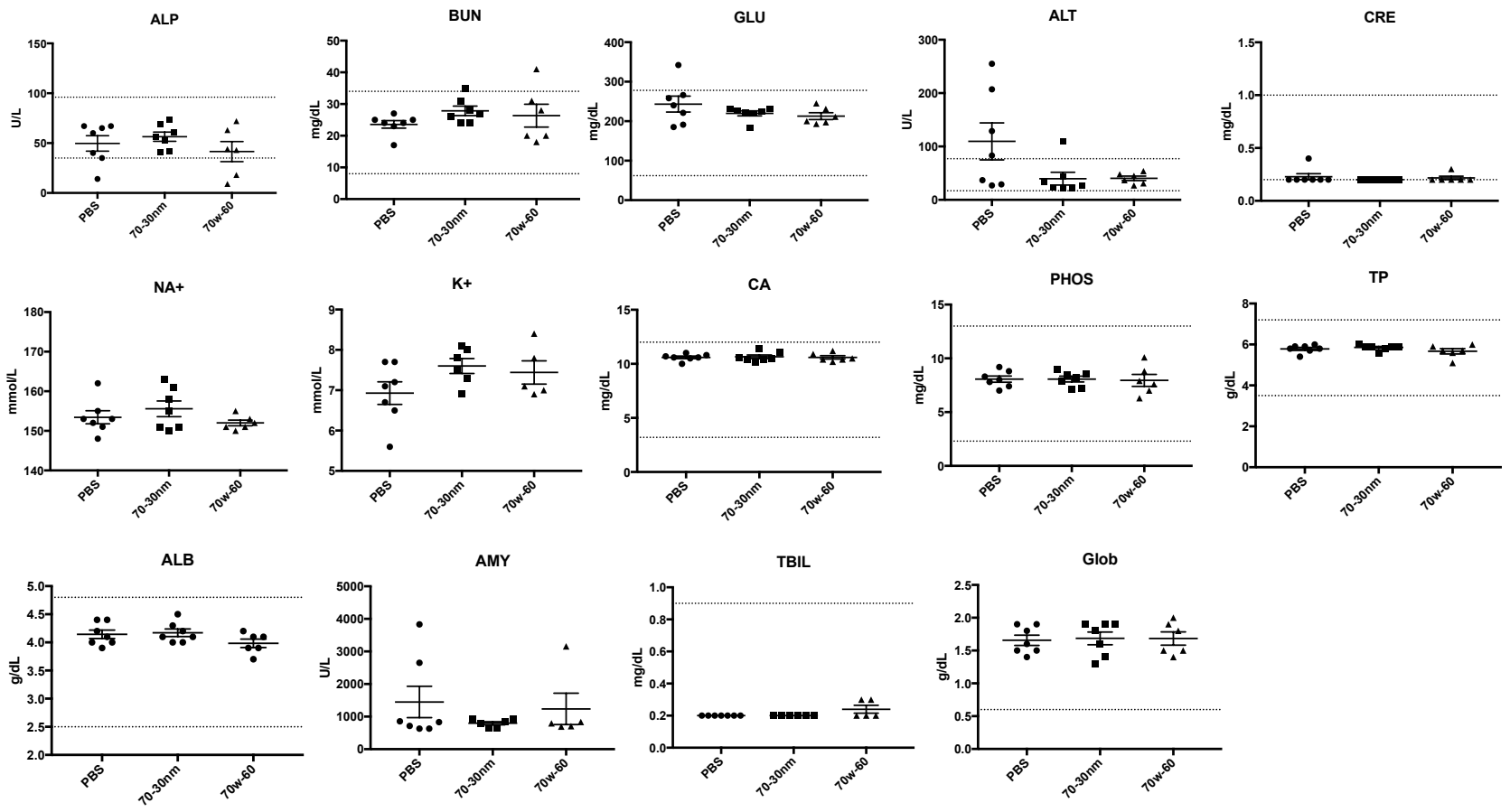


Figure 4.12: Blood chemistry results from the **ASO 70** and **ASO 70 - Wings 1** groups. Normal bounds are indicated by horizontal dashed lines.
'70-30nm' is the group that was injected with 30 nmol of **ASO 70**, '70w-60' is the group that was injected with 60 nmol **ASO 70- Wings 1**

Pleasingly, most of the diagnostic markers remained within the normal range and those that showed variation beyond these boundaries were not significant to one group or another. In all cases blood globulin was increased above the high bound of the normal range; it is presumed that this was as a result of the incision on the head not being completely healed.

4.12 Towards the Clinic

The **ASO70 - Wings 2** compound is being advanced forwards, satisfied that it elicits significant silencing ability in the transgenic mice, a toxicity trial in non-human primates will be performed. Presuming that an unexpected toxic event does not occur, it is anticipated that this compound will be met with FDA approval for compassionate use.

4.13 Future Work

ASO Name	**Sequence (5′ – 3′)**
ASO 70- Wings 3	&G*&C+C&C&C*T*A*G*^{Me}C*G*^{Me}C*G*^{Me}C*&G&A+C&T*&C

Table 4.7: An intended future iteration of the lead compound

A valuable experiment would be to synthesise a compound such as **ASO 70- Wings 3** that contains a high-affinity LNA monomer in each wing. The seemingly dramatic improvement in toxicological properties following inclusion of the phosphodiester linkages may allow for additional inclusion of high-affinity modifications that proved too toxic in previous iterations. A compound resembling this structure may be able to be administered at a lower dose and retain potency without exacerbating any toxic effects.

While replacing phosphorothioate linkages with phosphodiester linkages has led to an improvement in the toxicological profile, it has almost certainly decreased the stability of the oligonucleotide. It would be prudent to explore the effects of incorporation of non-phosphorothioate, non-phosphodiester linkages. Neutral functional groups such as certain amides[297] and triazoles[298] have been shown to be phosphate mimics and point-substitutions of these analogues are well tolerated in ASOs and siRNAs, though they are not suitable for full modification of ASOs. Their selective incorporation at a subset of the linkages, in combination with PS modification of the remaining linkages, may enable the preparation of stable, low-toxicity antisense oligonucleotides for use in the CNS.

4.14 Experimental

4.14.1 Oligonucleotide Synthesis

LNA phosphoramidites were synthesised in house. MOE, DNA and special phosphoramidites were purchased (ChemGenes). Modified nucleoside phosphoramidites (LNA or MOE) were coupled for 10 minutes and standard DNA phosphoramidites for 1 minute.

Screening scale (200 nmol) oligonucleotides were synthesised on a Dr Oligo 48 oligonucleotide synthesiser (Biolytic) on Unylinker CPG (Chemgenes, pre-packed in ABI 3900 columns). 0.1M DDTT (ChemGenes) was used as the sulfurising reagent and 0.25M BTT (AIC) as the activator. Oligonucleotides were characterised by LCMS and were purified by diafiltration in an Amicon centricon spin filter.

Intermediate scale (1 µmol) oligonucleotides were synthesised on an ABI394 oligonucleotide synthesiser on specified CPG (ChemGenes, packed into twist top columns). 0.1M DDTT (ChemGenes) was used as the sulfurising reagent and 0.25M BTT (AIC) as the activator. Oligonucleotides were deprotected using concentrated ammonia at 55 °C for 18 h and purified using ion-exchange chromatography (eluting

with 30% acetonitrile in water containing increasing gradients of $NaClO_4$). Oligonucleotides were characterised by LCMS and were purified by diafiltration in an Amicon centricon spin filter.

Preliminary *in vivo* scale oligonucleotides were synthesised at 10 µmol using an AKTA Oligopilot 10 (GE Life Sciences). 0.1M DDTT (ChemGenes) was used as the sulfurising reagent and 0.25 M BTT (AIC) as activator. The oligonucleotide was deprotected using concentrated ammonia at 55 °C for 18 h and purified using ion-exchange chromatography (eluting with 30% acetonitrile in water containing increasing gradients of $NaClO_4$). The purified oligonucleotide was desalted with Sephadex G25 and concentrated.

Experimental *in vivo* scale oligonucleotides were synthesised at 100 µmol using an AKTA Oligopilot 100 (GE Life Sciences). 0.1M DDTT (ChemGenes) was used as the sulfurising reagent and 0.25 M BTT (AIC) as activator. The oligonucleotide was deprotected using concentrated ammonia at 55 °C for 18 h and purified using ion-exchange chromatography (eluting with 30% acetonitrile in water containing increasing gradients of $NaClO_4$). The purified oligonucleotide was dialysed and concentrated using Amicon spin filters.

4.14.2 Mouse Work

All animal work was undertaken in accordance with the University of Massachusetts Medical School Institutional Animal Care and Use Committee (protocols A-2591-17 (Rabbit) and A-2551-16 (Rodent)). Institutional Biosafety Committee approval was also granted under protocol I-656-16. Wild type C57BL/6J animals were purchased from Jackson Labs. Transgenic *C9ORF72* BAC111 animals were purpose bred under protocol A-1964 (Robert Brown) and were maintained on standard rodent chow and acidified water *ad libitum*.

4.14.2.1 ICV Injections

Mice were anaesthetised either with an intraperitoneal injection of a fentanyl/midazolam/dexmedetomidine cocktail ($20\,\mu g\,kg^{-1}$/ $5\,mg\,kg^{-1}$/$2.5\,mg\,kg^{-1}$) or by inhalation of vaporised isoflurane (3% in pure oxygen for induction, 2% for maintenance). Anaesthetised animals were transferred to a Kopf small animal stereotaxic frame, ear bars placed and a hand-warmer was placed underneath the animal to preserve core body-temperature. Ophthalmic lubricant was placed over each eye and the fur covering the skull removed. The scalp was aseptically prepared by thorough swabbing with 3 changes of betadine/ 70% isopropanol and allowed to dry before a medial incision was made to expose the skull. Periosteum was dried with a sterile cotton swab and the syringe containing oligonucleotide was moved to bregma. A point was marked on the skull 1 mm dextrolaterally and 0.2 mm posterially from bregma and a 1 mm diameter hole drilled at this location. The tip of the needle was advanced 3 mm ventrally through this hole and after 2 minutes, the oligonucleotide injected. The needle was left in place for 3 minutes post-injection, then removed and the skin closed with 5-0 vicryl suture. For animals anaesthetised with the fentanyl/midazolam/dexmedetomidine cocktail, buprenorphine ($300\,\mu g\,kg^{-1}$) was administered subcutaneously and flumazenil and atipamezole given intraperitoneally. Only buprenorphine was given to animals anaesthetised with isoflurane. Animals were removed from the stereotaxic frame and allowed to recover in a warm cage, wet food and gel were provided, and the animals were returned to the vivarium when they were sternal.

4.14.2.2 ICV Pump Implantation

Alzet pumps were prepared as indicated by the manufacturer. Mice were anaesthetised by inhalation of vaporised Isoflurane (3% in pure oxygen for induction, 2% for maintenance). Anaesthetised animals were transferred to a Kopf small animal stereotaxic frame, ear bars placed and a hand-warmer was placed underneath the animal to preserve core body-temperature. Opthalmic lubricant was placed over each eye and the fur covering the

skull removed. The scalp was aseptically prepared by through swabbing with 3 changes of betadine/ 70% isopropanol and allowed to dry before a medial incision was made to expose the skull. Periosteum was dried with a sterile cotton swab and a subcutaneous pouch made into which the saline-lubricated pump was placed. The cannula was fitted into the cannula-introducing adapter on the frame and the tip moved to bregma. A point was marked on the skull 1 mm dextrolaterally and 0.2 mm posterially from bregma and a 1 mm diameter hole drilled at this location. The cannula floor was covered in cyanoacrylate (Vetbond) glue and the tip driven through the drilled hole. The cannula was held in place for 2 minutes for the glue to set and the skin then closed with 5-0 vicryl suture. Buprenorphine ($300\,\mu g\,kg^{-1}$) was administered subcutaneously, the animal was removed from the stereotaxic frame and allowed to recover in a warm cage, wet food and gel were provided, and the animals were returned to the vivarium when they were sternal.

4.14.2.3 Euthanasia and Tissue Harvest

Sodium pentobarbital (>800 mg/kg as Fatal Plus, administered intraperitoneally as a dilution in saline) or an isoflurane overdose was used for terminal anaesthesia. Animals were immediately perfused transcardially (chest fur wetted with 70% EtOH, thoracic incision made and diaphragm cut to expose the heart. The right atrium was snipped and perfusate introduced through a needle in the left ventricle), then decapitated. The brain was removed by careful craniotomy and the spinal cord removed by transecting the whole spine at the lumbar level and saline injection in to the spinal canal to force the intact cord out through the cervical opening.

4.14.2.4 FFPE-IF

Following PBS perfusion, 10% neutral buffered formalin (Sigma) was perfused until fixation was complete. Tissues of interest were excised and post-fixed in NBF at 4 °C for at least 24 h. Once the tissue was removed from NBF, it was placed in a cassette,

paraffin embedded and sectioned to 4 µm before mounting to charged slide and dewaxing at 60 °C for 1 hour. Slides were then deparaffinized and rehydrated (two washes in xylene, two washes in 100% ethanol, two washes in 95% ethanol, one wash in water). Slides were treated with 3% H_2O_2 in methanol for 10 min followed by two washes in TBST. Sections were treated with Proteinase K (Dako) for 10 min (Dako) then washed twice with TBST. Sections were treated with Cyto-Q Background Buster (Innovex Biosciences) then washed twice. The diluted antisera was applied (1:5000 in 2% BSA 5% normal goat serum in TBST). The slides were incubated with antisera overnight at 4 °C, washed 3 times for 5 min each with TBST, AF488/555/647 goat anti-rabbit IgG (CST) at 1:2000 in 2% BSA 5% normal goat serum in TBST For 1h at room temp. The slides were then washed 3 times for 5 min each with TBST. Sections were covered with SignalStain Boost with DAPI and coverslipped with Leica Surgipath glass slides.

4.14.2.5 Microscopy

Fluorescent images were obtained with a Leica DMi8 inverted fluorescence microscope equipped with a DAPI, GFP, Y3 and Y5 filter cube and HC PL FLUOTAR 5x/0.15, HC PL FL 10x/0.3 PH1, HC PLAN APO 20x/0.7 PH2, HC PL FL 20x/0.4 CORR PH1, HCX PL FL L 40x/0.6 CORR PH2, HCX PL APO 40x/1.3 OIL, HCX PL APO 63x/1.4-0.6 OIL objectives.

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Appendix A

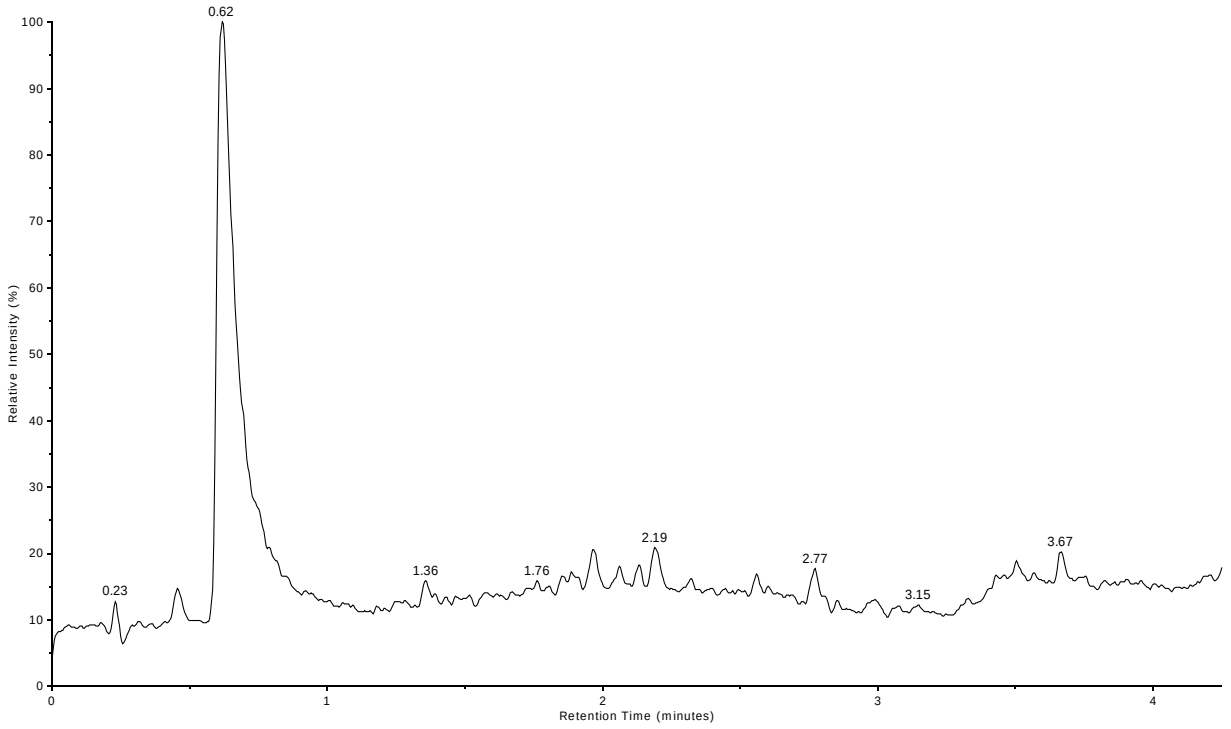
Analytical Data

A.1 2,3,3-trimethyl-3H-indole

Top 5 Peak Report

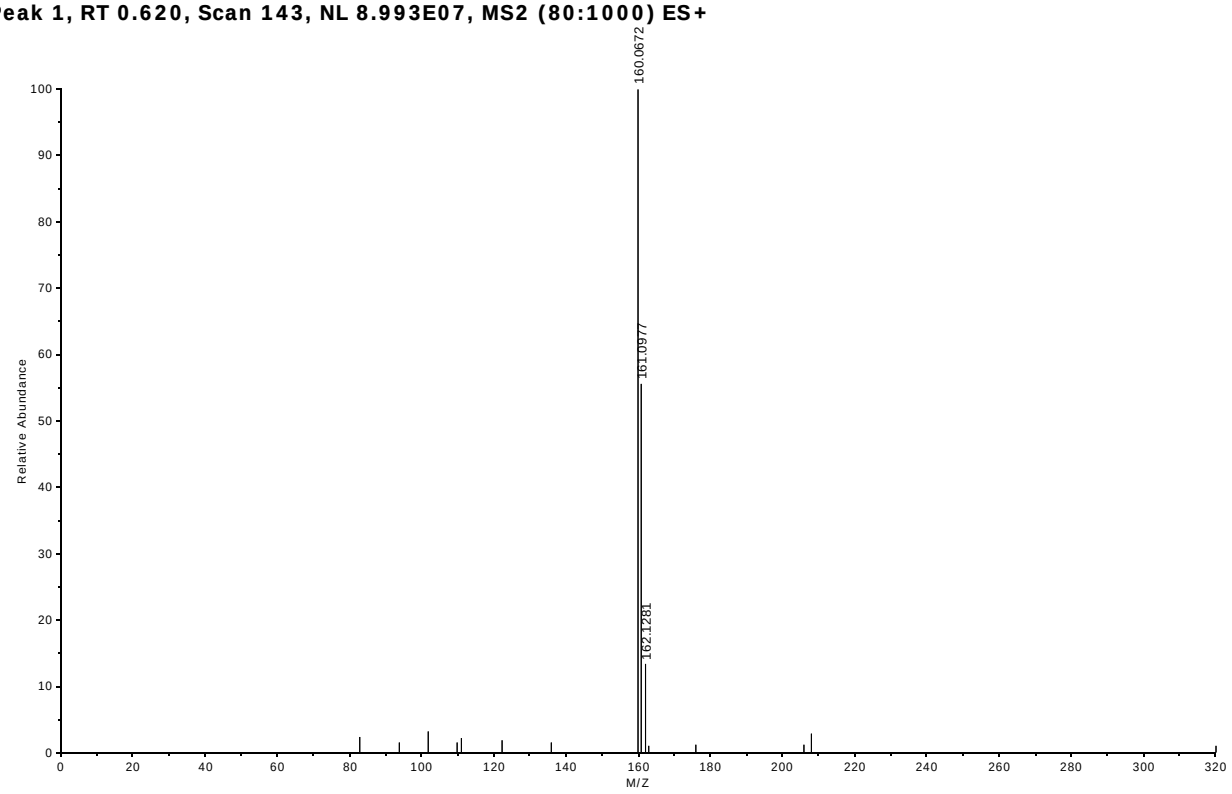
Group: Watts, J
Submitter: Michael Moazami
Project: RP-LC MW 80-1000
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Experiment: B ESIPOS C18 Acid MW 80-1000
Filename: Watts_J_mm731618_MM_299.pdf
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Acquisition Date: 22/07/2014 13:07:39

TIC, NL 1.847E08, MS2 (80:1000) ES+

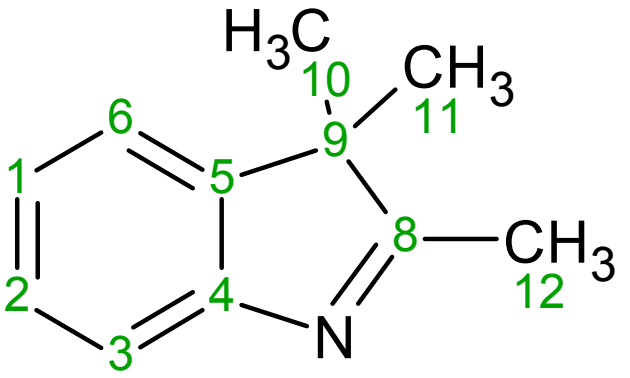


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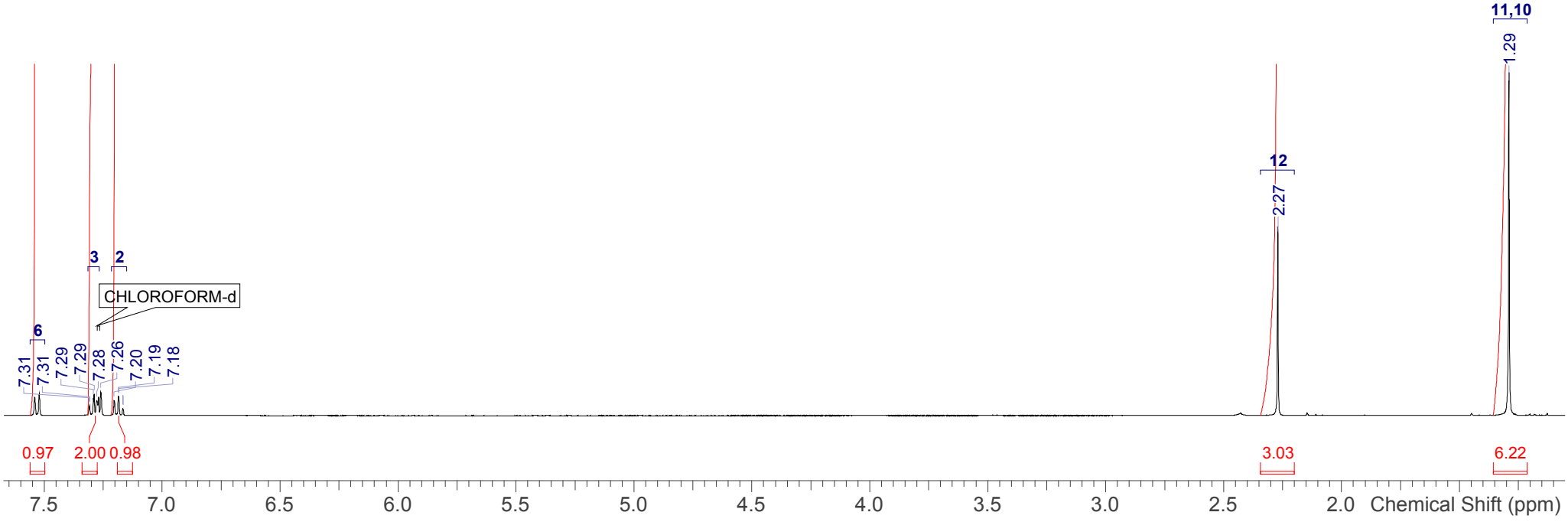
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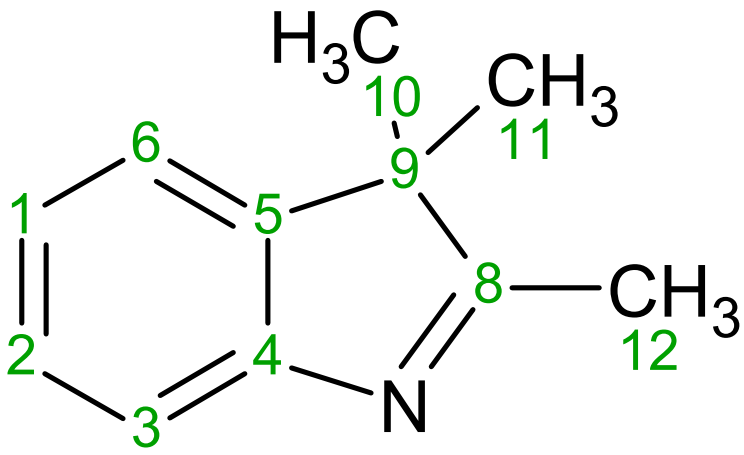
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Date Stamp	21 Jul 2014 12:48:20		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\jy2114mm1 Trimethylindolenine\10\PDATA\1\1r		
Frequency (MHz)	400.1300	***Nucleus***	1H
Number of Transients	16	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	1048576	***Pulse Sequence***	zg30
Receiver Gain	31.69	***SW(cyclical) (Hz)***	8012.82
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	2465.1301	***Spectrum Type***	standard
Sweep Width (Hz)	8012.81	***Temperature (degree C)***	24.989



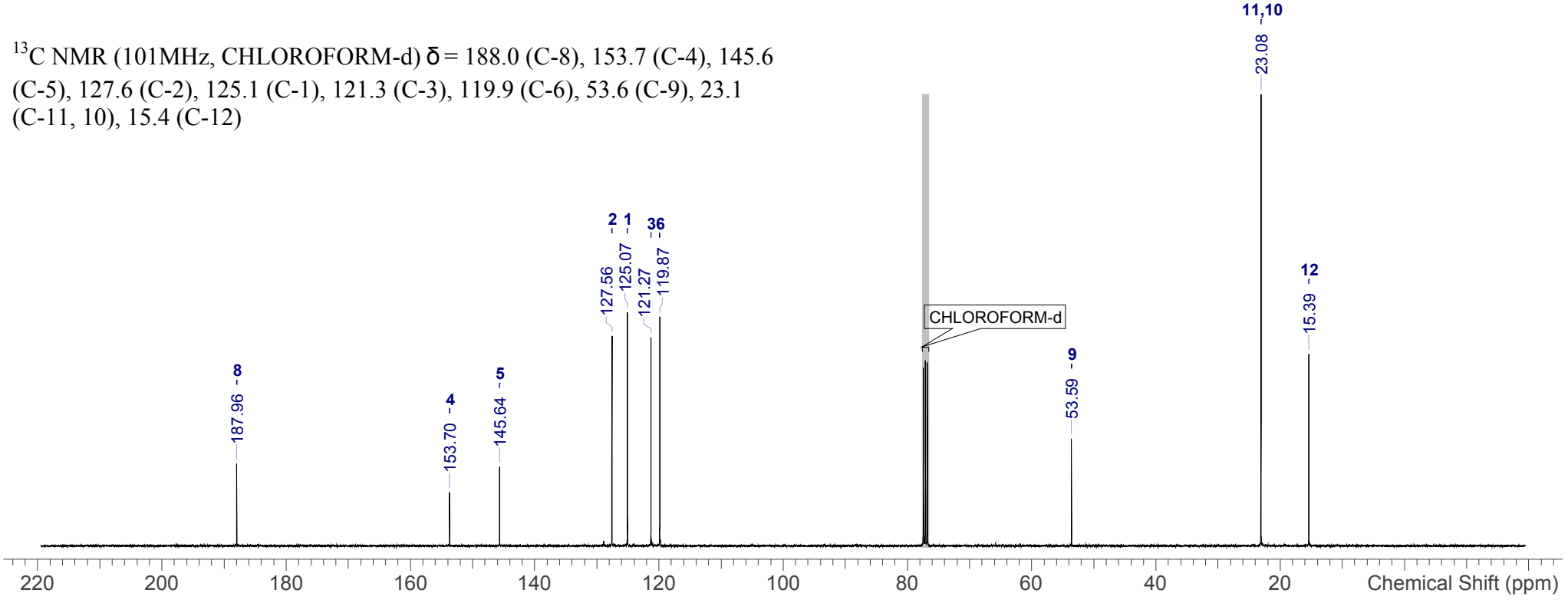
^{1}H NMR (400MHz, CHLOROFORM-d) δ = 7.53 (1H, d, J = 7.6 Hz, H-6), 7.29 (1H, td, J = 7.6 Hz, J = 1.4 Hz, H-3), 7.27 (1H, d, J = 7.0 Hz, M07), 7.19 (1H, td, J = 7.4 Hz, J = 1.0 Hz, H-2), 2.27 (3H, s, H-12), 1.29 (6H, s, H-11, 10)



Acquisition Time (sec)	1.3631	***Comment***	mm/7316/18
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Date Stamp	21 Jul 2014 13:18:45		
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Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	10060.3320	***Spectrum Type***	standard
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	24.992



^{13}C NMR (101MHz, CHLOROFORM-d) δ = 188.0 (C-8), 153.7 (C-4), 145.6 (C-5), 127.6 (C-2), 125.1 (C-1), 121.3 (C-3), 119.9 (C-6), 53.6 (C-9), 23.1 (C-11, 10), 15.4 (C-12)

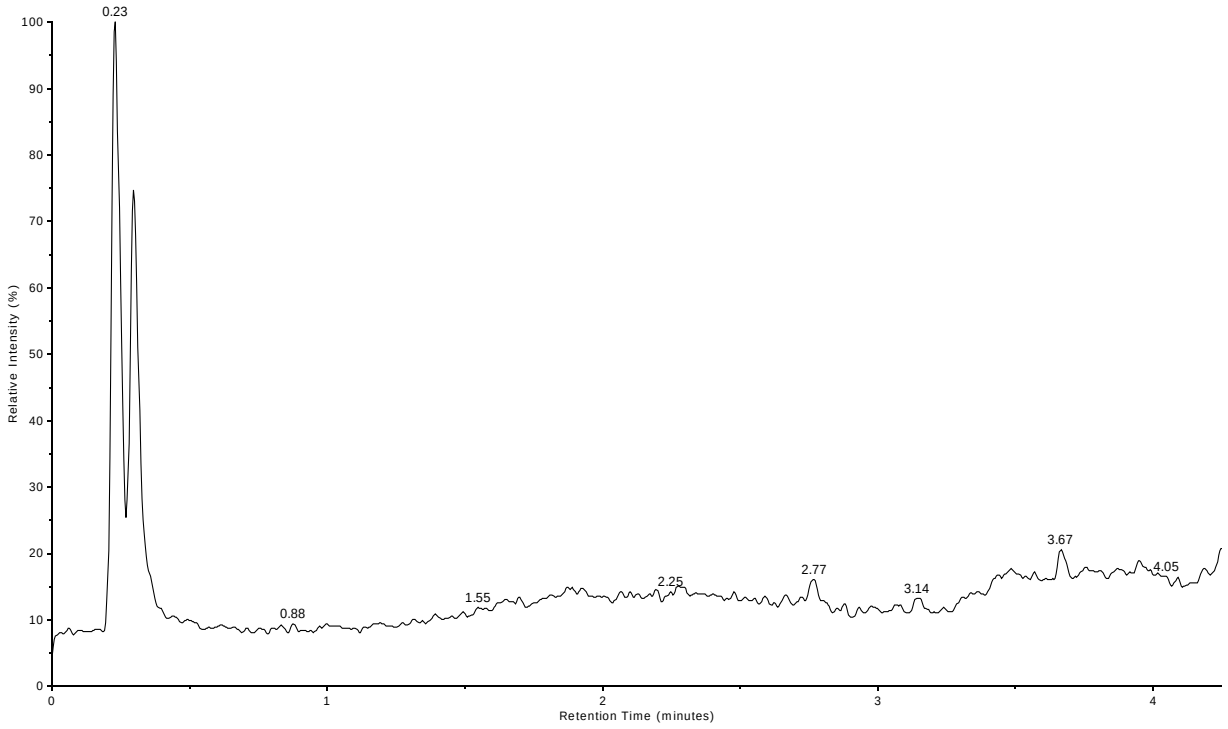


A.2 1,2,3,3-tetramethyl-3H-indol-1-ium iodide

Top 5 Peak Report

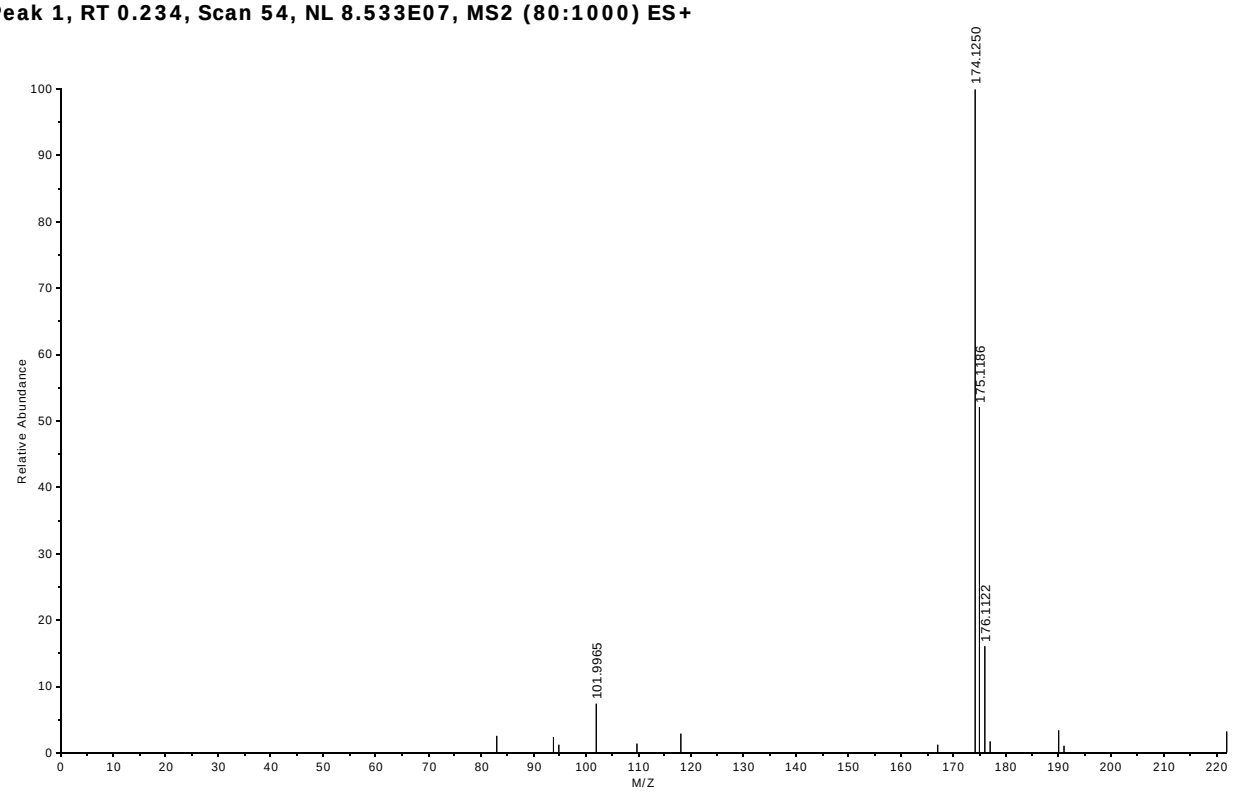
Group: Watts, J
Submitter: Michael Moazami
Project: RP-LC MW 80-1000
Sample ID: Mm/7316/19
Experiment: B ESIPOS C18 Acid MW 80-1000
Filename: Watts_J_Mm731619_MM_298.pdf
Instrument: Blue RP UHPLC-MS (B30:1023)
Acquisition Date: 22/07/2014 11:11:37

TIC, NL 1.650E08, MS2 (80:1000) ES+

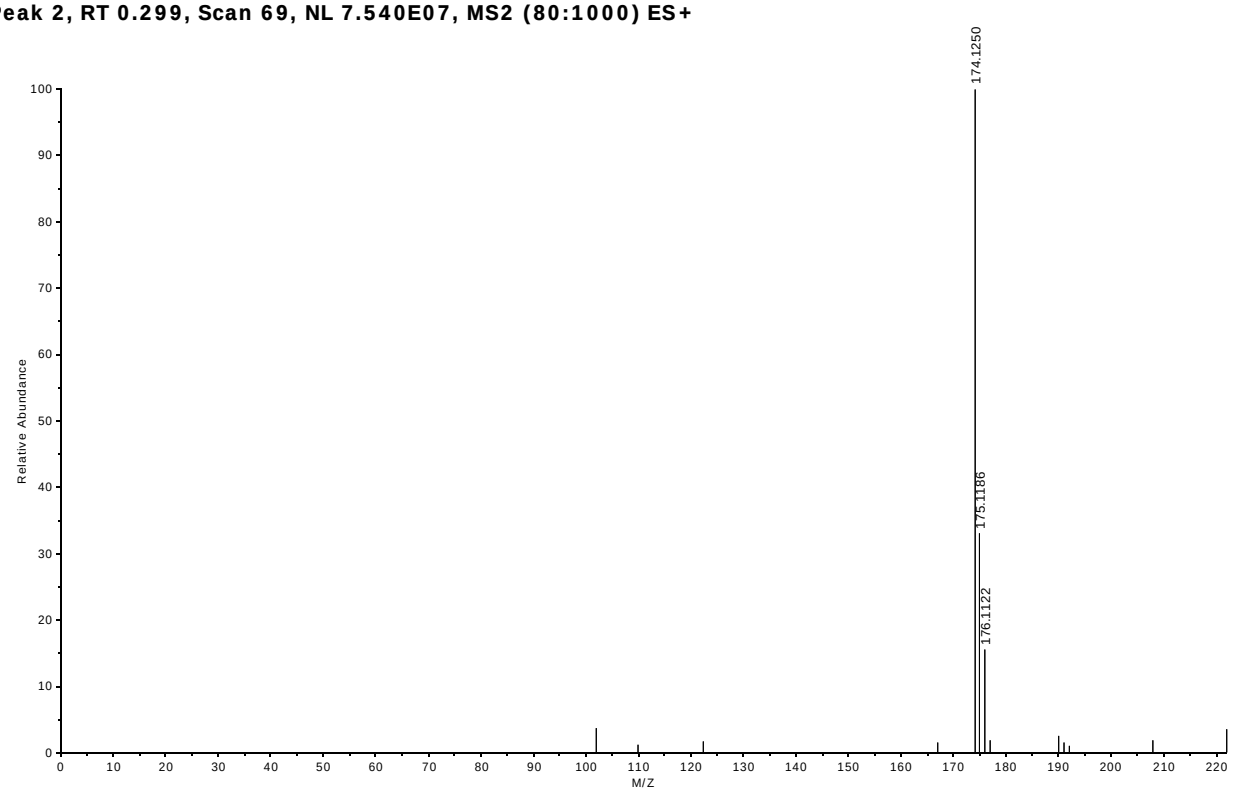


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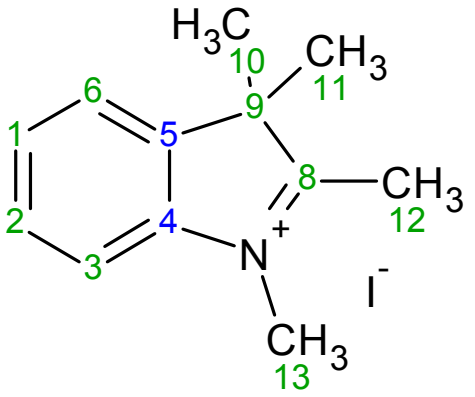
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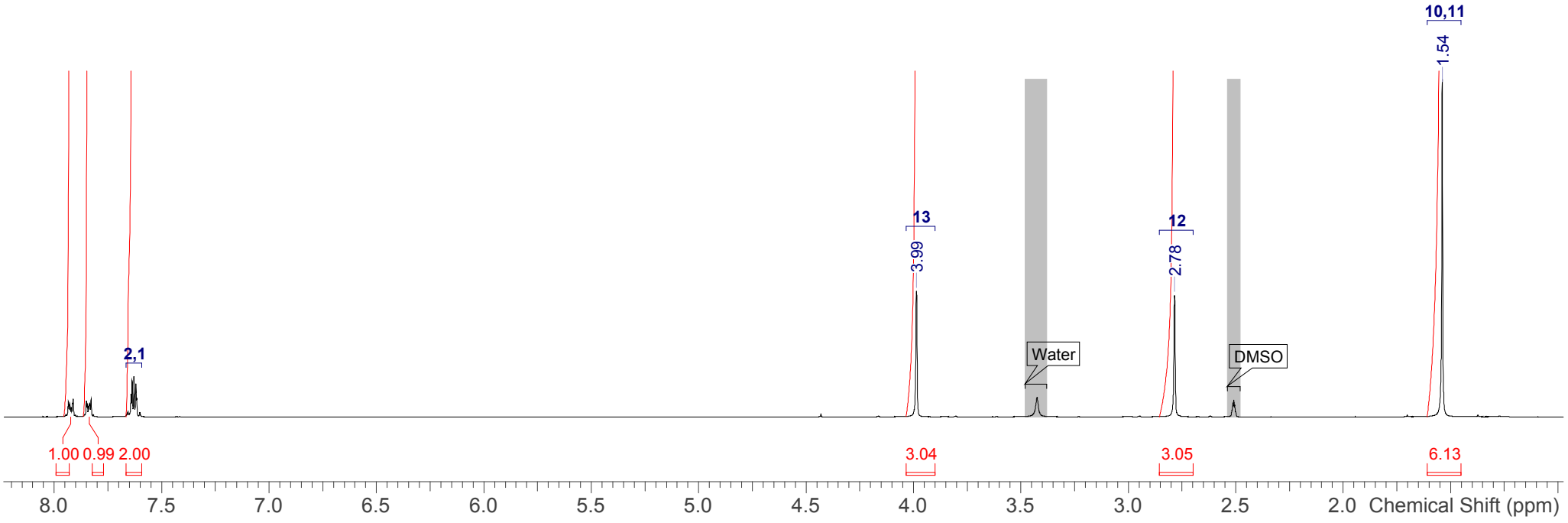
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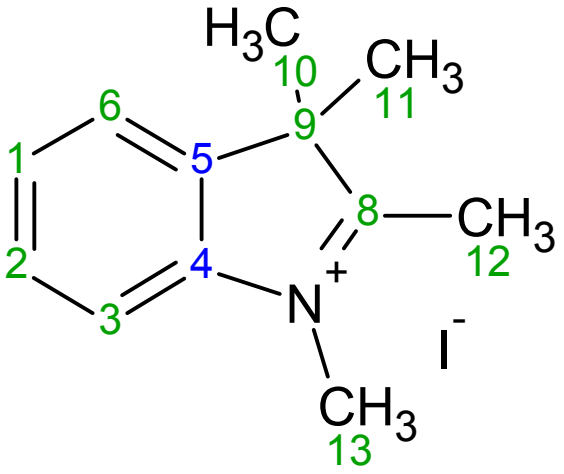
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Frequency (MHz)	400.1300	***Nucleus***	1H
Number of Transients	16	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	131072	***Pulse Sequence***	zg30
Receiver Gain	64.46	***SW(cyclical) (Hz)***	8012.82
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Spectrum Type	standard	***Sweep Width (Hz)***	8012.76
Temperature (degree C)	25.004		



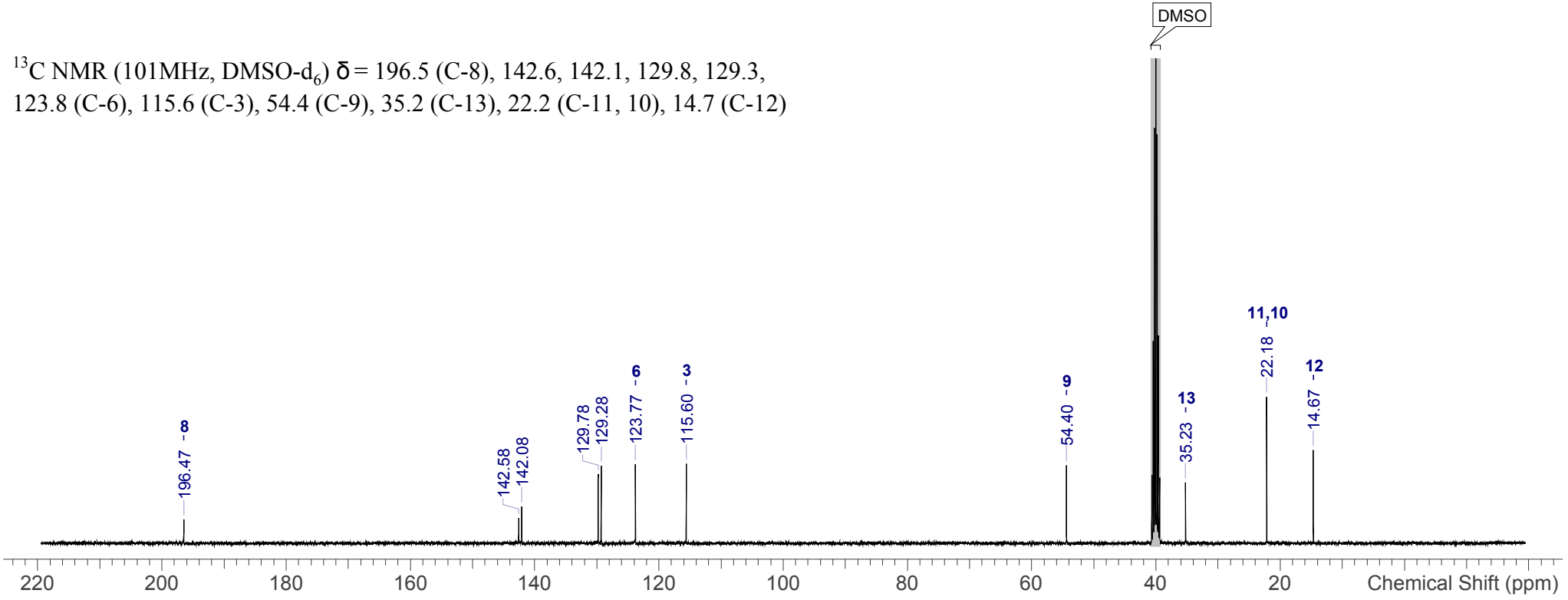
^{1}H NMR (400MHz, DMSO-d_6) δ = 7.89 - 7.96 (1H, m, M04), 7.81 - 7.86 (1H, m, M05), 7.59 - 7.67 (2H, m, H-2, 1), 3.99 (3H, s, H-13), 2.78 (3H, s, H-12), 1.54 (6H, s, H-10, 11)



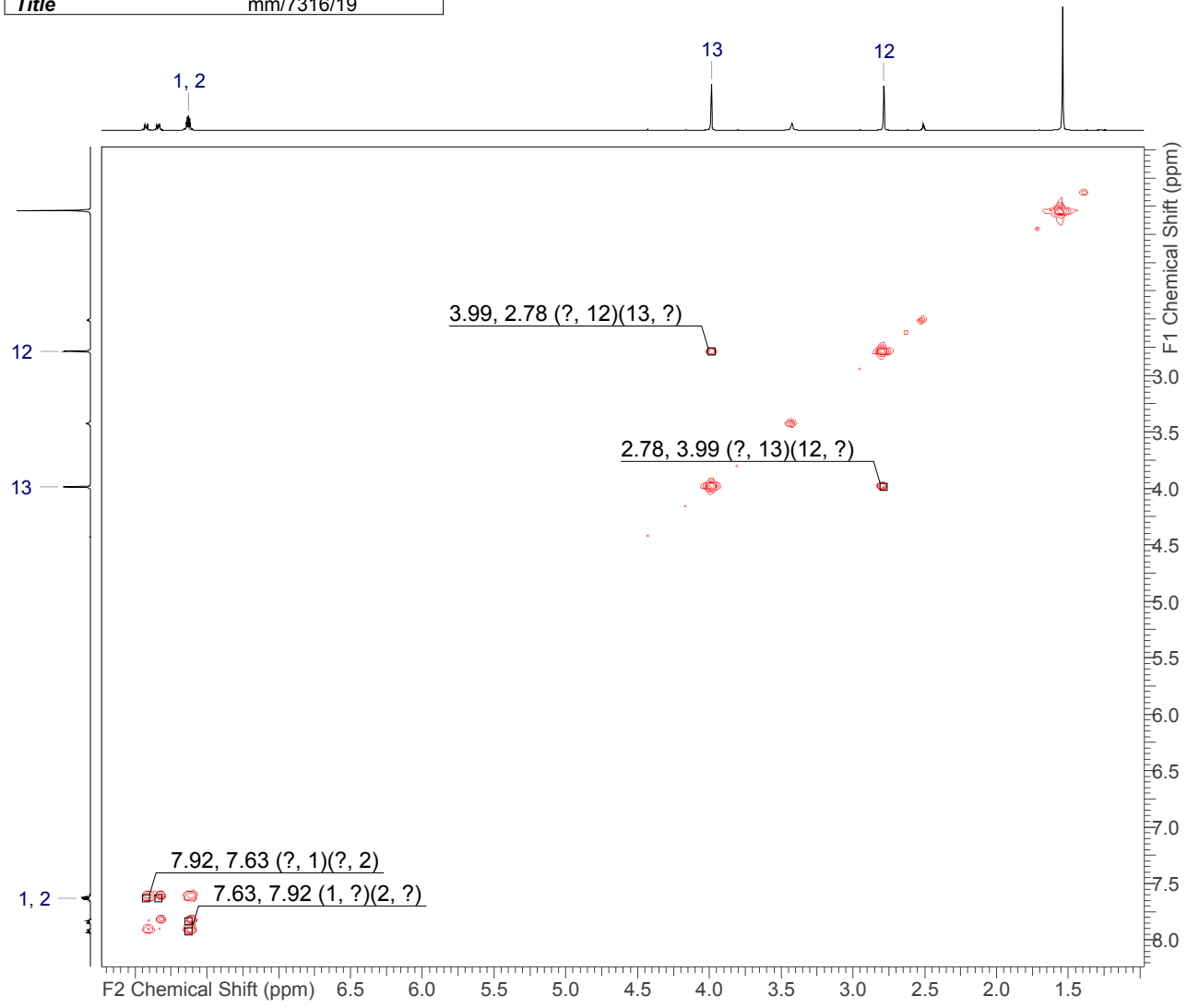
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Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	10060.3320
Spectrum Type	standard	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	24.992		

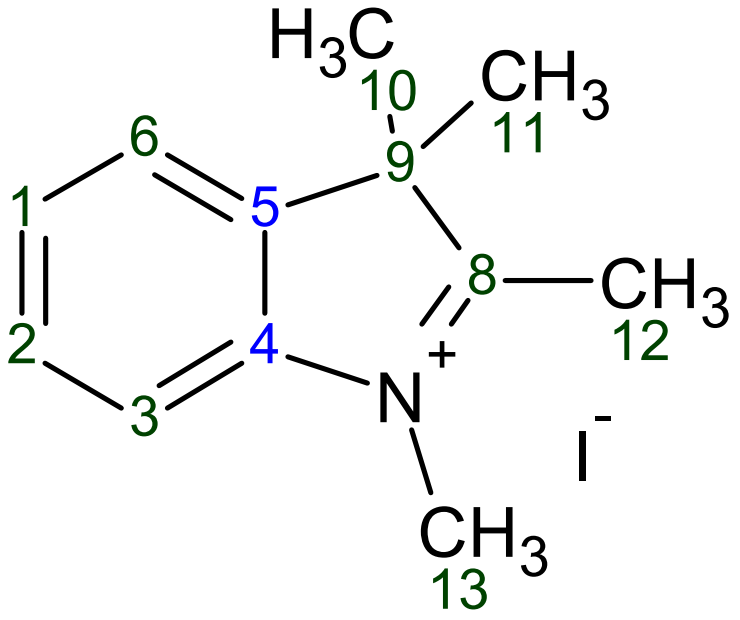


^{13}C NMR (101MHz, DMSO-d_6) δ = 196.5 (C-8), 142.6, 142.1, 129.8, 129.3, 123.8 (C-6), 115.6 (C-3), 54.4 (C-9), 35.2 (C-13), 22.2 (C-11, 10), 14.7 (C-12)



Acquisition Time (sec)	(0.3133, 0.0783)	***Comment***	mm/7316/19
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Number of Transients	2	***Origin***	spect
Original Points Count	(1024, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	cosygpqf
Solvent	DMSO-d6	***Spectrum Type***	COSY
Sweep Width (Hz)	(3264.78, 3264.78)	***Temperature (degree C)***	24.986
Title	mm/7316/19		



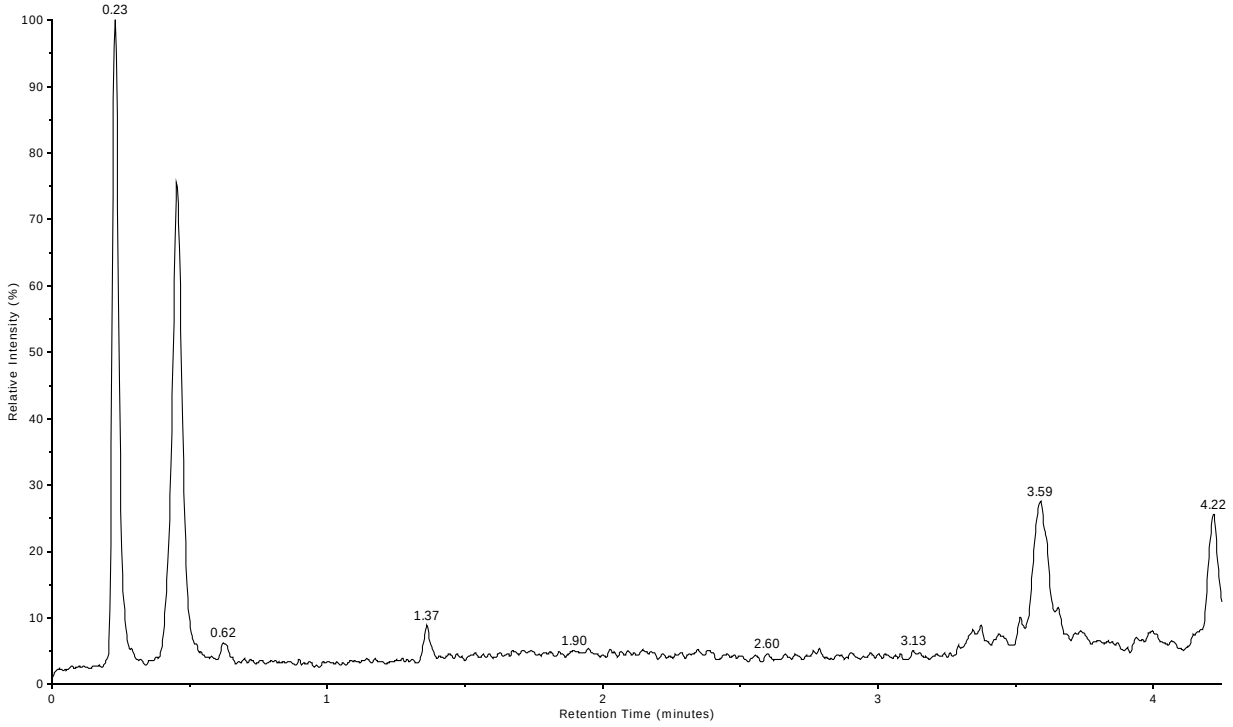


A.3 1-(5-carboxypentyl)-2,3,3-trimethyl-3H-indol-1-ium bromide

Top 5 Peak Report

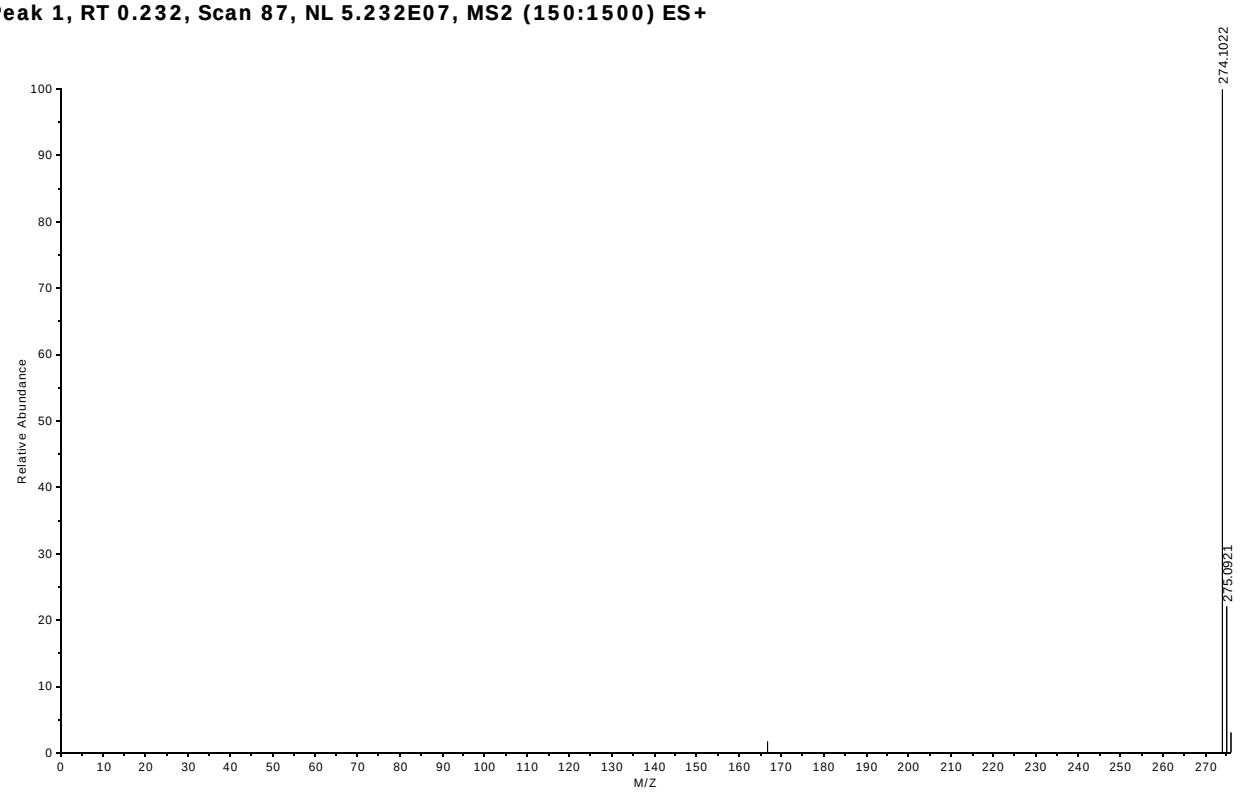
Group: Watts, J
Submitter: Michael Moazami
Project: RP-LC MW 150-1500
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Experiment: B ESIPOS (simple mix) C18 Acid
Filename: Watts_J_mm731626_MM_322.pdf
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Acquisition Date: 29/07/2014 14:26:37

TIC, NL 7.651E07, MS2 (150:1500) ES+

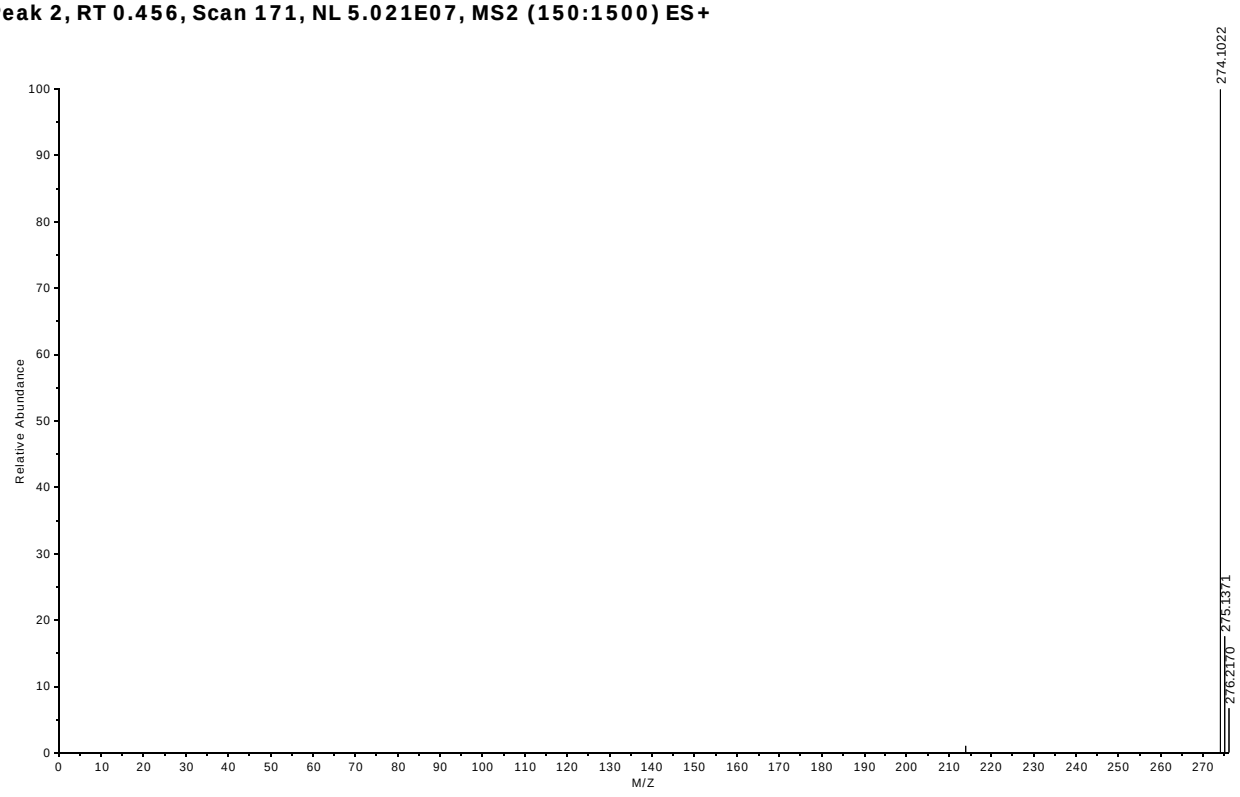


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3	1.37	4091052	5.56	7899962	5.36
4	3.59	13677870	18.60	49052222	33.29
5	4.22	11981379	16.30	34307365	23.28

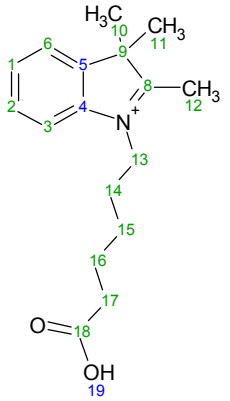
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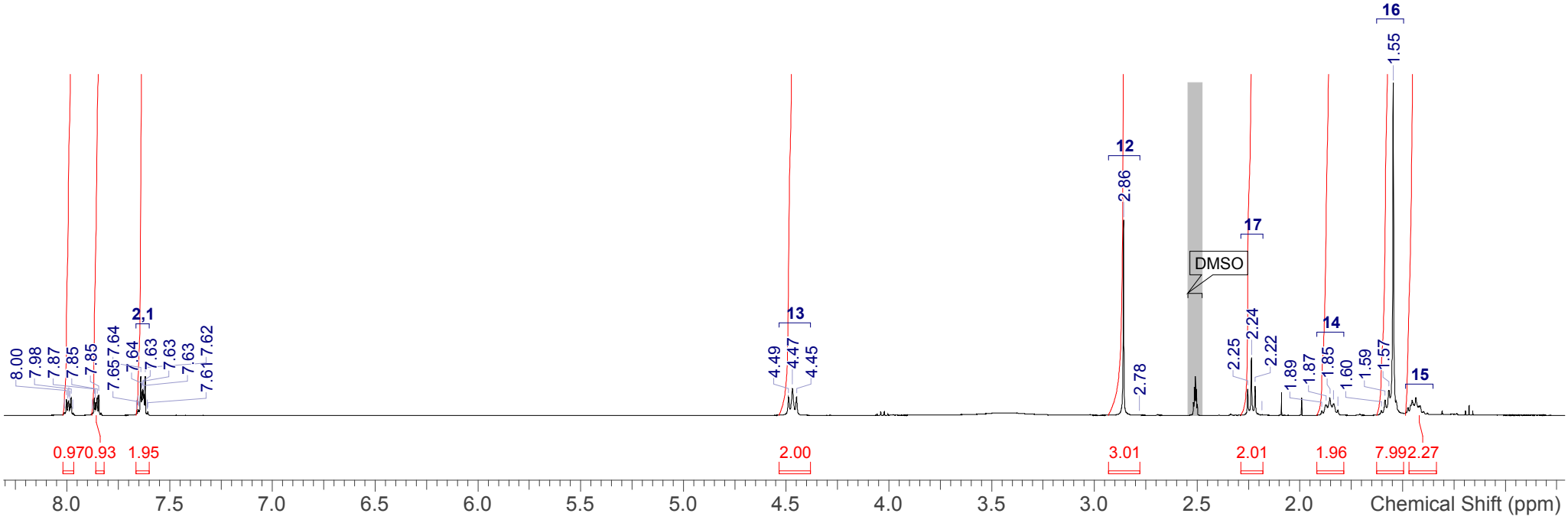
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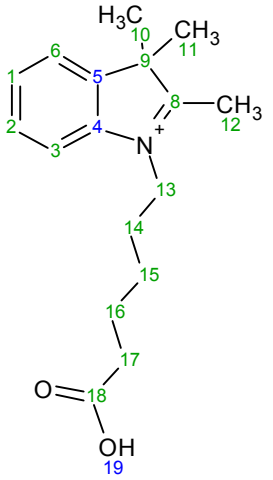
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Number of Transients	16	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	131072	***Pulse Sequence***	zg30
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Solvent	DMSO-d6	***Spectrum Offset (Hz)***	2470.9973
Spectrum Type	standard	***Sweep Width (Hz)***	8012.76
Temperature (degree C)	25.004		



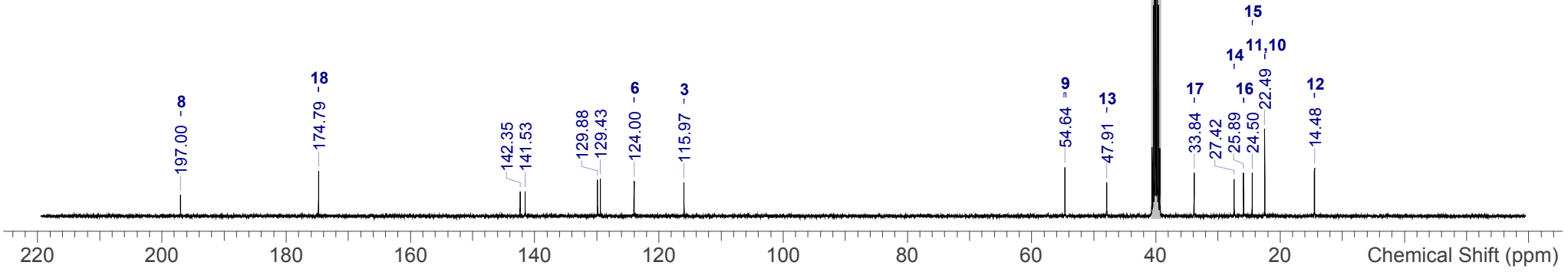
^{1}H NMR (400MHz, DMSO-d_6) δ = 7.97 - 8.02 (1H, m, M07), 7.84 - 7.88 (1H, m, M08), 7.60 - 7.66 (2H, m, H-2, 1), 4.47 (2H, br t, J = 7.7 Hz, H-13), 2.86 (3H, s, H-12), 2.24 (2H, t, J = 7.2 Hz, H-17), 1.85 (2H, quin, J = 7.7 Hz, H-14), 1.50 - 1.63 (8H, m, H-16), 1.35 - 1.49 (2H, m, H-15)



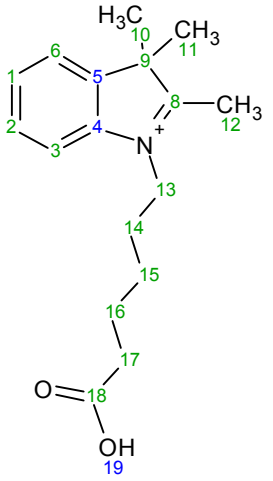
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Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	10060.3320
Spectrum Type	standard	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	24.993		



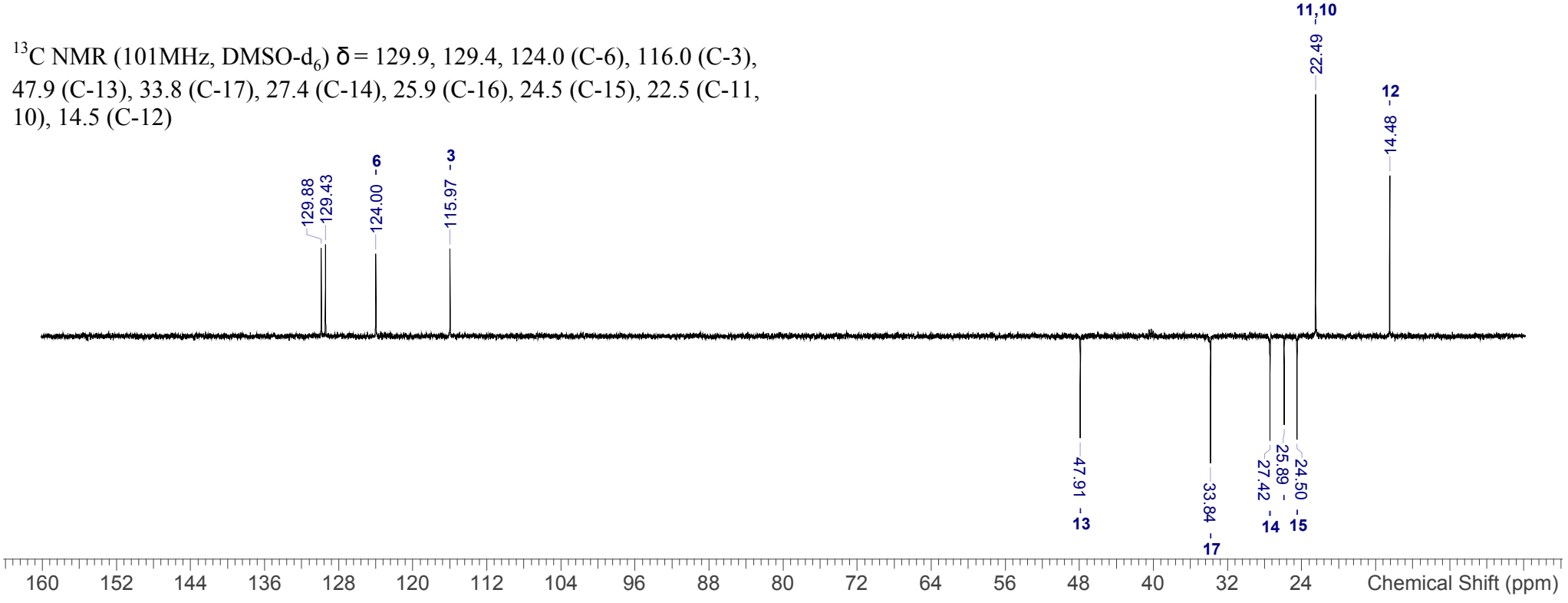
^{13}C NMR (101MHz, DMSO-d_6) δ = 197.0 (C-8), 174.8 (C-18), 142.3, 141.5, 129.9, 129.4, 124.0 (C-6), 116.0 (C-3), 54.6 (C-9), 47.9 (C-13), 33.8 (C-17), 27.4 (C-14), 25.9 (C-16), 24.5 (C-15), 22.5 (C-11, 10), 14.5 (C-12)



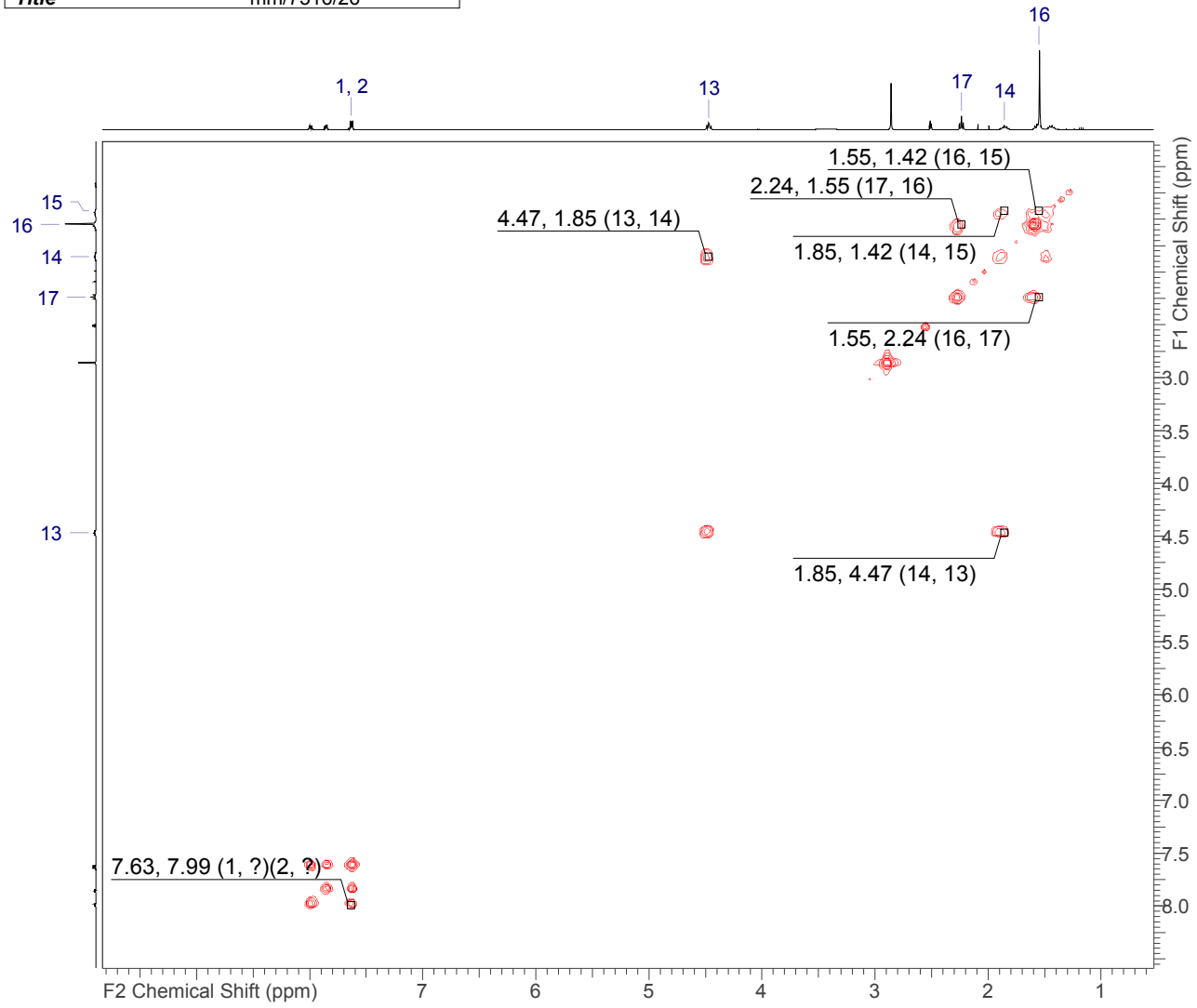
Acquisition Time (sec)	2.0316	***Comment***	mm/7316/26
Date	29 Jul 2014 16:14:44		
Date Stamp	29 Jul 2014 16:14:44		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data Reprocessed\jy2914mm1 trimethylindolenine hexanoic acid bromide\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	8047.8379
Spectrum Type	DEPT135	***Sweep Width (Hz)***	16128.54
Temperature (degree C)	25.001		

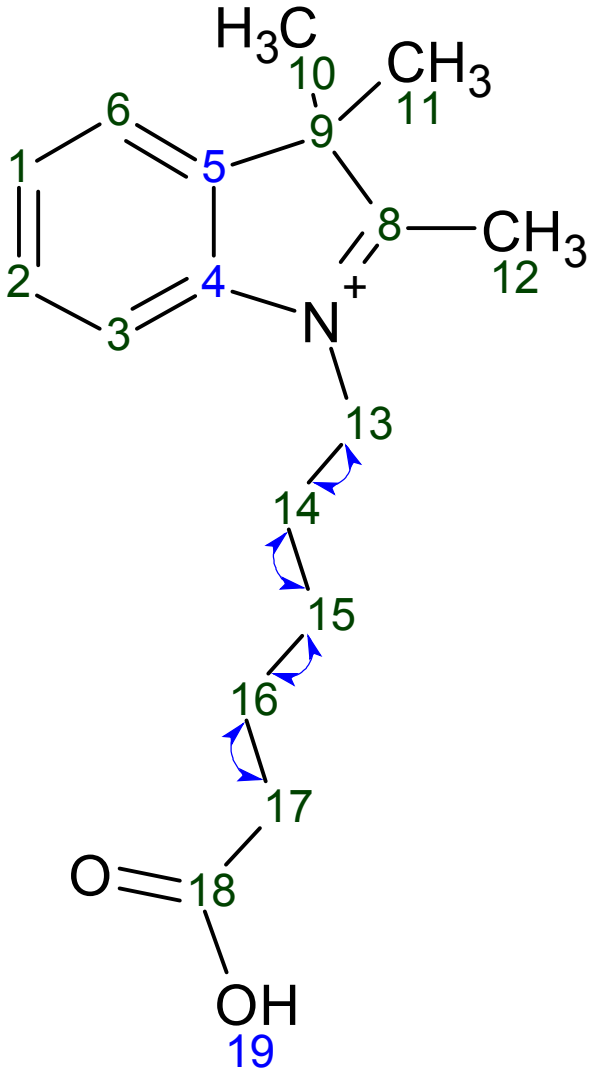


^{13}C NMR (101MHz, DMSO-d_6) δ = 129.9, 129.4, 124.0 (C-6), 116.0 (C-3), 47.9 (C-13), 33.8 (C-17), 27.4 (C-14), 25.9 (C-16), 24.5 (C-15), 22.5 (C-11, 10), 14.5 (C-12)



Acquisition Time (sec)	(0.1761, 0.0440)	***Comment***	mm/7316/26
Date	29 Jul 2014 15:30:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data Reprocessed\jy2914mm1 trimethylindolenine hexanoic acid bromide\13\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)	***Nucleus***	(1H, 1H)
Number of Transients	2	***Origin***	spect
Original Points Count	(1024, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	cosygpqf
Solvent	DMSO-d6	***Spectrum Type***	COSY
Sweep Width (Hz)	(5808.28, 5808.28)	***Temperature (degree C)***	24.994
Title	mm/7316/26		



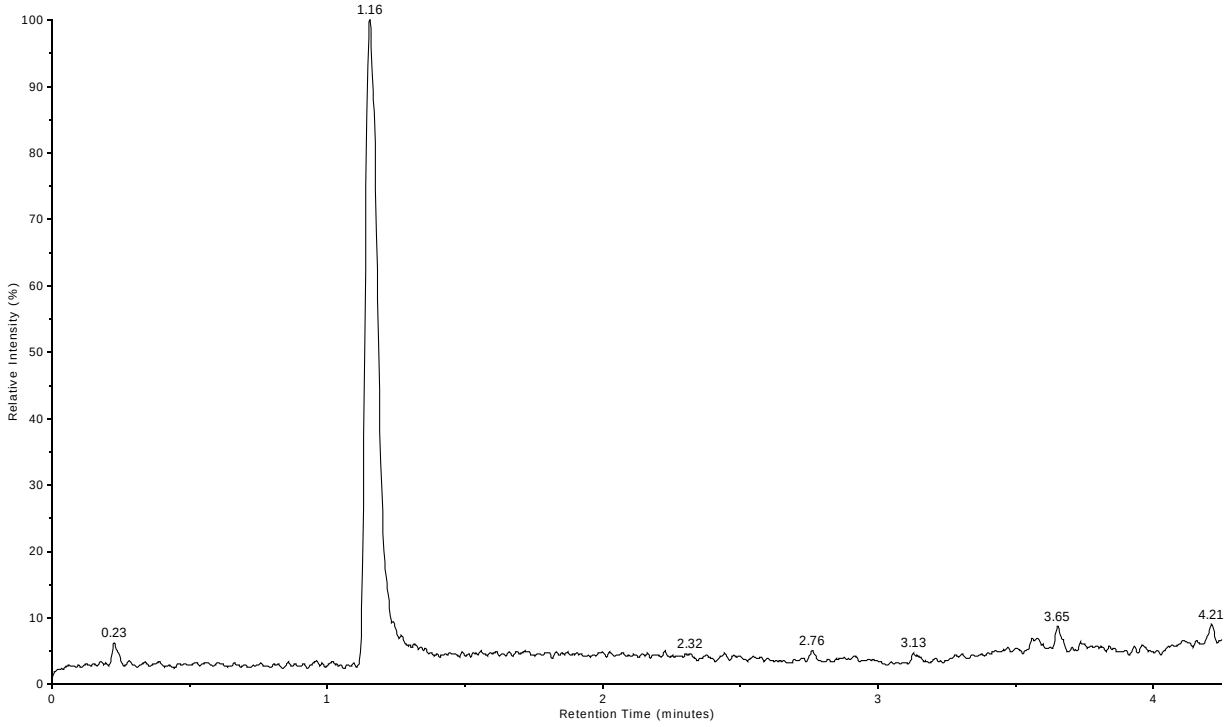


A.4 N-((1Z,3E)-3-(phenylimino)prop-1-en-1-yl)benzenaminium chloride

Top 5 Peak Report

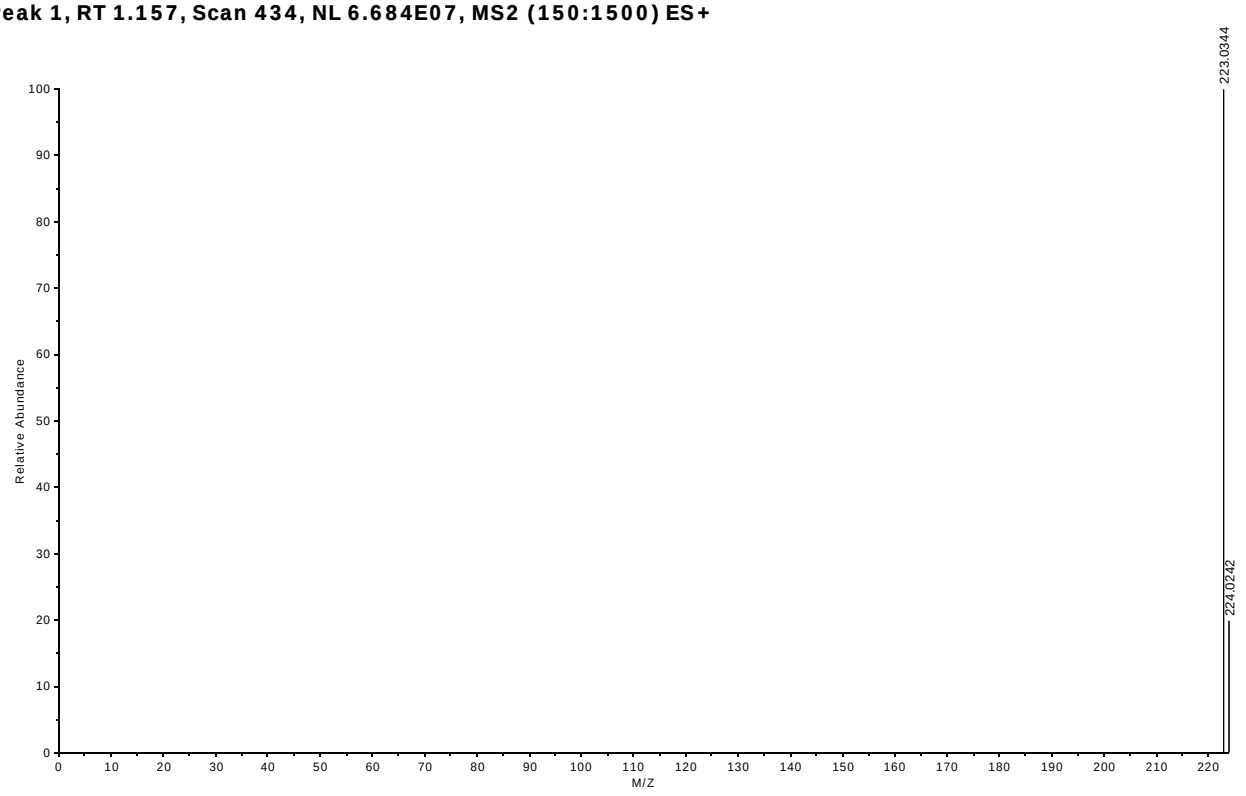
Group: Watts, J
Submitter: Michael Moazami
Project: RP-LC MW 150-1500
Sample ID: mm/7316/22
Experiment: B ESIPOS (simple mix) C18 Acid
Filename: Watts_J_mm731622_MM_323.pdf
Instrument: Blue RP UHPLC-MS (B30:1023)
Acquisition Date: 29/07/2014 14:36:26

TIC, NL 8.612E07, MS2 (150:1500) ES+

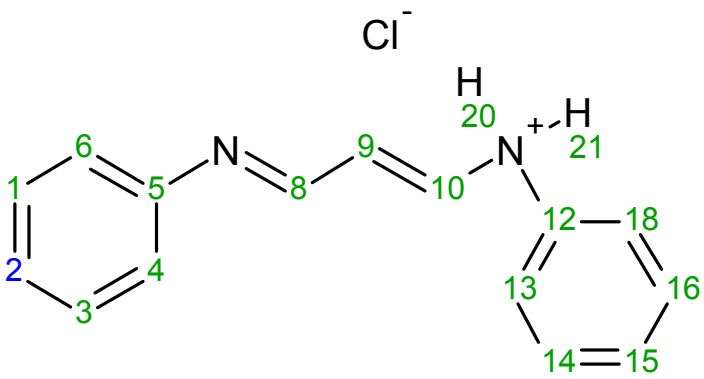


	RT Mins	Height	Height %	Area	Area %
1	1.16	81069286	100.00	270499563	100.00

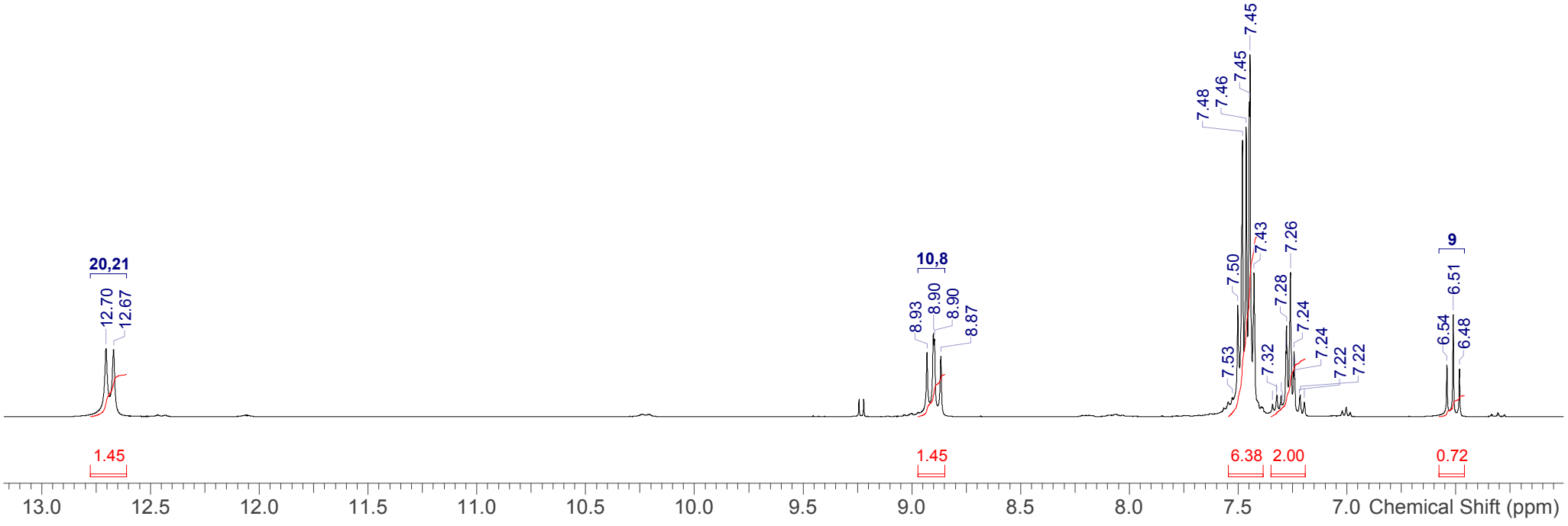
Peak 1, RT 1.157, Scan 434, NL 6.684E07, MS2 (150:1500) ES+



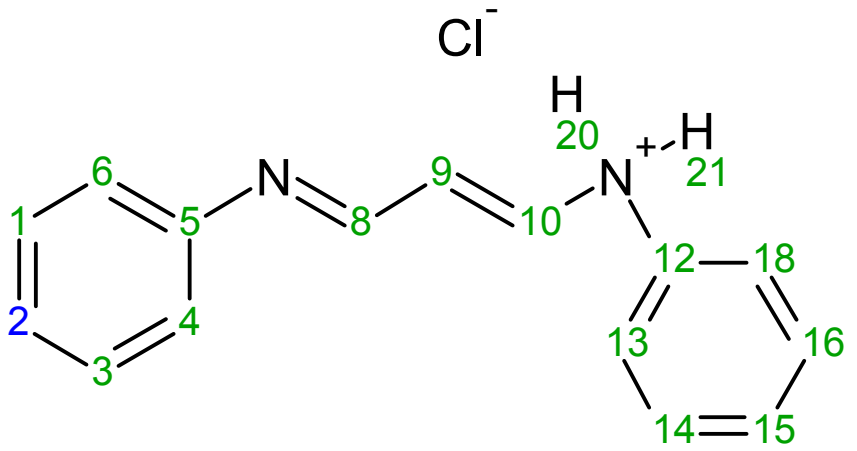
Acquisition Time (sec)	4.0894	***Comment***	mm/7316/22		
Date	29 Jul 2014 16:37:44				
Date Stamp	29 Jul 2014 16:37:44				
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\jy2914mm2 malondialdehyde dianil HCl\10\PDATA\1\1r				
Frequency (MHz)	400.1300	***Nucleus***	1H	***Number of Transients***	16
Origin	spect	***Original Points Count***	32768	***Owner***	nmr
Points Count	65536	***Pulse Sequence***	zg30	***Receiver Gain***	64.46
SW(cyclical) (Hz)	8012.82	***Solvent***	DMSO-d6	***Spectrum Offset (Hz)***	2470.9668
Spectrum Type	standard	***Sweep Width (Hz)***	8012.70	***Temperature (degree C)***	24.992



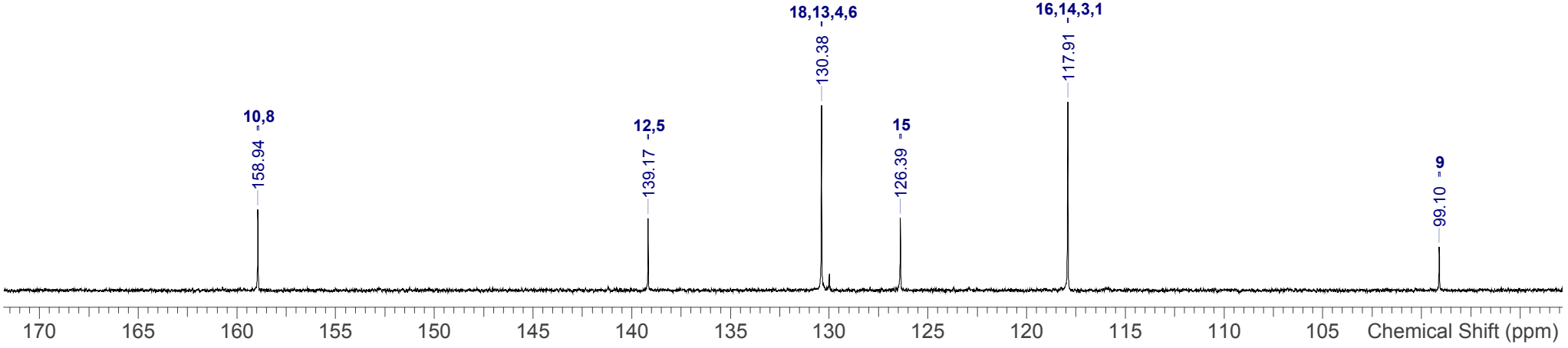
^{1}H NMR (400MHz, DMSO-d_6) δ = 12.69 (1H, br d, J = 13.8 Hz, H-20, 21), 8.90 (1H, dd, J = 13.7 Hz, J = 11.7 Hz, H-10, 8), 7.38 - 7.55 (6H, m, M03), 7.19 - 7.35 (2H, m, M04), 6.51 (1H, t, J = 11.6 Hz, H-9)



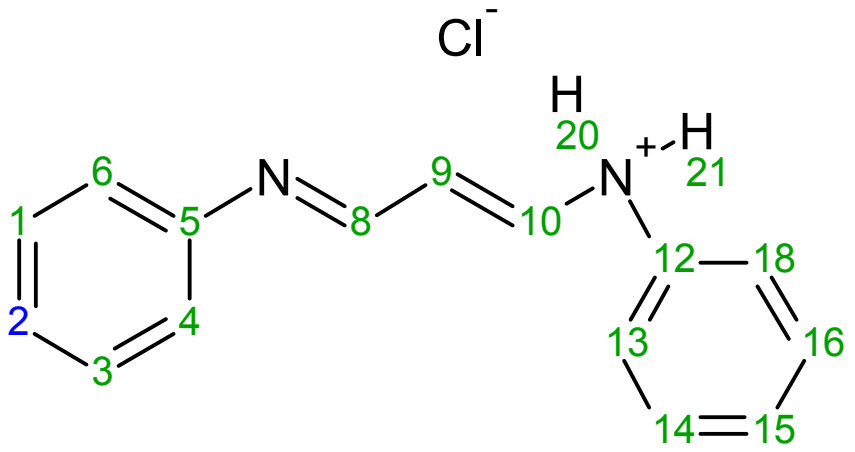
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/22
Date	29 Jul 2014 17:08:14		
Date Stamp	29 Jul 2014 17:08:14		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\jy2914mm2 malondialdehyde dianil HCl\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	10060.3320
Spectrum Type	standard	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	25.001		

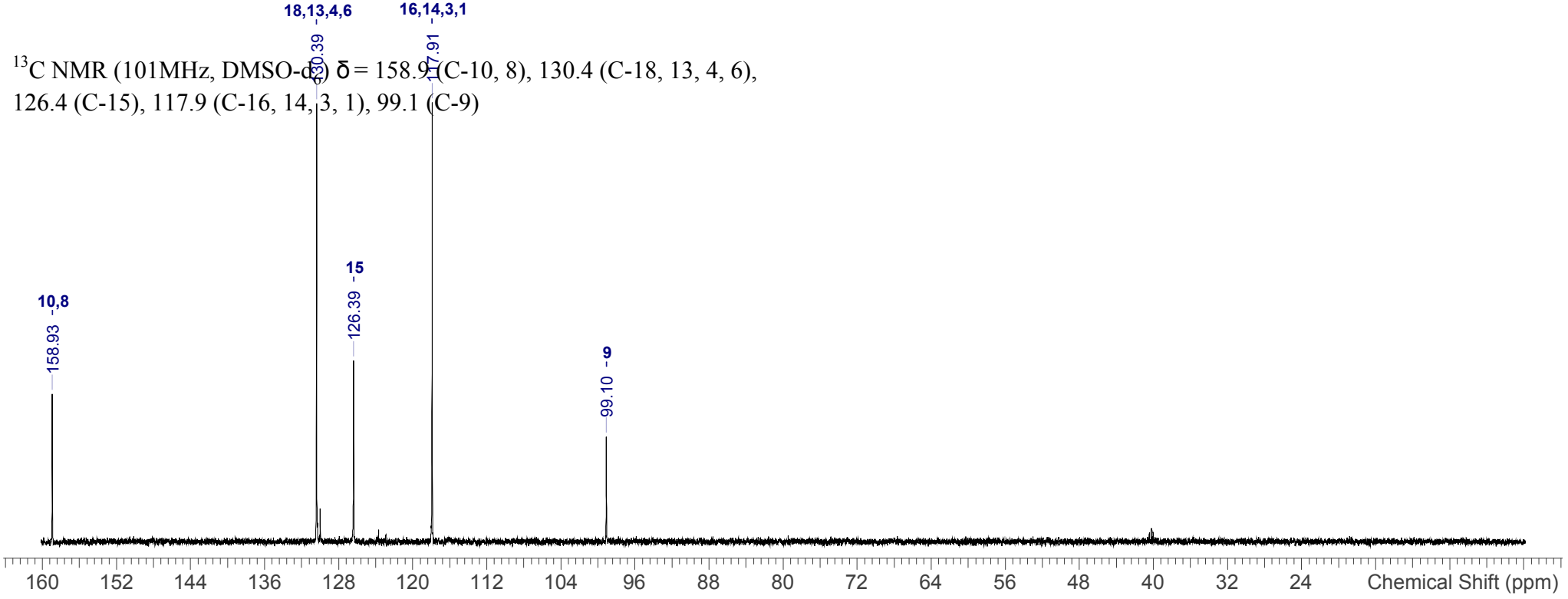


^{13}C NMR (101MHz, DMSO-d_6) δ = 158.9 (C-10, 8), 139.2 (C-12, 5), 130.4 (C-18, 13, 4, 6), 126.4 (C-15), 117.9 (C-16, 14, 3, 1), 99.1 (C-9)



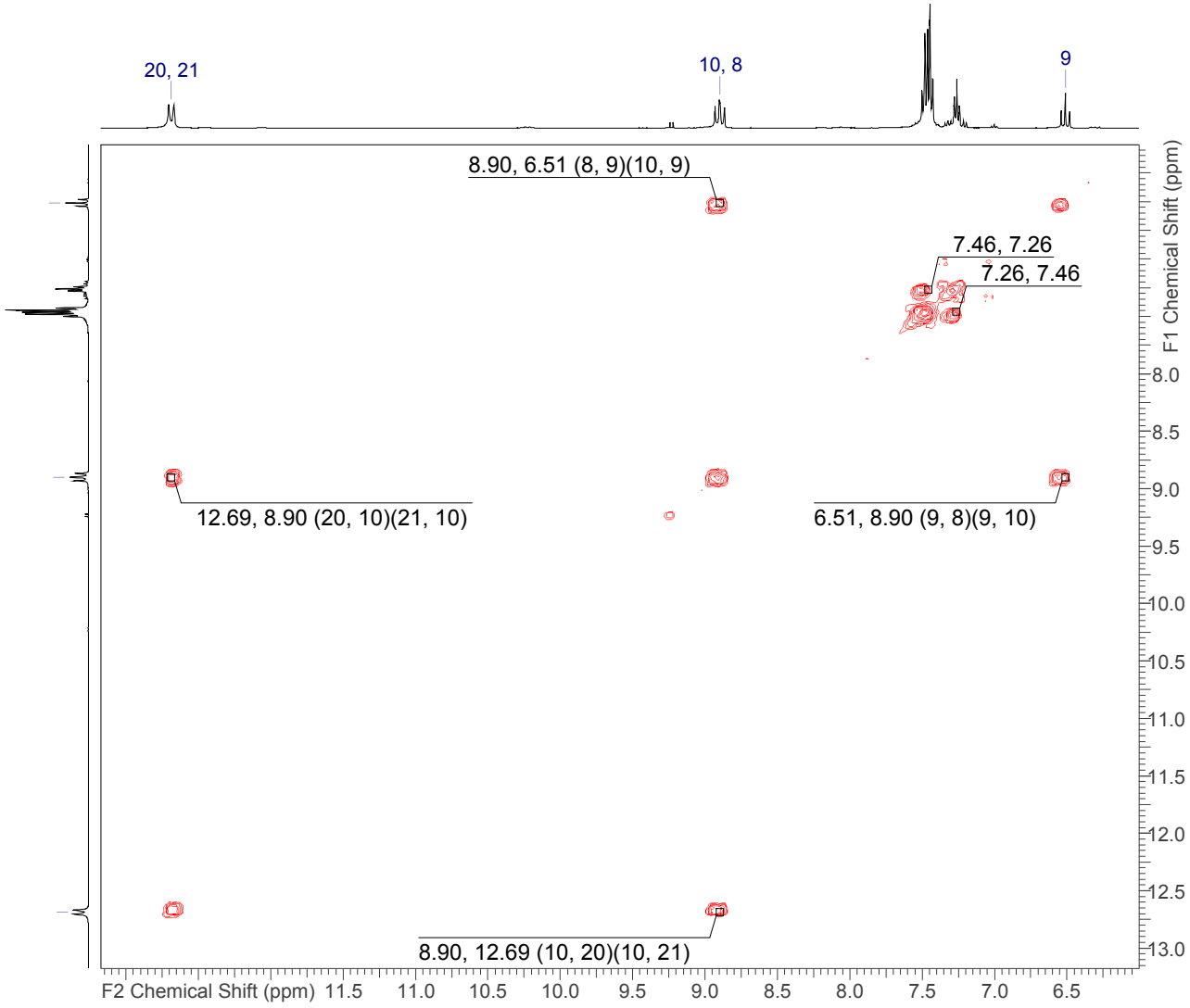
Acquisition Time (sec)	2.0316	***Comment***	mm/7316/22
Date	29 Jul 2014 17:26:52		
Date Stamp	29 Jul 2014 17:26:52		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\jy2914mm2 malondialdehyde dianil HCl\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	8047.9146
Spectrum Type	DEPT135	***Sweep Width (Hz)***	16128.54
Temperature (degree C)	25.001		

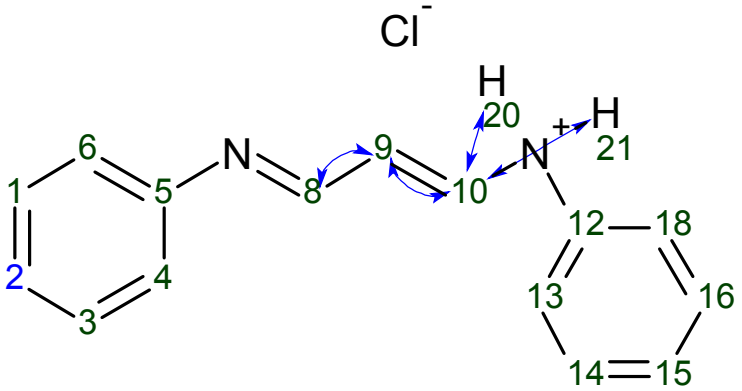




^{13}C NMR (101MHz, DMSO-d6) δ = 158.9 (C-10, 8), 130.4 (C-18, 13, 4, 6), 126.4 (C-15), 117.9 (C-16, 14, 3, 1), 99.1 (C-9)

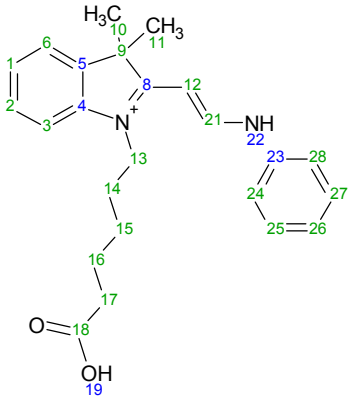
Acquisition Time (sec)	(0.2171, 0.0543)	***Comment***	5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218
Date	29 Jul 2014 17:43:00		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\jy2914mm2 malondialdehyde dianil HCl\13\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)	***Nucleus***	(1H, 1H)
Number of Transients	2	***Origin***	spect
Original Points Count	(1024, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	cosygpqf
Solvent	DMSO-d6	***Spectrum Type***	COSY
Sweep Width (Hz)	(4712.37, 4712.37)	***Temperature (degree C)***	24.994
Title	mm/		



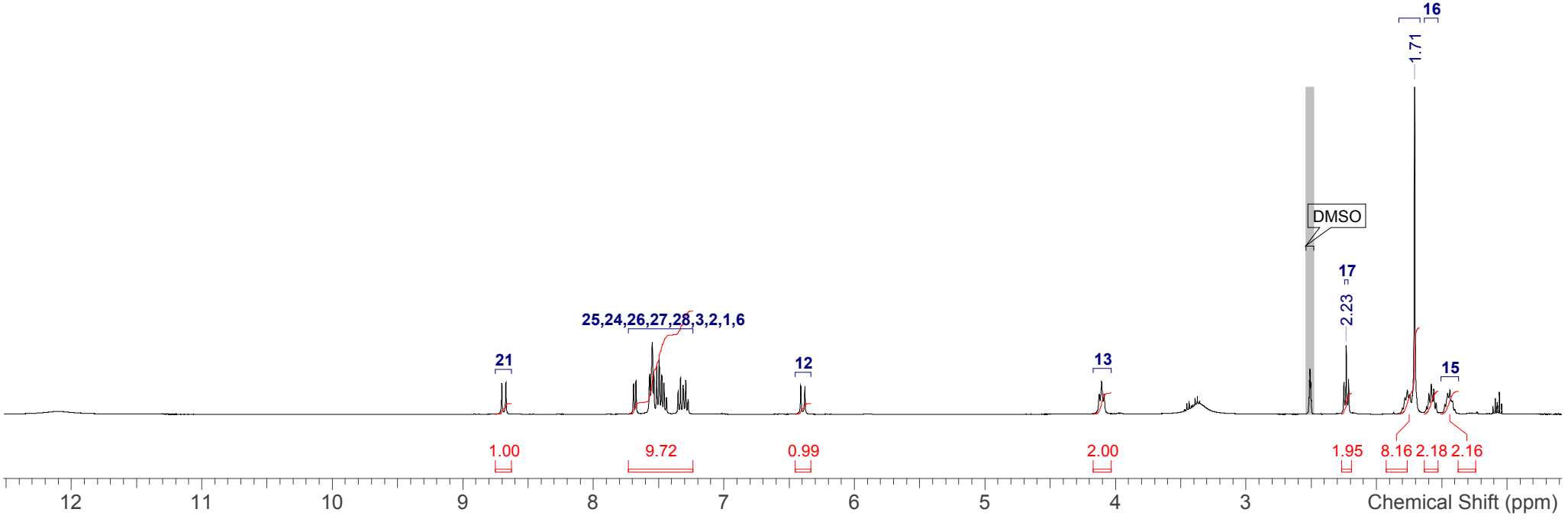


A.5 (E)-1-(5-carboxypentyl)-3,3-dimethyl-2-(2-(phenylamino)vinyl)-3H-indol-1-ium bromide

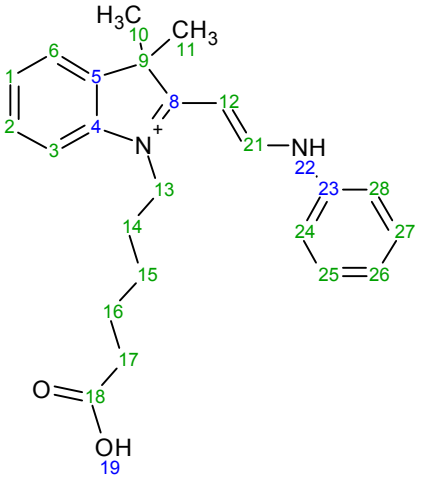
Acquisition Time (sec)	4.0894	***Comment***	mm/7361/29		
Date	31 Jul 2014 14:58:11				
Date Stamp	31 Jul 2014 14:58:11				
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\jy3114mm1 Hexanoic acid indole hemiCy3\10\PDATA\1\1r				
Frequency (MHz)	400.1300	***Nucleus***	1H	***Number of Transients***	16
Origin	spect	***Original Points Count***	32768	***Owner***	nmr
Points Count	65536	***Pulse Sequence***	zg30	***Receiver Gain***	50.16
SW(cyclical) (Hz)	8012.82	***Solvent***	DMSO-d6	***Spectrum Offset (Hz)***	2470.9668
Spectrum Type	standard	***Sweep Width (Hz)***	8012.70	***Temperature (degree C)***	25.006



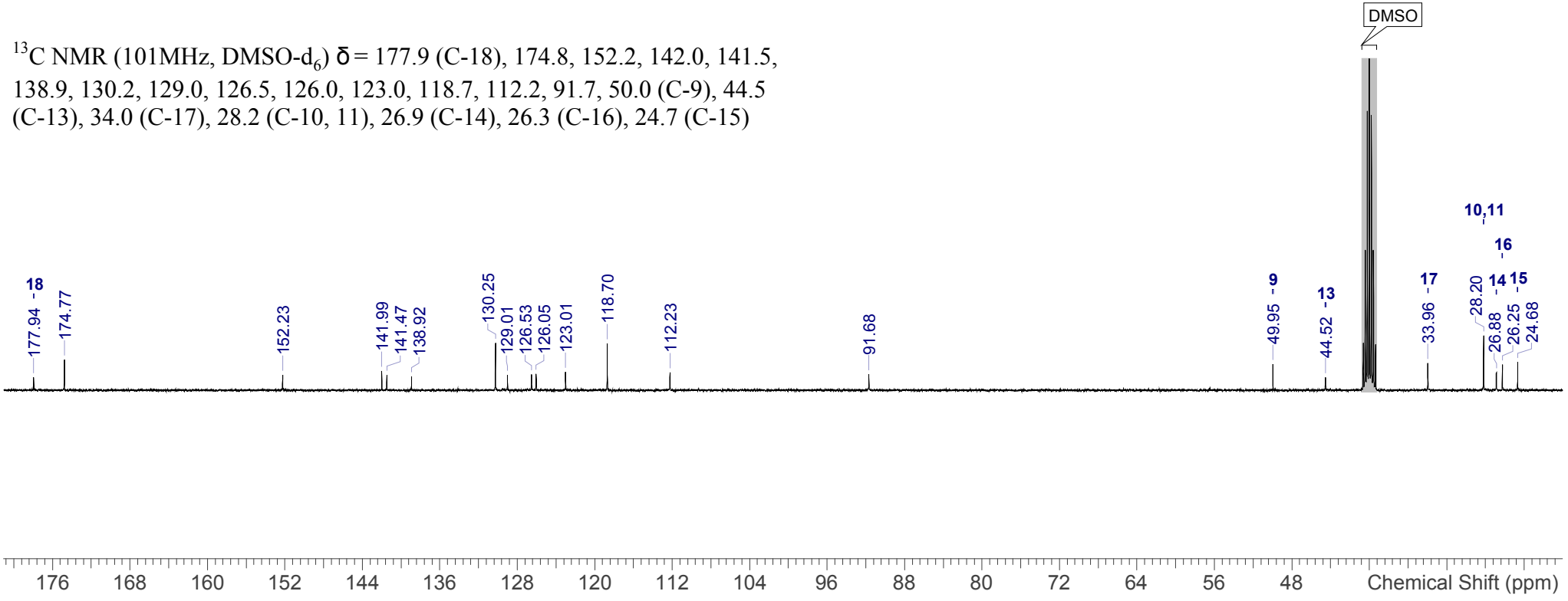
^{1}H NMR (400MHz, DMSO-d_6) δ = 8.63 - 8.75 (1H, m, H-21), 7.24 - 7.73 (10H, m, H-25, 24, 26, 27, 28, 3, 2, 1, 6), 6.33 - 6.45 (1H, m, H-12), 4.03 - 4.17 (2H, m, H-13), 2.23 (1H, s, H-17), 2.19 - 2.27 (2H, m, M02), 1.71 (8H, s, H-14, 10, 11), 1.53 - 1.63 (2H, m, H-16), 1.37 - 1.50 (2H, m, H-15)



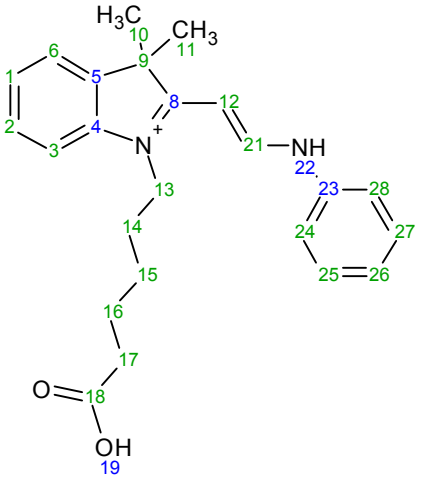
Acquisition Time (sec)	1.3631	***Comment***	mm/7361/29
Date	31 Jul 2014 15:28:56		
Date Stamp	31 Jul 2014 15:28:56		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\jy3114mm1 Hexanoic acid indole hemiCy3\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	10060.3320
Spectrum Type	standard	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	25.007		



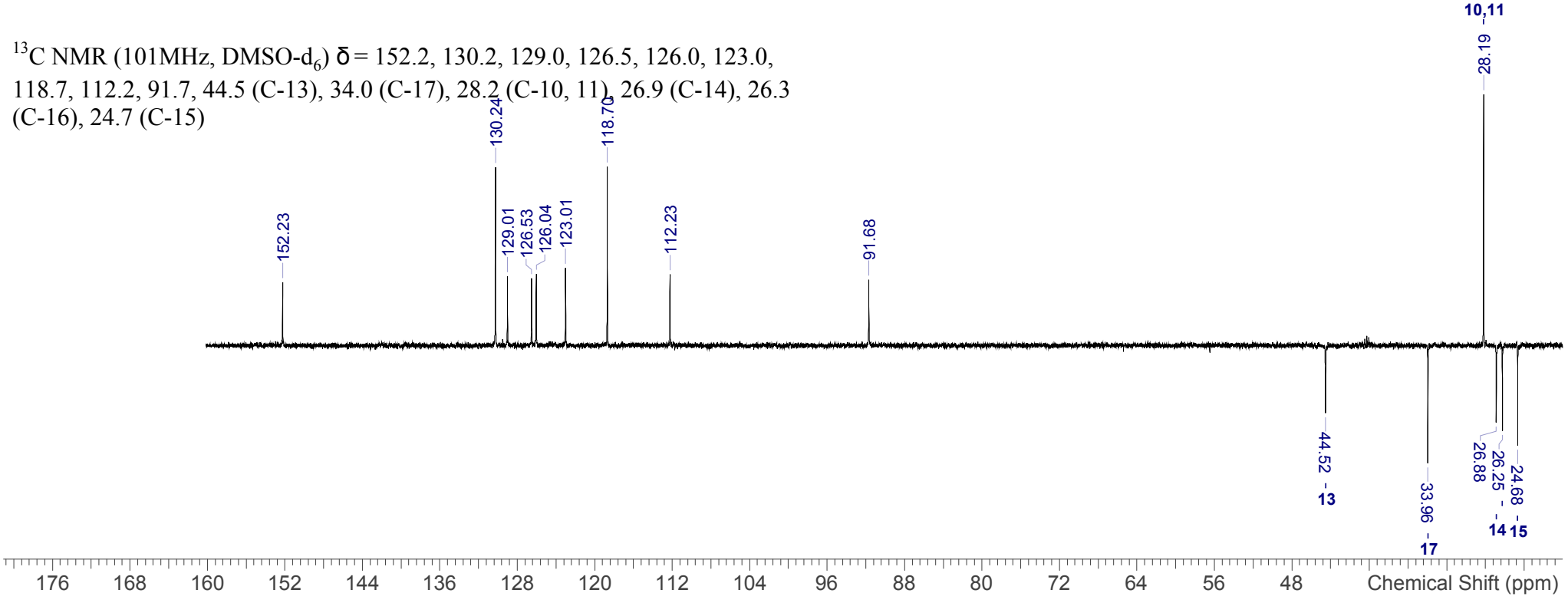
^{13}C NMR (101MHz, DMSO-d_6) δ = 177.9 (C-18), 174.8, 152.2, 142.0, 141.5, 138.9, 130.2, 129.0, 126.5, 126.0, 123.0, 118.7, 112.2, 91.7, 50.0 (C-9), 44.5 (C-13), 34.0 (C-17), 28.2 (C-10, 11), 26.9 (C-14), 26.3 (C-16), 24.7 (C-15)



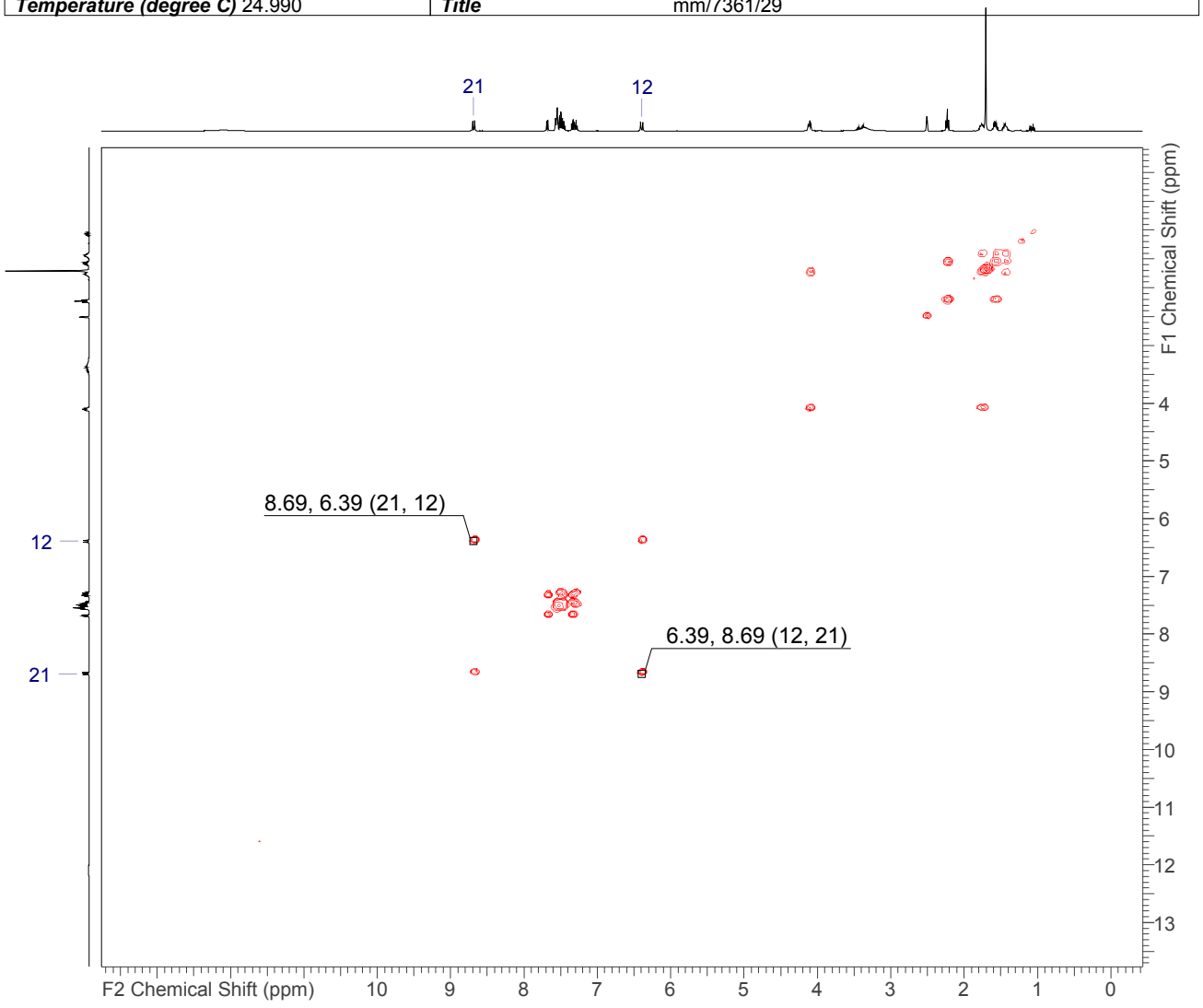
Acquisition Time (sec)	2.0316	***Comment***	mm/7361/29
Date	31 Jul 2014 15:47:30		
Date Stamp	31 Jul 2014 15:47:30		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\jy3114mm1 Hexanoic acid indole hemiCy3\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	8047.9146
Spectrum Type	DEPT135	***Sweep Width (Hz)***	16128.54
Temperature (degree C)	24.993		

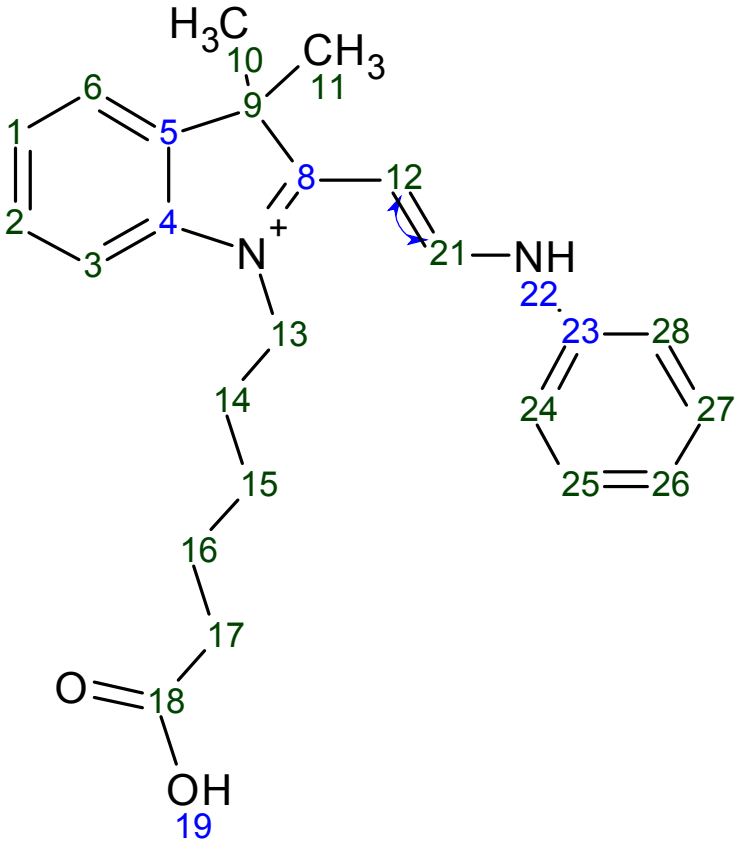


^{13}C NMR (101MHz, DMSO-d$_6$) δ = 152.2, 130.2, 129.0, 126.5, 126.0, 123.0, 118.7, 112.2, 91.7, 44.5 (C-13), 34.0 (C-17), 28.2 (C-10, 11), 26.9 (C-14), 26.3 (C-16), 24.7 (C-15)



Acquisition Time (sec)	(0.1802, 0.0451)	***Comment***	mm/7361/29
Date	31 Jul 2014 14:56:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\jy3114mm1 Hexanoic acid indole hemiCy3\13\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)		
Nucleus	(1H, 1H)	***Number of Transients***	2
Origin	spect	***Original Points Count***	(1024, 256)
Owner	nmr	***Points Count***	(1024, 1024)
Pulse Sequence	cosygpqf	***Solvent***	DMSO-d6
Spectrum Type	COSY	***Sweep Width (Hz)***	(5676.27, 5676.27)
Temperature (degree C)	24.990	***Title***	mm/7361/29



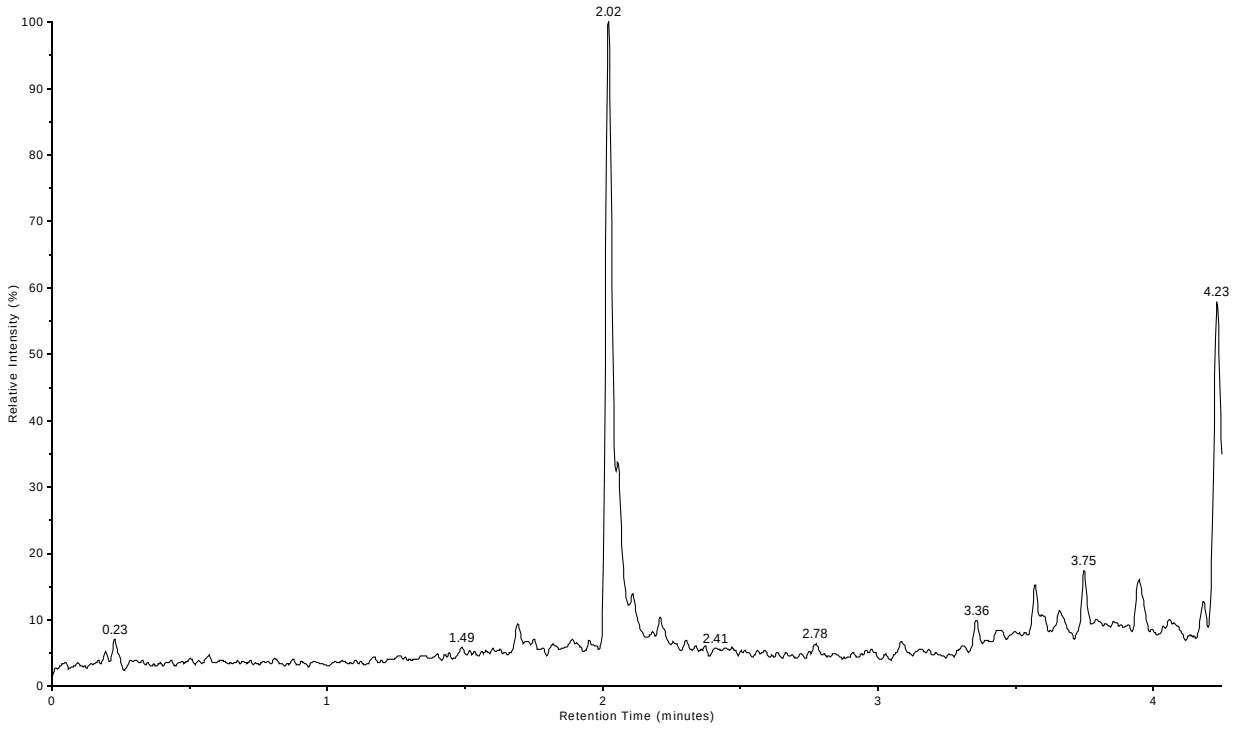


A.6 1-(5-carboxypentyl)-3,3-dimethyl-2-((1E,3E)-3-(1,3,3-trimethylindolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium bromide

Top 5 Peak Report

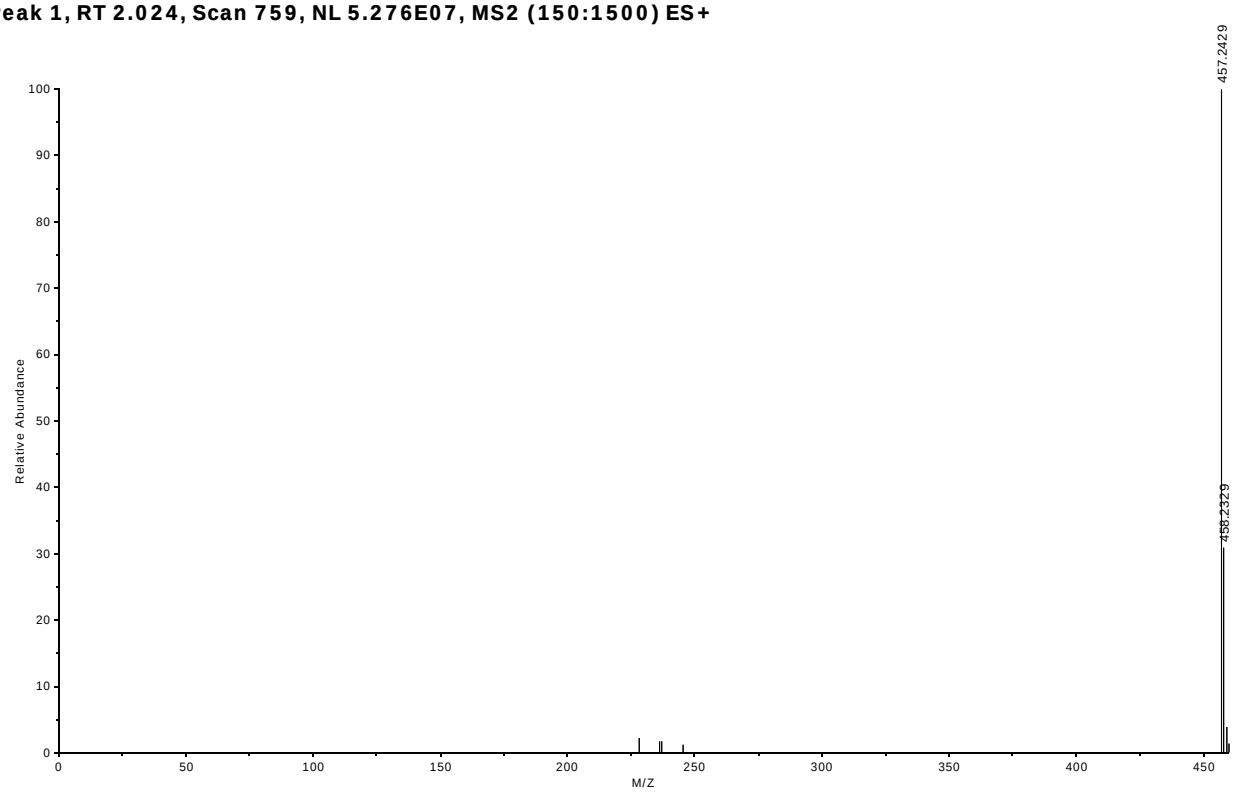
Group: Watts, J
Submitter: Michael Moazami
Project: RP-LC MW 150-1500
Sample ID: mm/7316/34 30-end
Experiment: B ESIPOS (simple mix) C18 Acid
Filename: Watts_J_mm731634_30end_MM_352.pdf
Instrument: Blue RP UHPLC-MS (B30:1023)
Acquisition Date: 05/08/2014 14:14:40

TIC, NL 8.098E07, MS2 (150:1500) ES+

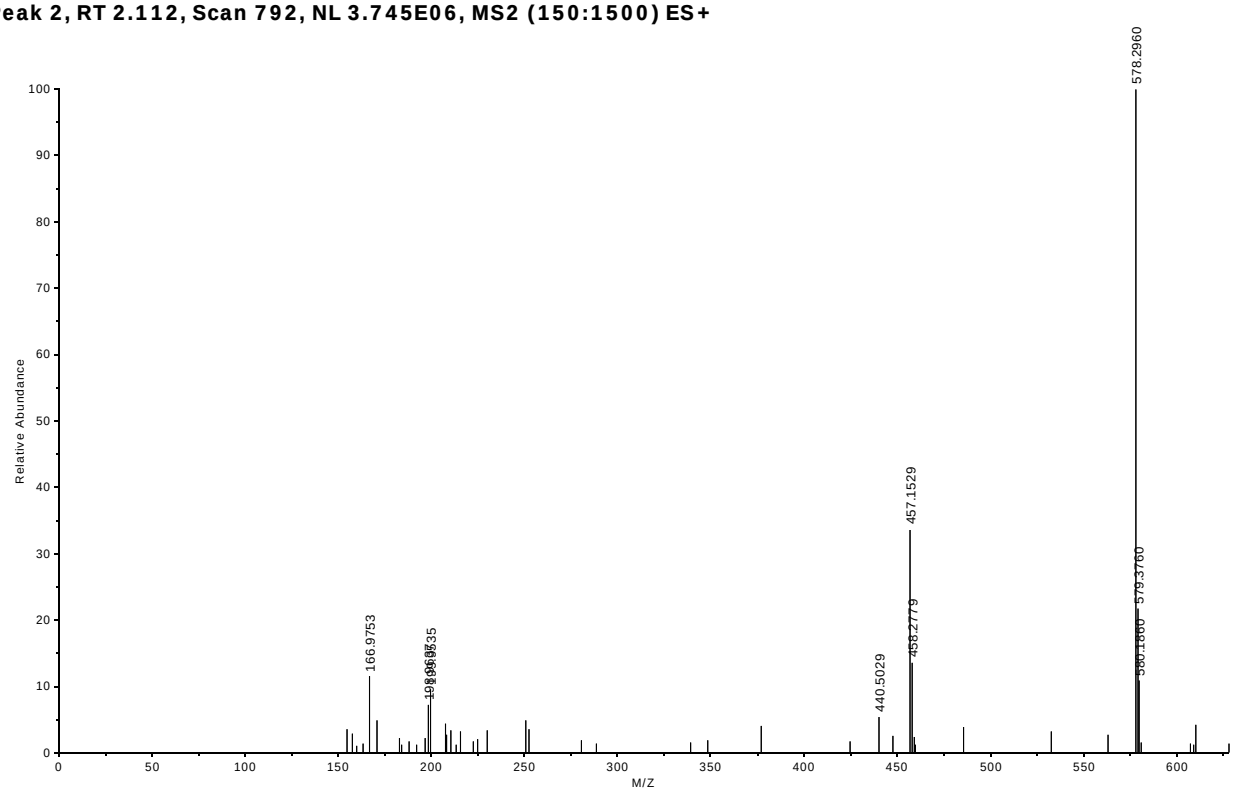


	RT Mins	Height	Height %	Area	Area %
1	2.02	65675350	100.00	132884277	100.00
2	2.11	5362119	8.16	9758048	7.34
3	3.75	7416494	11.29	12983743	9.77
4	3.95	6321127	9.62	11995564	9.03
5	4.23	29064877	44.26	66013018	49.68

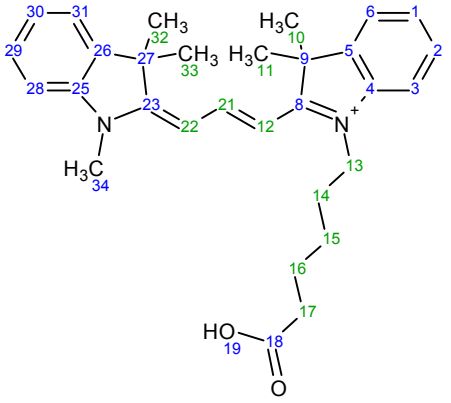
Peak 1, RT 2.024, Scan 759, NL 5.276E07, MS2 (150:1500) ES+



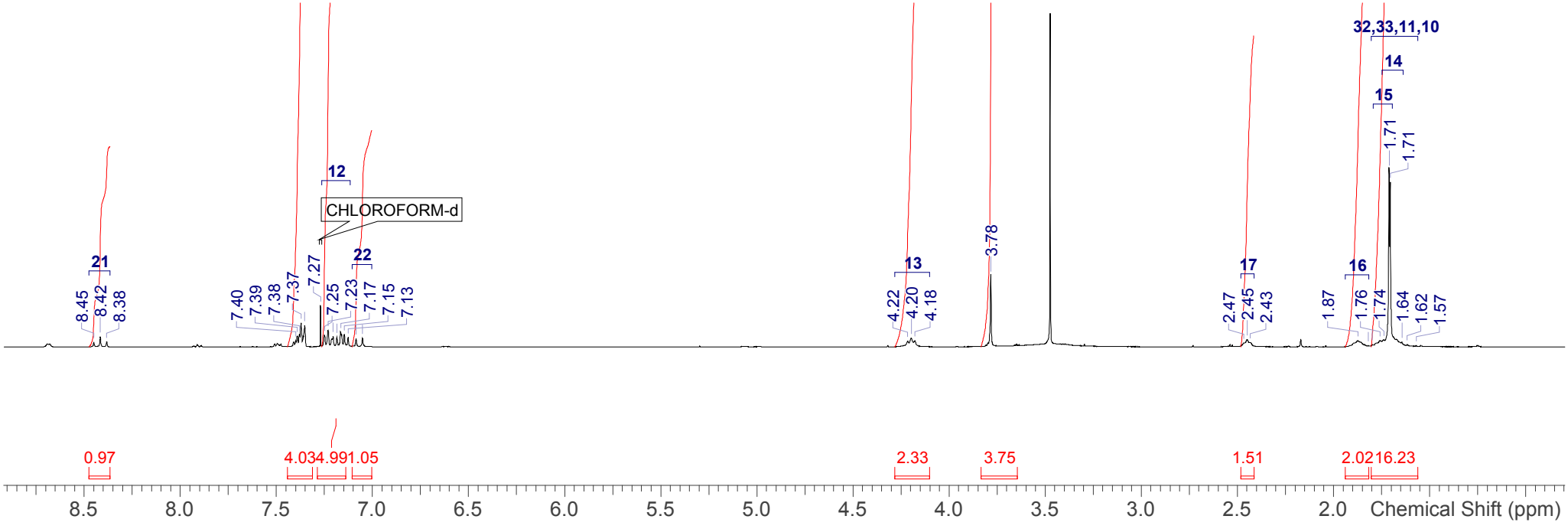
Peak 2, RT 2.112, Scan 792, NL 3.745E06, MS2 (150:1500) ES+



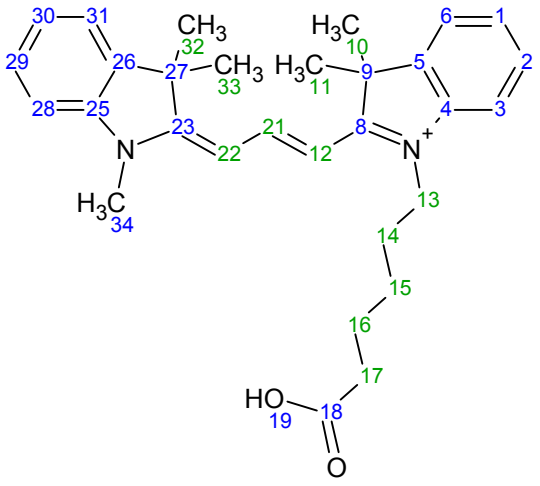
Acquisition Time (sec)	4.0894	***Comment***	mm/7316/34 30-flask fractions		
Date	05 Aug 2014 16:16:33			***Date Stamp***	05 Aug 2014 16:16:33
File Name	C:\Users\Mike Moazami\Desktop\au0514mm1- Cy3\10\PDATA\1\1r				
Frequency (MHz)	400.1300	***Nucleus***	1H	***Number of Transients***	16
Origin	spect	***Original Points Count***	32768	***Owner***	nmr
Points Count	262144	***Pulse Sequence***	zg30	***Receiver Gain***	64.46
SW(cyclical) (Hz)	8012.82	***Solvent***	CHLOROFORM-d		
Spectrum Offset (Hz)	2464.9822	***Spectrum Type***	standard	***Sweep Width (Hz)***	8012.79
Temperature (degree C)	25.004				



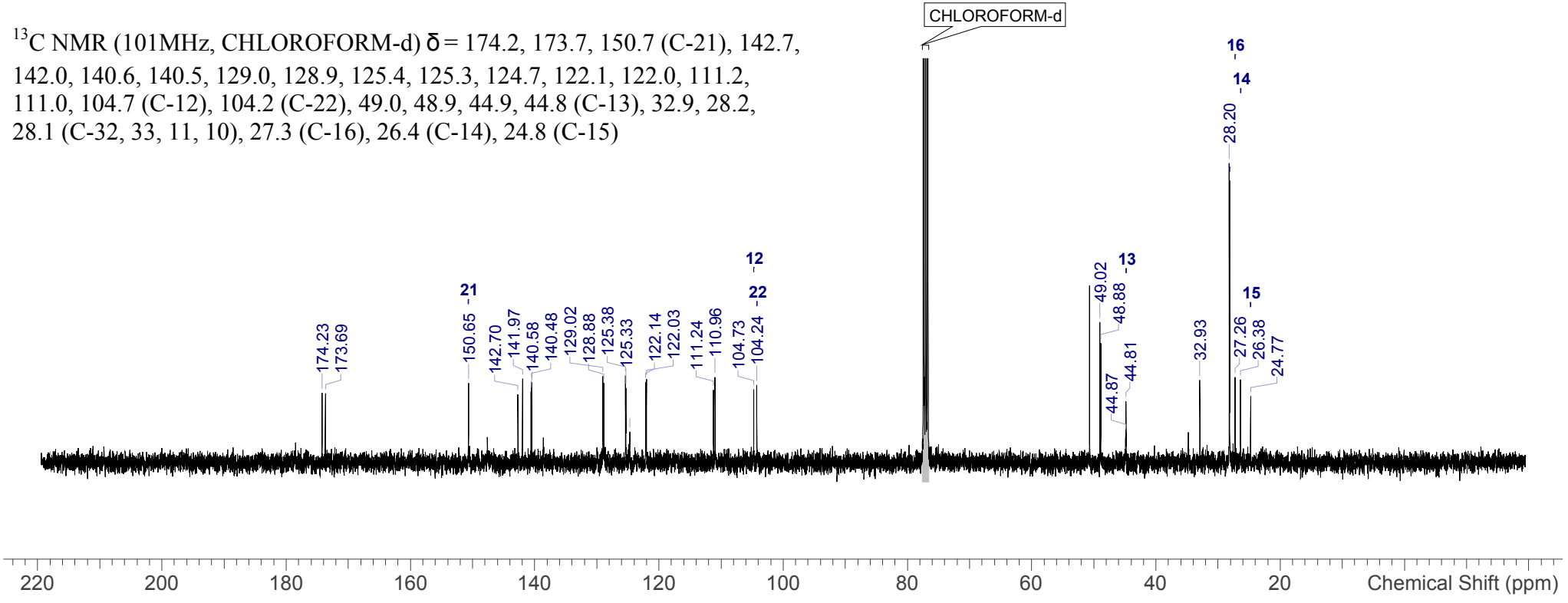
^{1}H NMR (400MHz, CHLOROFORM-d) δ = 8.42 (1H, t, J = 13.4 Hz, H-21), 7.31 - 7.44 (4H, m, M08), 7.12 - 7.26 (5H, m, H-12), 7.07 (1H, d, J = 13.4 Hz, H-22), 4.20 (2H, br t, J = 7.5 Hz, H-13), 3.78 (4H, s, M04), 2.45 (2H, br t, J = 6.4 Hz, H-17), 1.82 - 1.94 (2H, m, H-16), 1.75 (2H, br d, J = 7.5 Hz, H-15), 1.64 (3H, br s, H-14), 1.71 (11H, d, J = 2.1 Hz, H-32, 33, 11, 10)



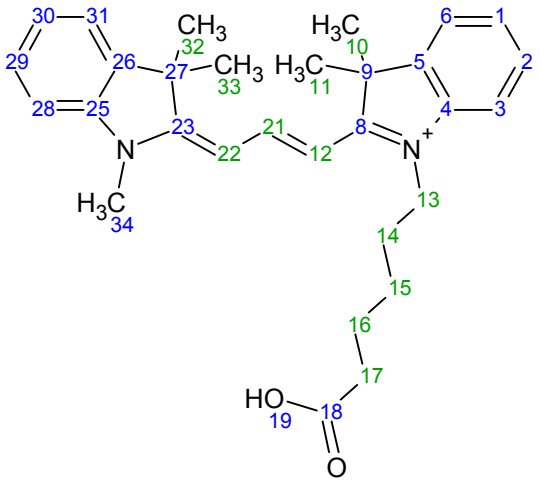
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/34 30-flask fractions
Date	05 Aug 2014 16:46:53		
Date Stamp	05 Aug 2014 16:46:53		
File Name	C:\Users\Mike Moazami\Desktop\au0514mm1- Cy3\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	10060.3320	***Spectrum Type***	standard
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	25.003



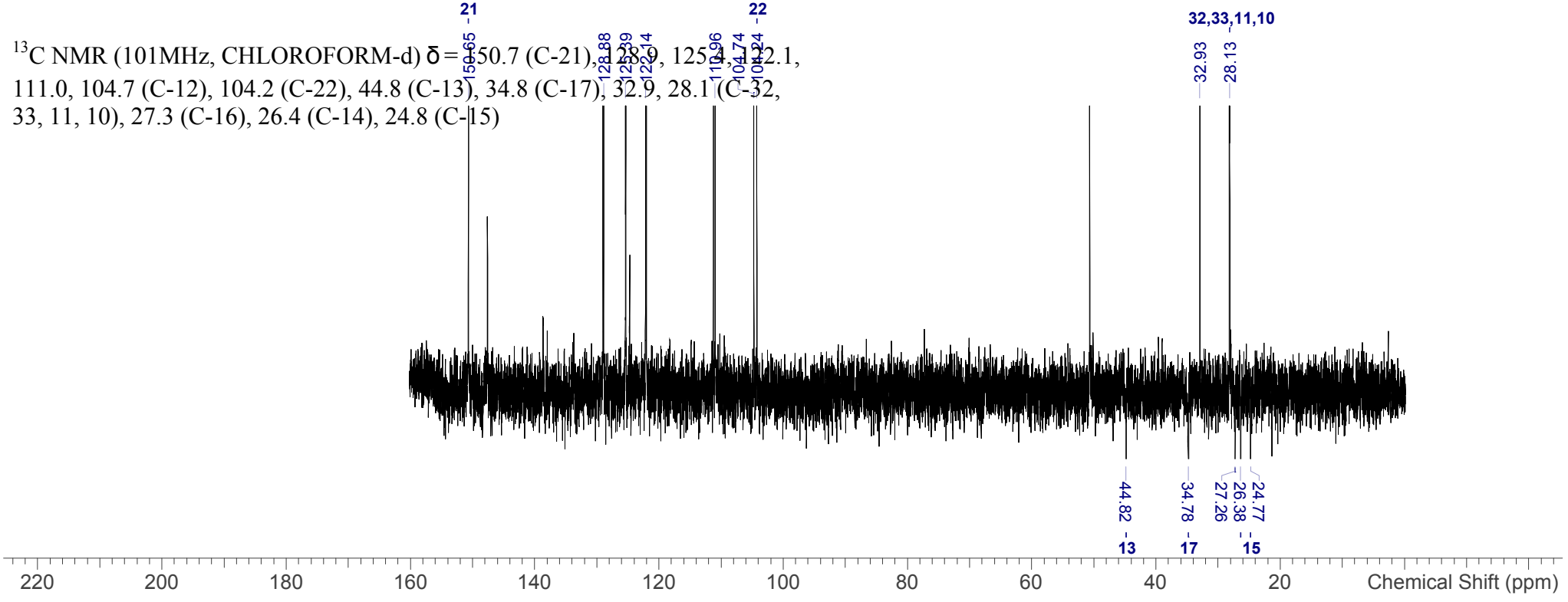
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 174.2, 173.7, 150.7 (C-21), 142.7, 142.0, 140.6, 140.5, 129.0, 128.9, 125.4, 125.3, 124.7, 122.1, 122.0, 111.2, 111.0, 104.7 (C-12), 104.2 (C-22), 49.0, 48.9, 44.9, 44.8 (C-13), 32.9, 28.2, 28.1 (C-32, 33, 11, 10), 27.3 (C-16), 26.4 (C-14), 24.8 (C-15)



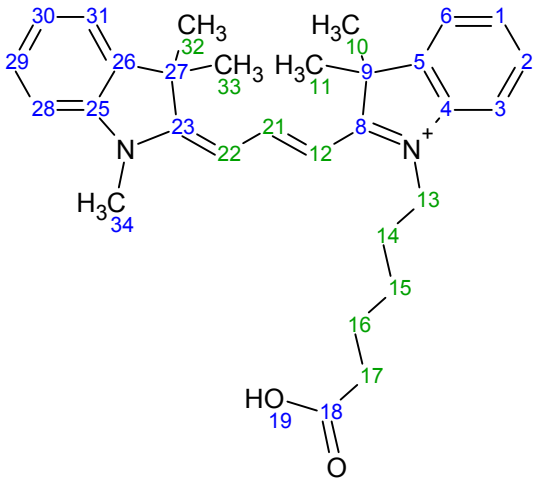
Acquisition Time (sec)	2.0316	***Comment***	mm/7316/34 30-flask fractions
Date	05 Aug 2014 17:05:29		
Date Stamp	05 Aug 2014 17:05:29		
File Name	C:\Users\Mike Moazami\Desktop\au0514mm1- Cy3\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	8047.7910	***Spectrum Type***	DEPT135
Sweep Width (Hz)	16128.54	***Temperature (degree C)***	24.991



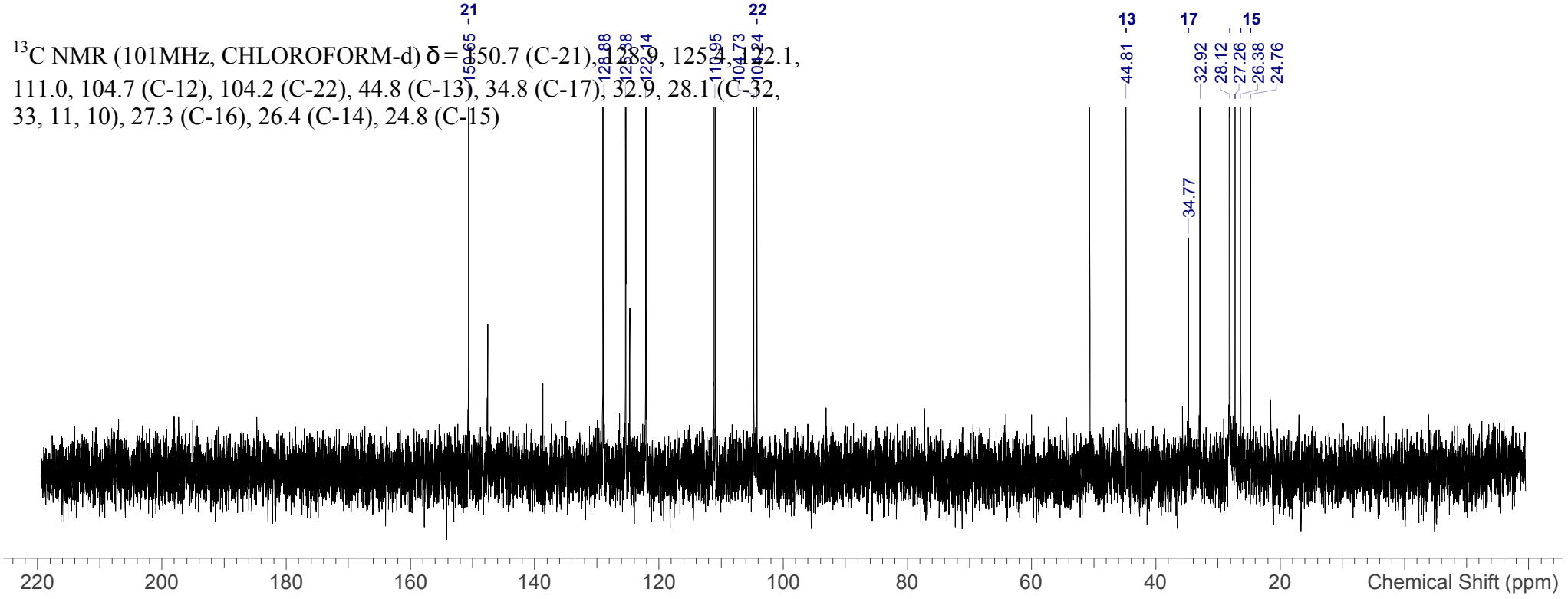
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 150.7 (C-21), 128.9, 125.4, 122.1, 111.0, 104.7 (C-12), 104.2 (C-22), 44.8 (C-13), 34.8 (C-17), 32.9, 28.1 (C-32, 33, 11, 10), 27.3 (C-16), 26.4 (C-14), 24.8 (C-15)



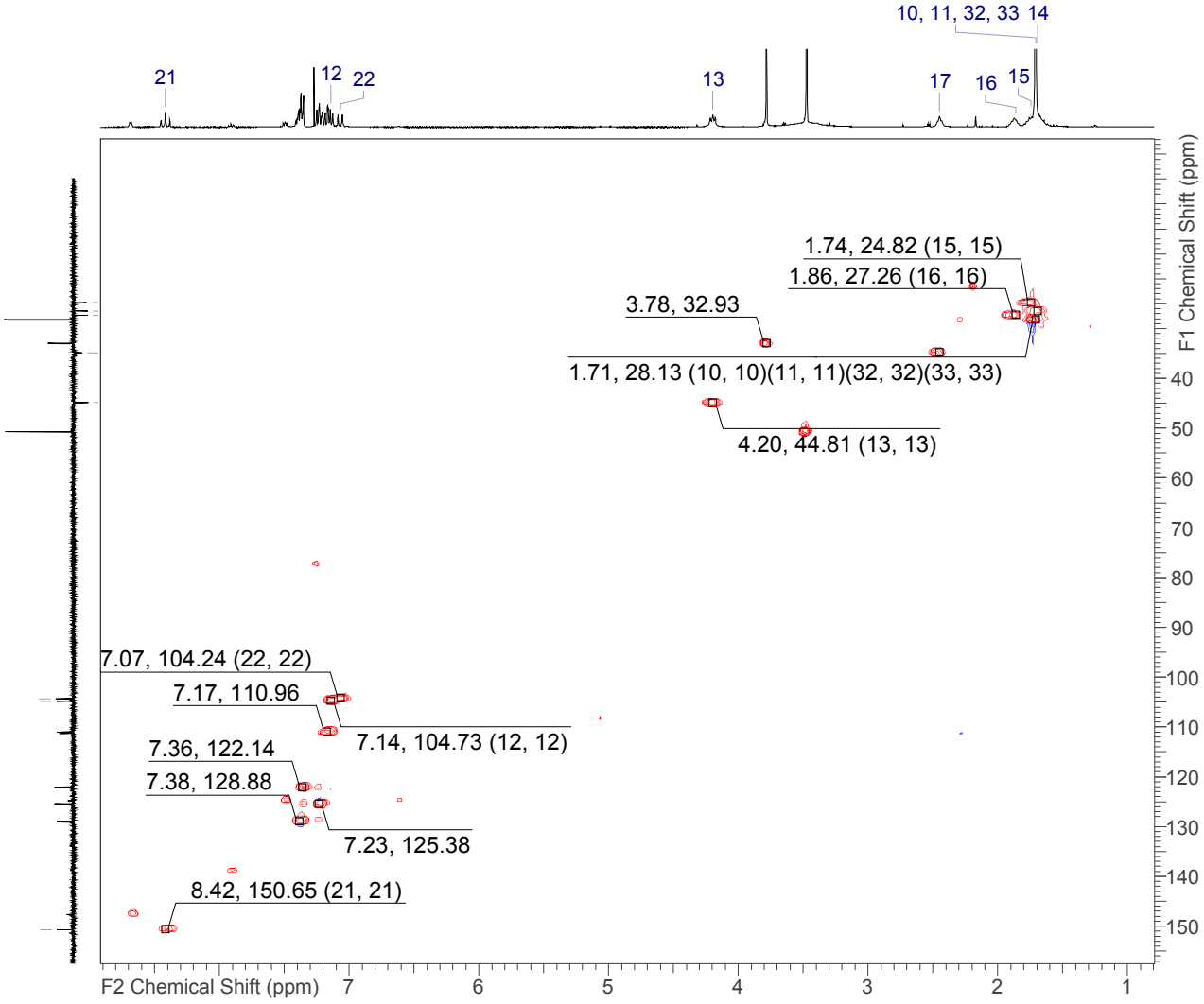
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/34 30-flask fractions
Date	05 Aug 2014 17:37:22		
Date Stamp	05 Aug 2014 17:37:22		
File Name	C:\Users\Mike Moazami\Desktop\au0514mm1- Cy3\14\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp45
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	10060.3135	***Spectrum Type***	DEPT45
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	24.990

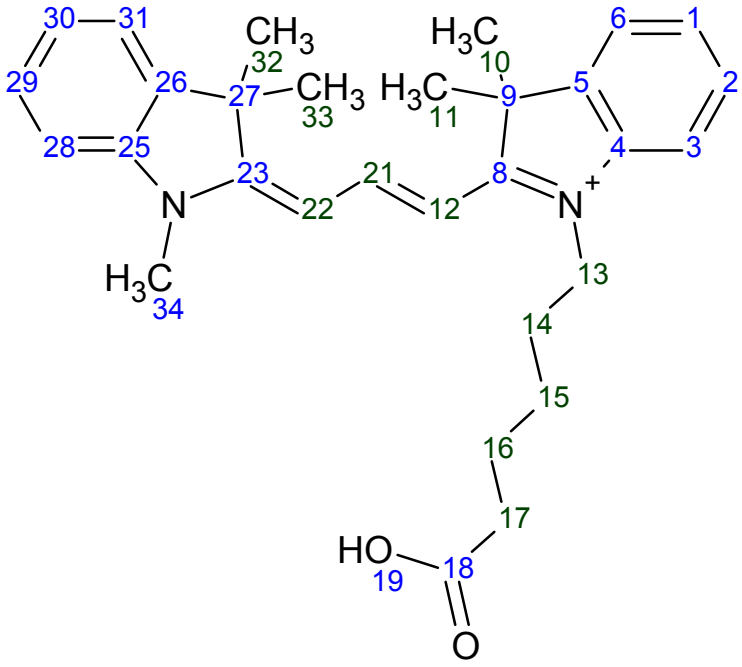


^{13}C NMR (101MHz, CHLOROFORM-d) δ = 150.7 (C-21), 128.9, 125.4, 122.1, 111.0, 104.7 (C-12), 104.2 (C-22), 44.8 (C-13), 34.8 (C-17), 32.9, 28.1 (C-32, 33, 11, 10), 27.3 (C-16), 26.4 (C-14), 24.8 (C-15)

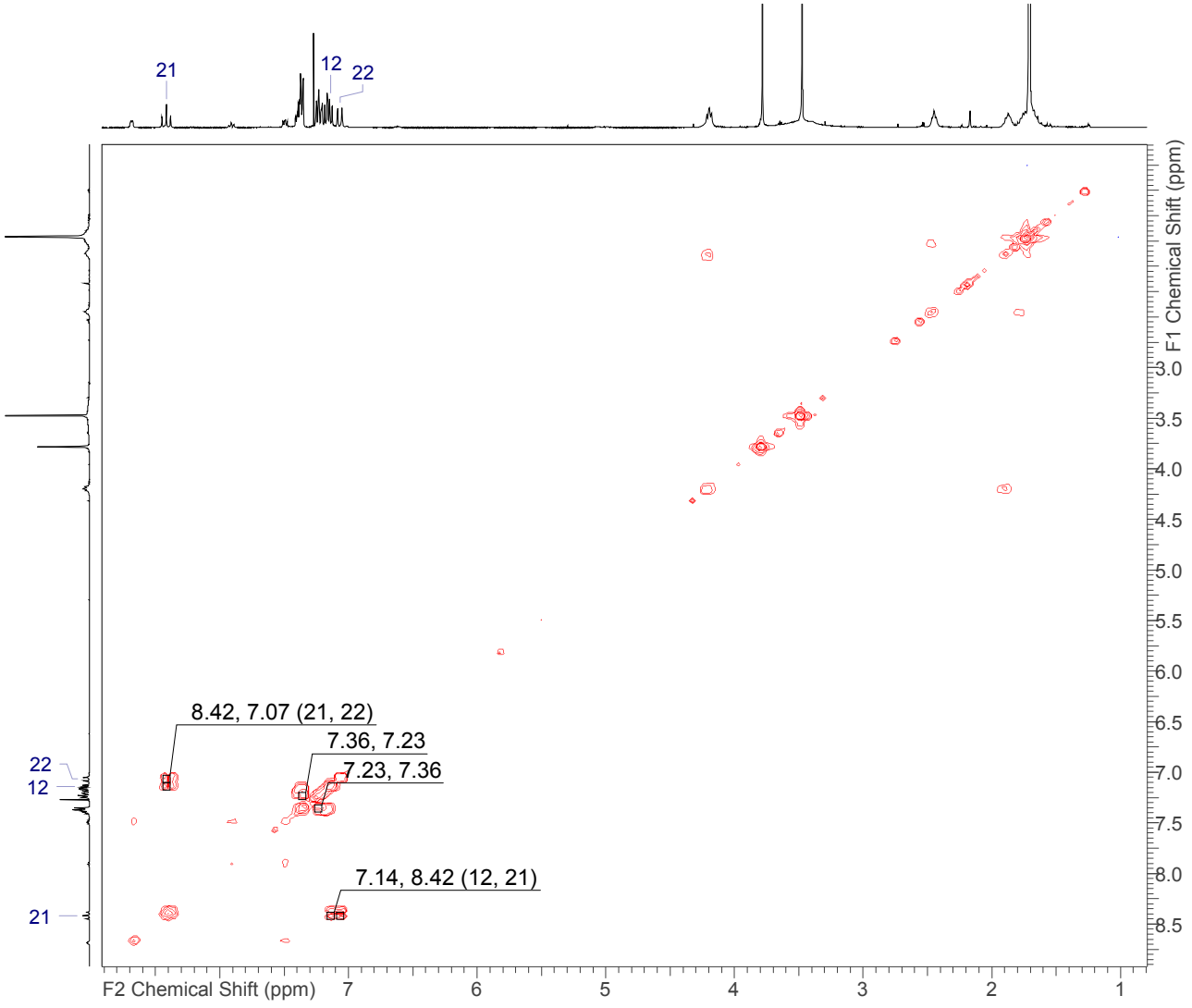


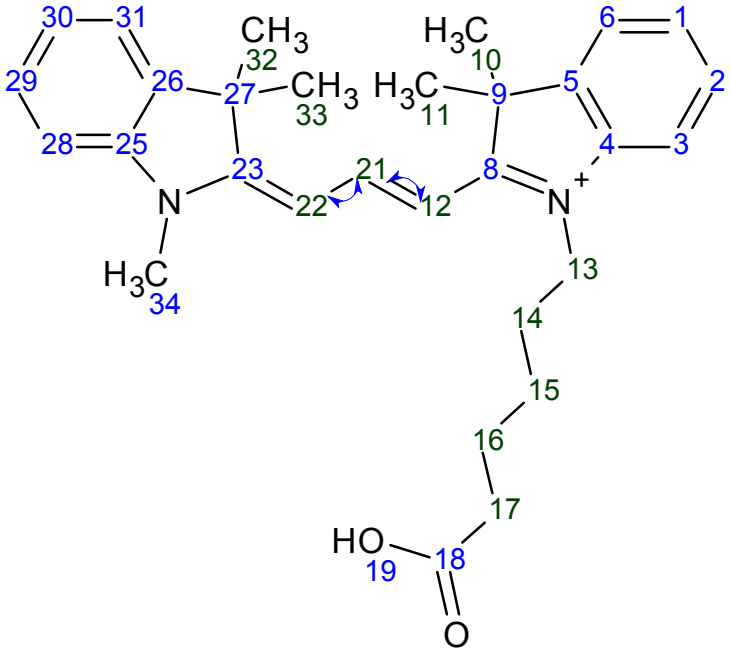
Acquisition Time (sec)	(0.1362, 0.0154)	***Comment***	5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218
Date	05 Aug 2014 17:52:00		
File Name	C:\Users\Mike Moazami\Desktop\au0514mm1- Cy3\15\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)		
Nucleus	(1H, 13C)	***Number of Transients***	2
Origin	spect	***Original Points Count***	(512, 256)
Owner	nmr	***Points Count***	(1024, 1024)
Pulse Sequence	hsqcetgp	***Solvent***	CHLOROFORM-d
Spectrum Type	HSQC	***Sweep Width (Hz)***	(3755.73, 16650.39)
Temperature (degree C)	25.037	***Title***	mm/





Acquisition Time (sec)	(0.2724, 0.0681)	***Comment***	5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218
Date	05 Aug 2014 17:21:00		
File Name	C:\Users\Mike Moazami\Desktop\au0514mm1- Cy3\13\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)		
Nucleus	(1H, 1H)	***Number of Transients***	2
Origin	spect	***Original Points Count***	(1024, 256)
Owner	nmr	***Points Count***	(1024, 1024)
Pulse Sequence	cosygpqf	***Solvent***	CHLOROFORM-d
Spectrum Type	COSY	***Sweep Width (Hz)***	(3755.73, 3755.73)
Temperature (degree C)	24.991	***Title***	mm/





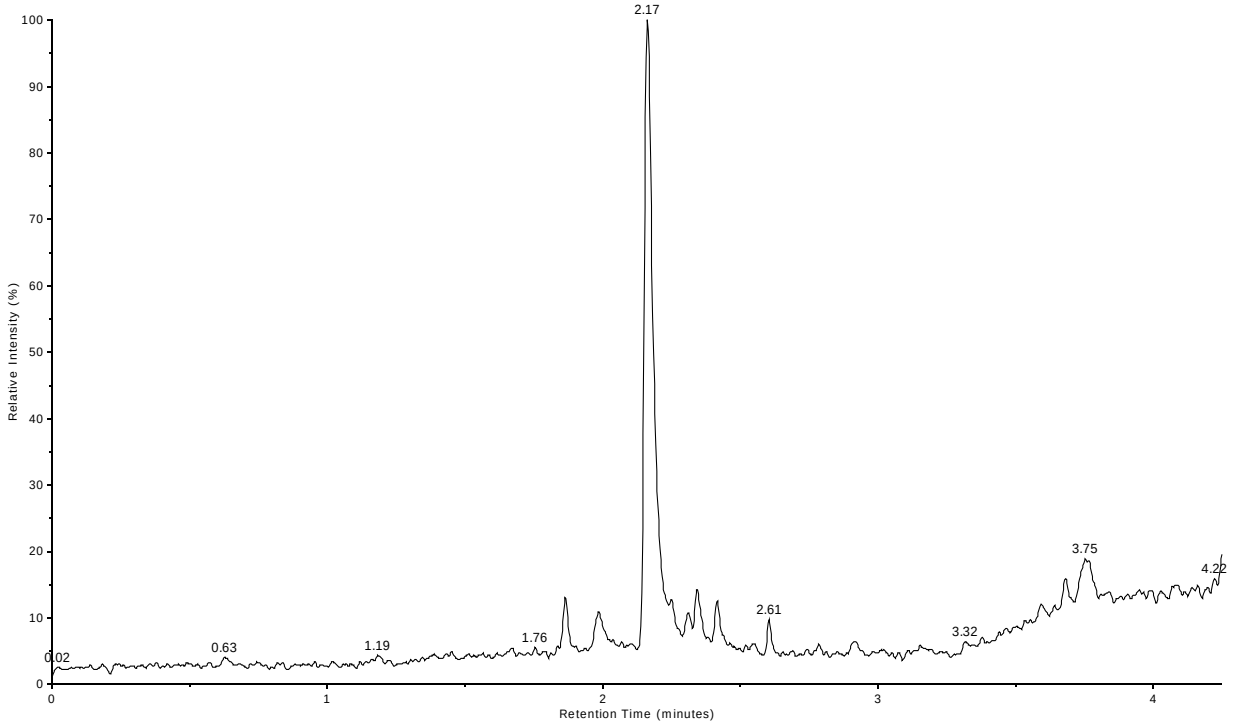
A.7 1-(5-carboxypentyl)-3,3-dimethyl-2-((1E,3E,5Z)-5-(1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3H-indol-1-ium bromide

Top 5 Peak Report

Group: Watts, J
Project: RP-LC MW 150-1500
Experiment: B ESIPOS (simple mix) C18 Acid
Instrument: Blue RP UHPLC-MS (B30:1023)

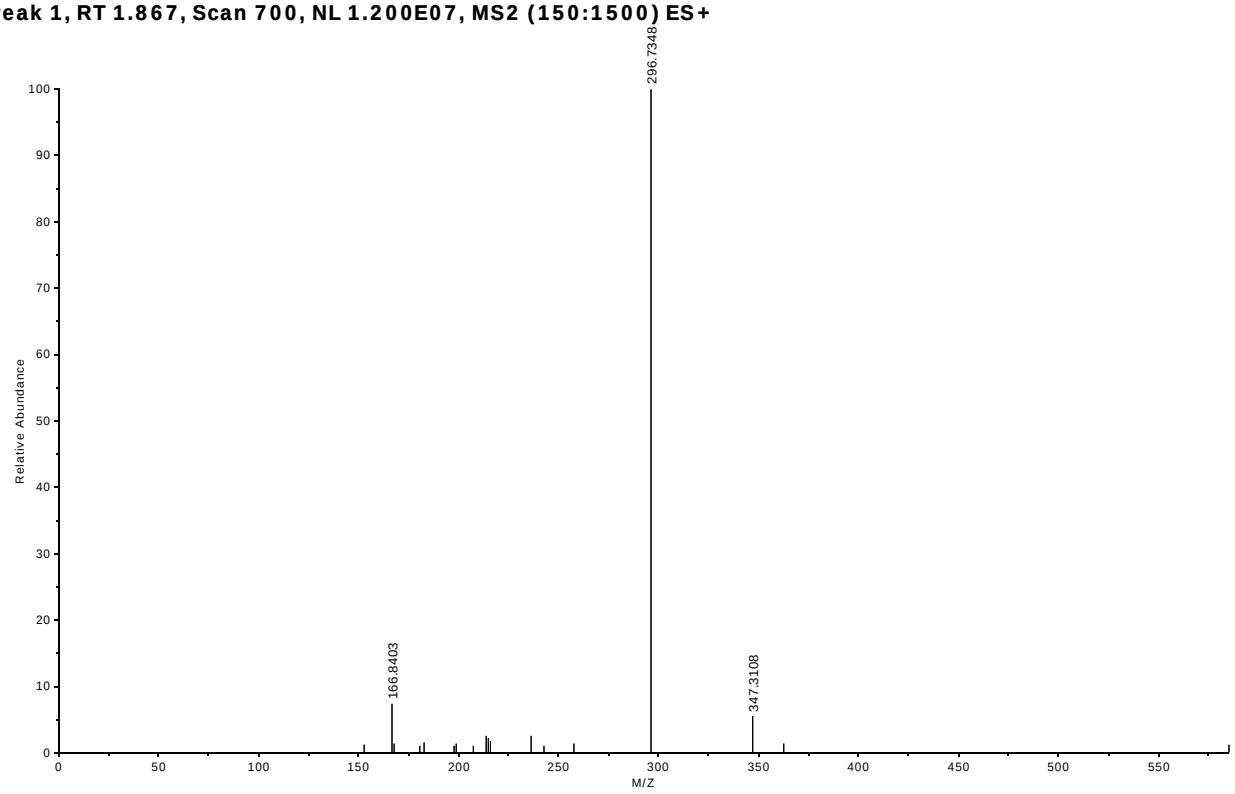
Submitter: Michael Moazami
Sample ID: mm/7316/35
Filename: Watts_J_mm731635_MM_369.pdf
Acquisition Date: 08/08/2014 14:16:16

TIC, NL 1.046E08, MS2 (150:1500) ES+

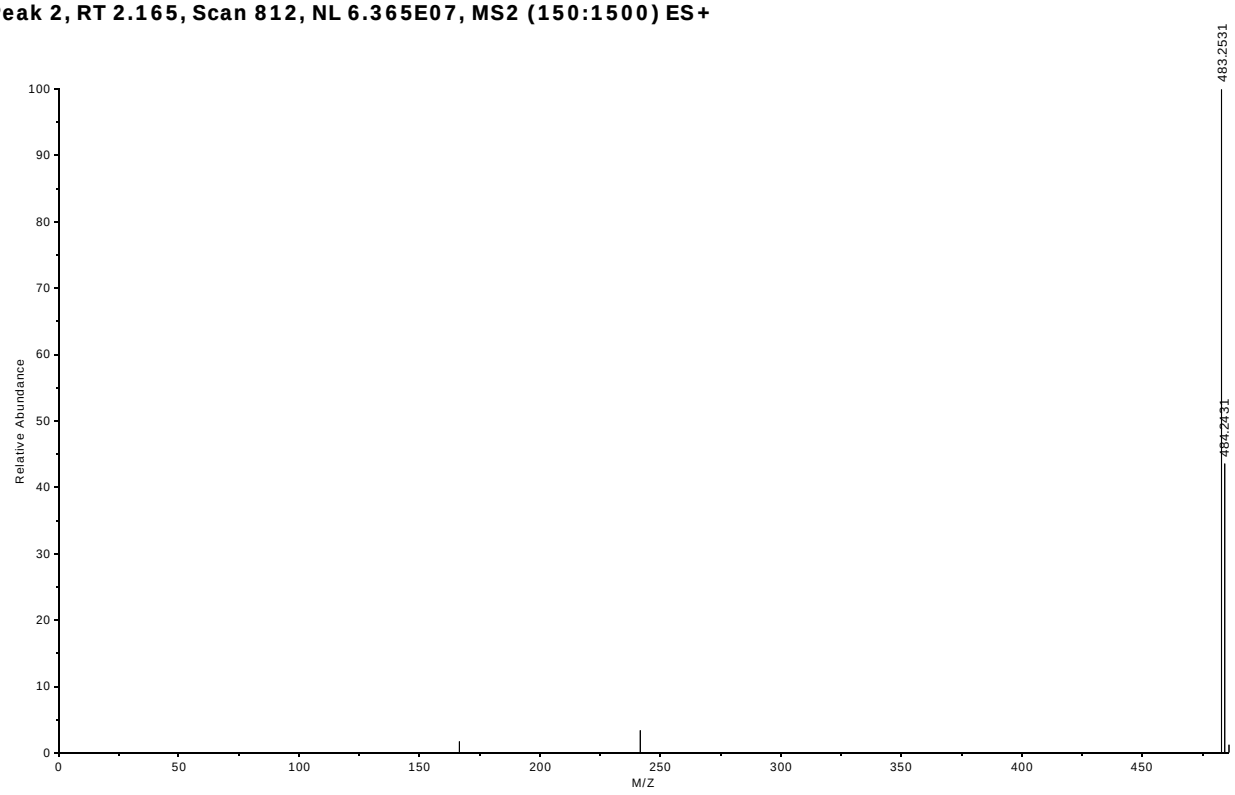


	RT Mins	Height	Height %	Area	Area %
1	1.87	7835581	8.19	9357258	3.89
2	2.17	95705492	100.00	240253845	100.00
3	2.34	6876925	7.19	11439244	4.76
4	2.42	6651817	6.95	9790186	4.07
5	3.75	6909198	7.22	11749282	4.89

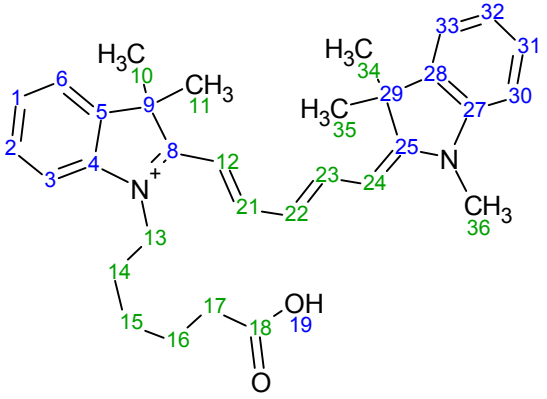
Peak 1, RT 1.867, Scan 700, NL 1.200E07, MS2 (150:1500) ES+



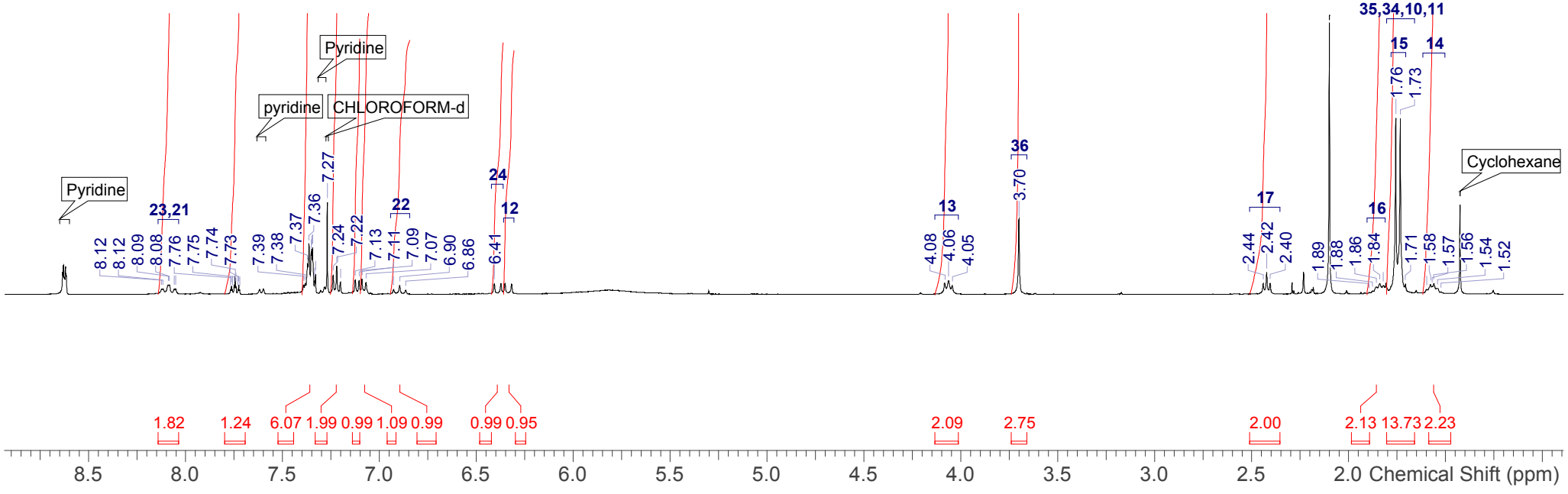
Peak 2, RT 2.165, Scan 812, NL 6.365E07, MS2 (150:1500) ES+



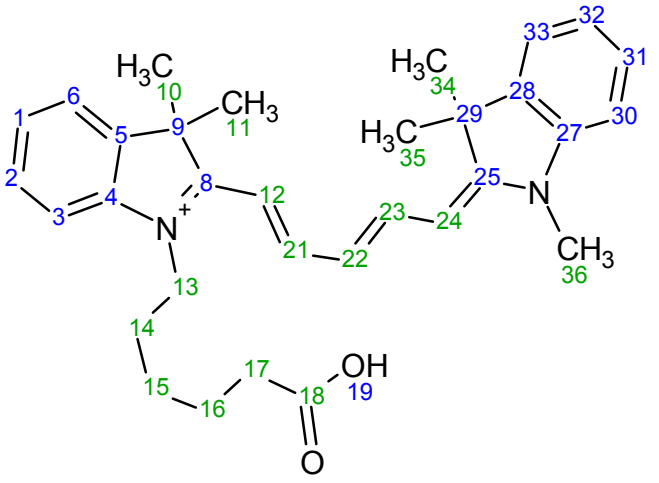
Acquisition Time (sec)	4.0894	***Comment***	mm/7316/35	***Date***	08 Aug 2014 18:17:43
Date Stamp	08 Aug 2014 18:17:43				
File Name	C:\Users\Mike Moazami\Desktop\au0814mm1\10\PDATA\1\1r				
Frequency (MHz)	400.1300	***Nucleus***	1H	***Number of Transients***	16
Origin	spect	***Original Points Count***	32768	***Owner***	nmr
Points Count	65536	***Pulse Sequence***	zg30	***Receiver Gain***	83.67
SW(cyclical) (Hz)	8012.82	***Solvent***	CHLOROFORM-d		
Spectrum Offset (Hz)	2464.9387	***Spectrum Type***	standard	***Sweep Width (Hz)***	8012.70
Temperature (degree C)	24.990				



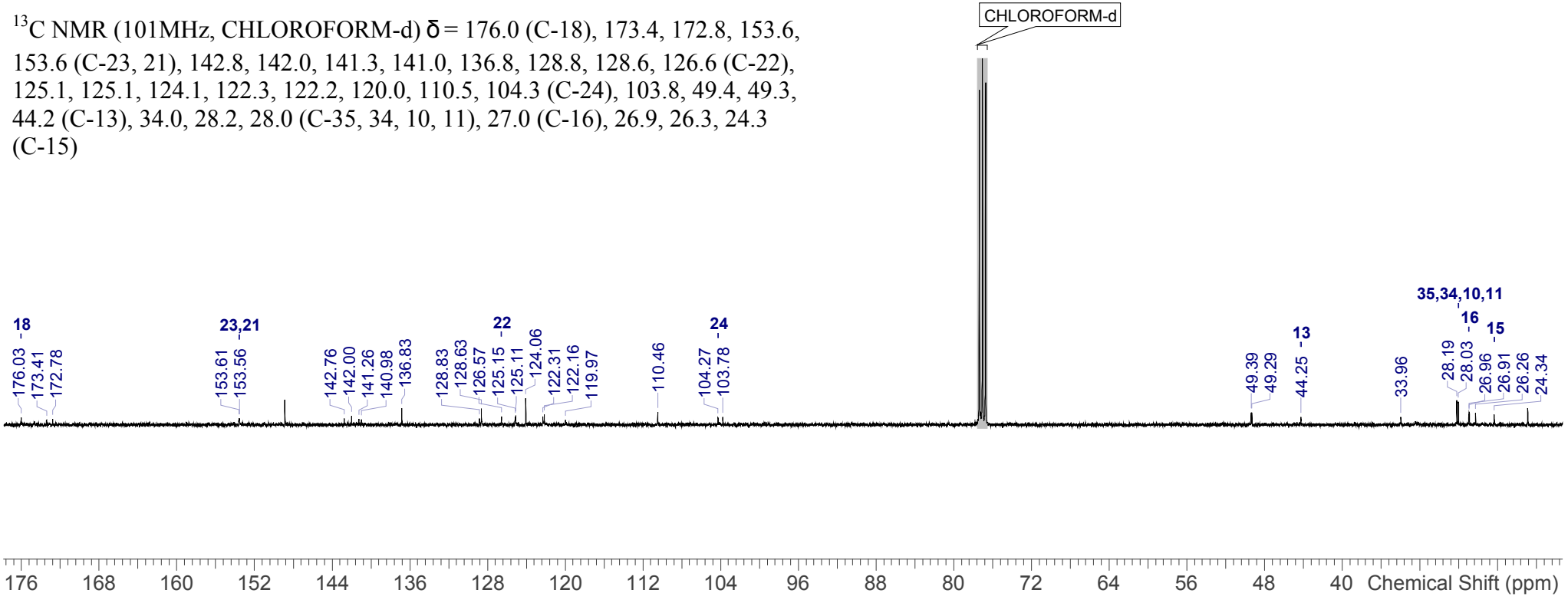
^{1}H NMR (400MHz, CHLOROFORM-d) δ = 8.09 (2H, td, J = 13.0 Hz, J = 2.9 Hz, H-23, 21), 7.74 (1H, tt, J = 7.7 Hz, J = 1.8 Hz, M07), 7.32 - 7.40 (6H, m, M10), 7.22 (2H, t, J = 7.3 Hz, M18), 7.12 (1H, d, J = 7.9 Hz, M17), 7.08 (1H, d, J = 8.1 Hz, M16), 6.90 (1H, br t, J = 12.5 Hz, H-22), 6.39 (1H, d, J = 13.6 Hz, H-24), 6.34 (1H, d, J = 13.6 Hz, H-12), 4.07 (2H, t, J = 7.6 Hz, H-13), 3.70 (3H, s, H-36), 2.42 (2H, t, J = 7.2 Hz, H-17), 1.81 - 1.91 (2H, m, H-16), 1.71 - 1.78 (2H, m, H-15), 1.74 (11H, d, J = 9.3 Hz, H-35, 34, 10, 11), 1.50 - 1.62 (2H, m, H-14)



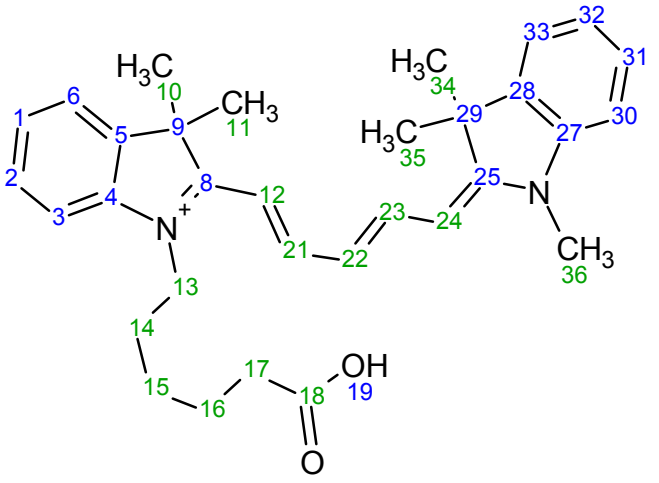
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Date	08 Aug 2014 18:48:07		
Date Stamp	08 Aug 2014 18:48:07		
File Name	C:\Users\Mike Moazami\Desktop\au0814mm1\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	10060.3320	***Spectrum Type***	standard
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	25.003



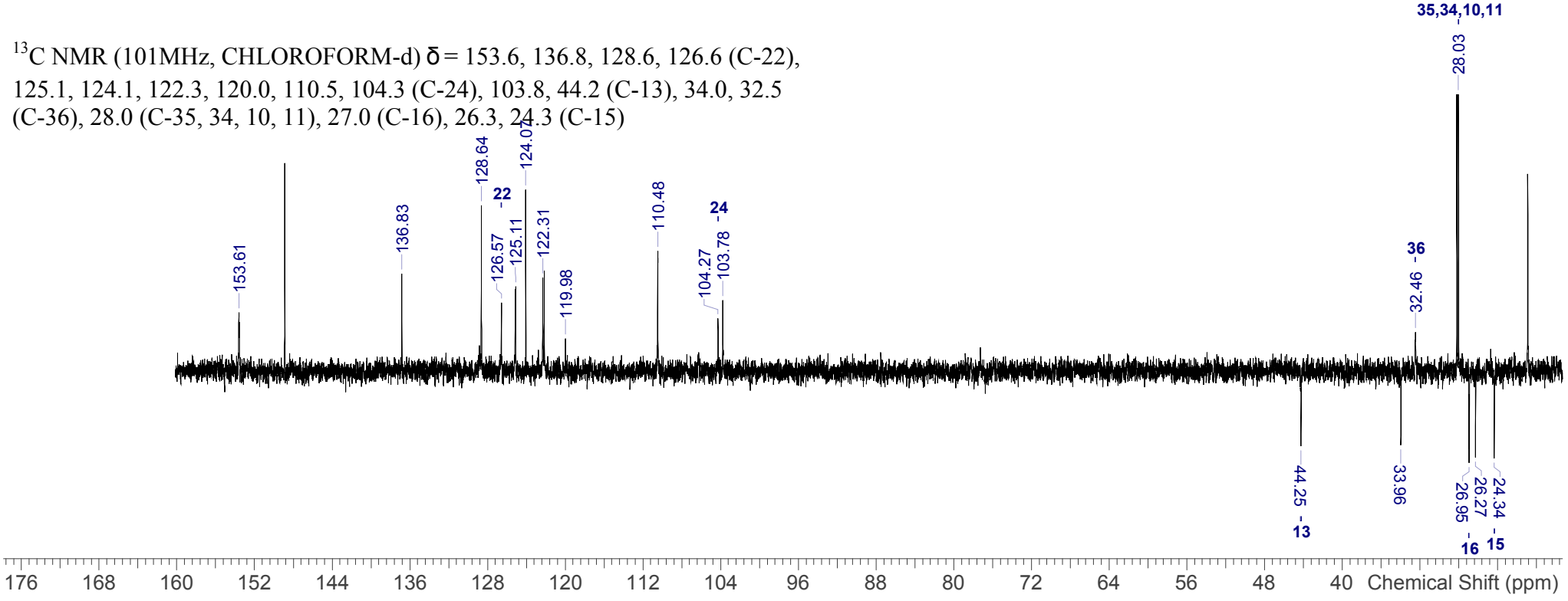
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 176.0 (C-18), 173.4, 172.8, 153.6, 153.6 (C-23, 21), 142.8, 142.0, 141.3, 141.0, 136.8, 128.8, 128.6, 126.6 (C-22), 125.1, 125.1, 124.1, 122.3, 122.2, 120.0, 110.5, 104.3 (C-24), 103.8, 49.4, 49.3, 44.2 (C-13), 34.0, 28.2, 28.0 (C-35, 34, 10, 11), 27.0 (C-16), 26.9, 26.3, 24.3 (C-15)



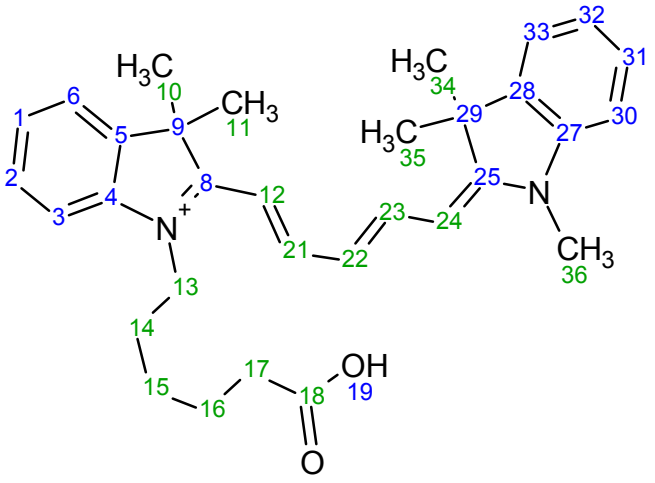
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Date	08 Aug 2014 19:06:44		
Date Stamp	08 Aug 2014 19:06:44		
File Name	C:\Users\Mike Moazami\Desktop\au0814mm1\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	8048.0767	***Spectrum Type***	DEPT135
Sweep Width (Hz)	16128.54	***Temperature (degree C)***	25.003



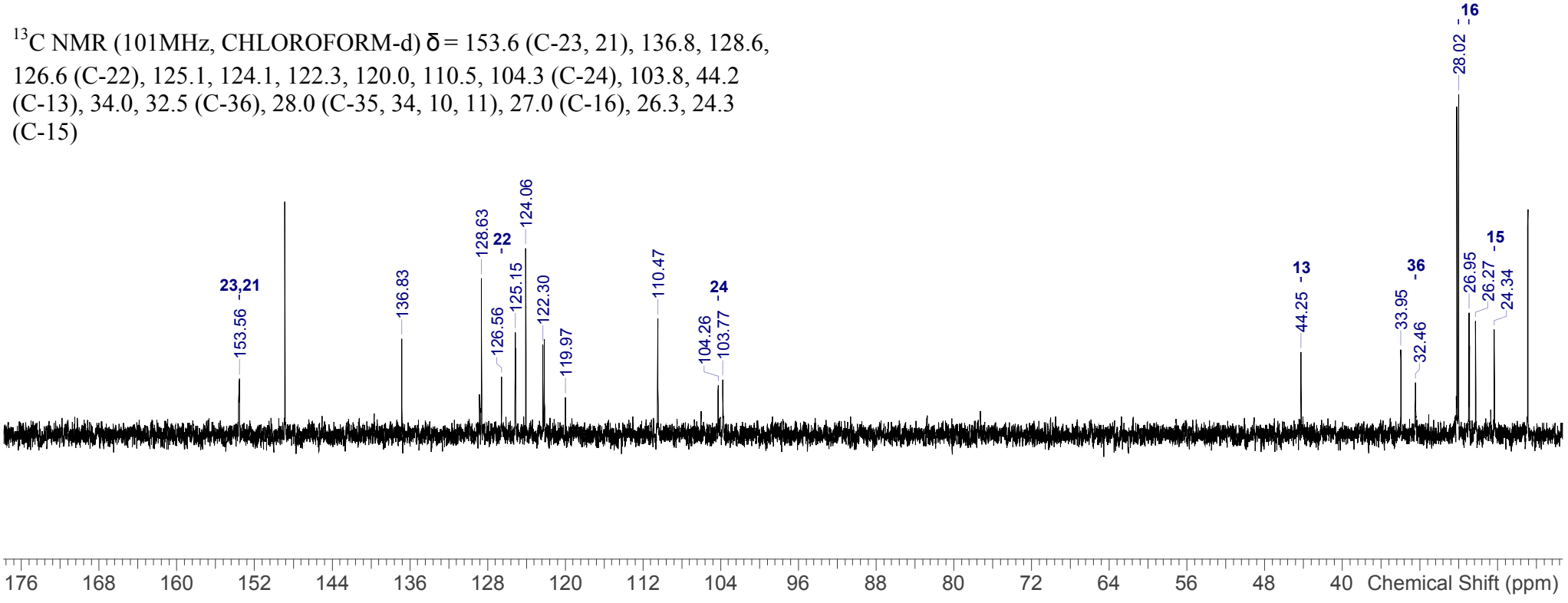
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 153.6, 136.8, 128.6, 126.6 (C-22), 125.1, 124.1, 122.3, 120.0, 110.5, 104.3 (C-24), 103.8, 44.2 (C-13), 34.0, 32.5 (C-36), 28.0 (C-35, 34, 10, 11), 27.0 (C-16), 26.3, 24.3 (C-15)



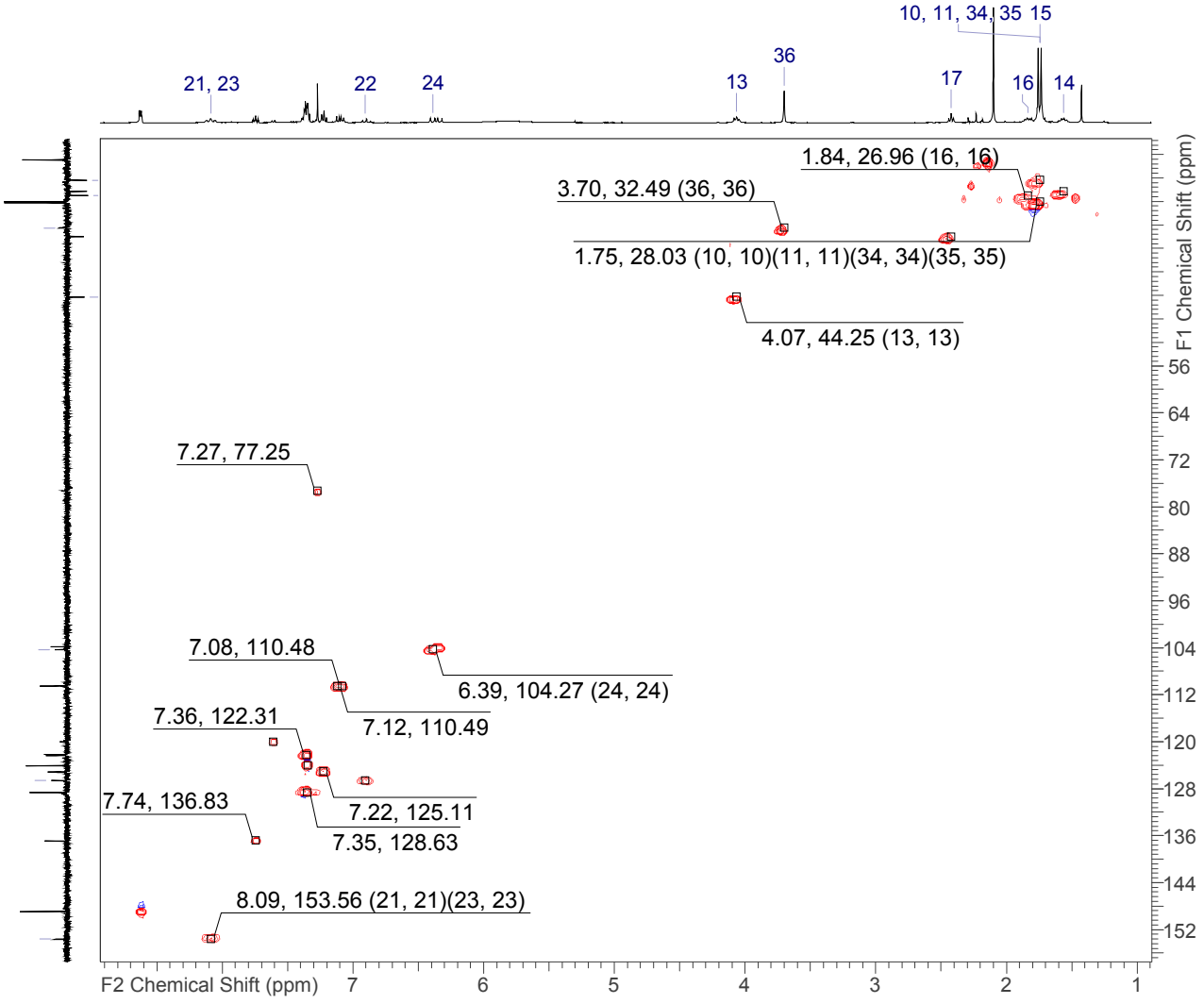
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Date	08 Aug 2014 20:00:18		
Date Stamp	08 Aug 2014 20:00:18		
File Name	C:\Users\Mike Moazami\Desktop\au0814mm1\15\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp45
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	10060.3564	***Spectrum Type***	DEPT45
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	24.990

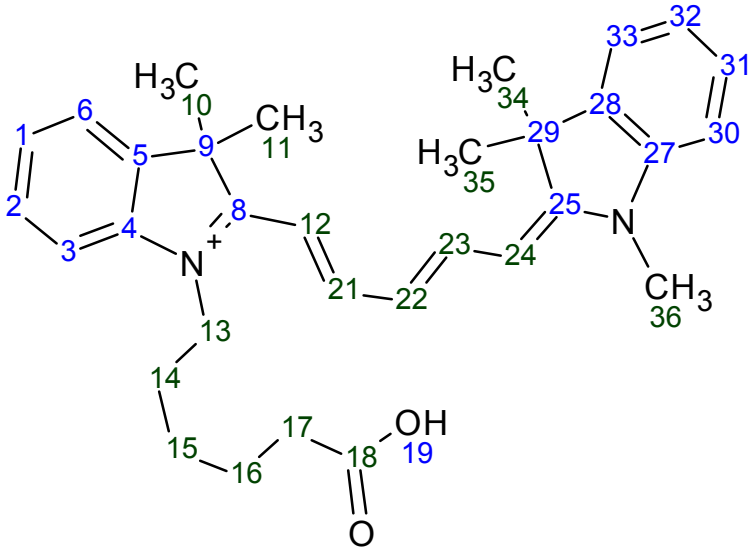


^{13}C NMR (101MHz, CHLOROFORM-d) δ = 153.6 (C-23, 21), 136.8, 128.6, 126.6 (C-22), 125.1, 124.1, 122.3, 120.0, 110.5, 104.3 (C-24), 103.8, 44.2 (C-13), 34.0, 32.5 (C-36), 28.0 (C-35, 34, 10, 11), 27.0 (C-16), 26.3, 24.3 (C-15)

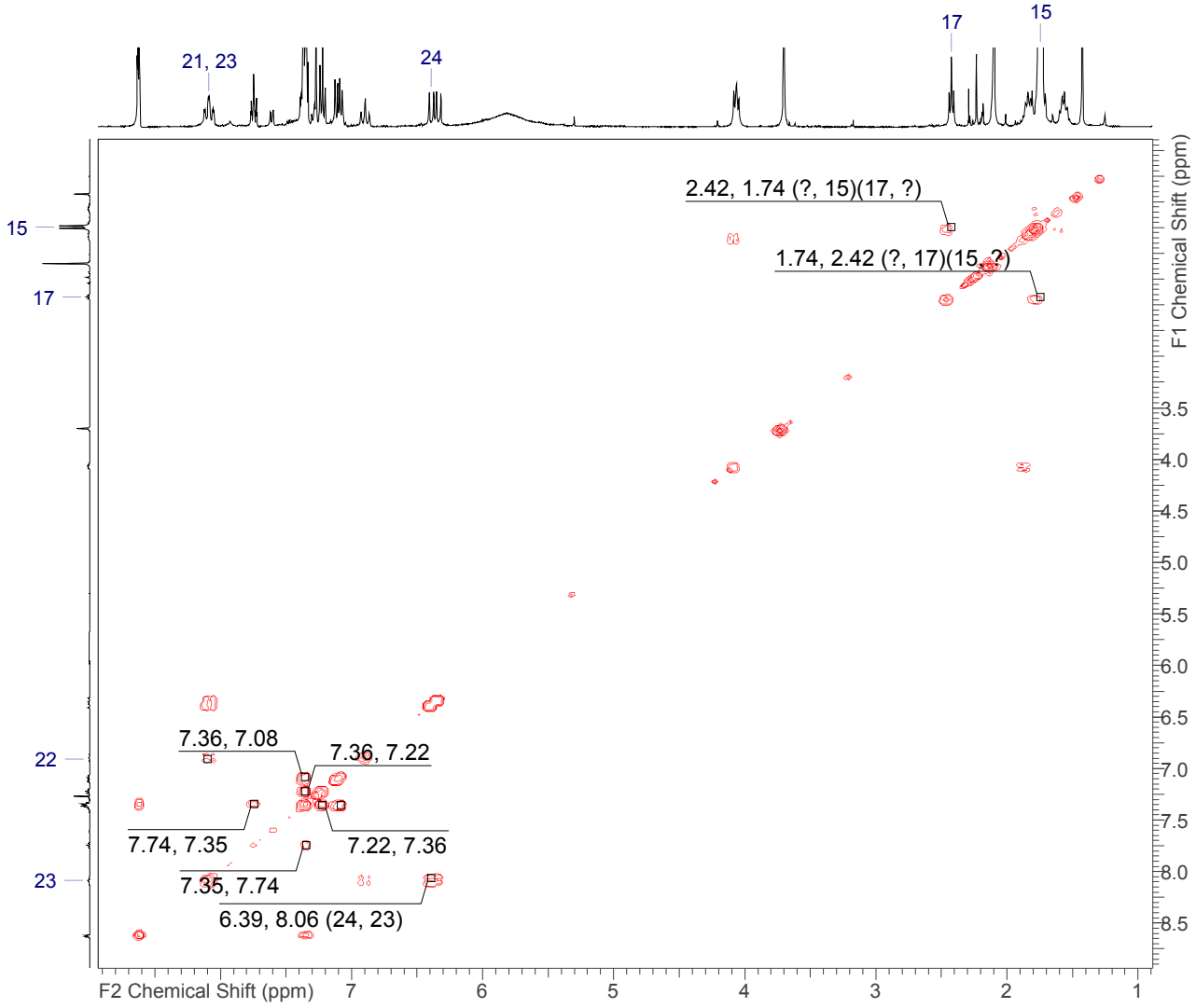


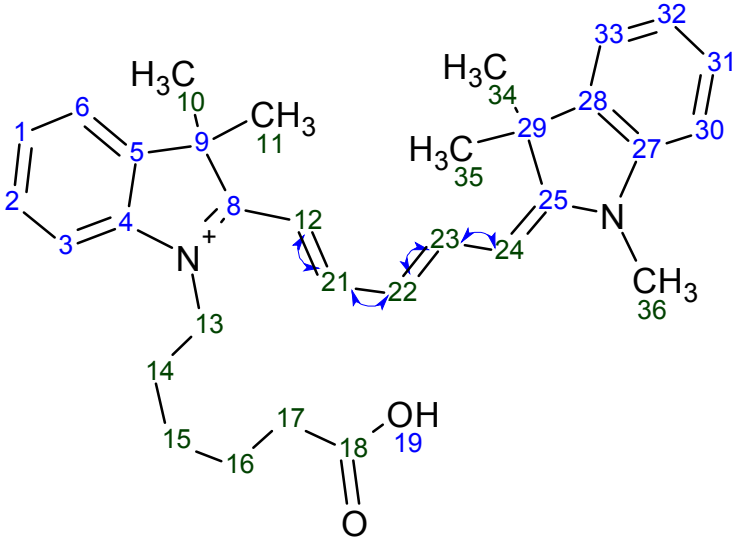
Acquisition Time (sec)	(0.1352, 0.0154)	***Comment***	5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218
Date	08 Aug 2014 20:15:00		
File Name	C:\Users\Mike Moazami\Desktop\au0814mm1\16\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)		
Nucleus	(1H, 13C)	***Number of Transients***	2
Origin	spect	***Original Points Count***	(512, 256)
Owner	nmr	***Points Count***	(1024, 1024)
Pulse Sequence	hsqcetgp	***Solvent***	CHLOROFORM-d
Spectrum Type	HSQC	***Sweep Width (Hz)***	(3784.18, 16650.39)
Temperature (degree C)	25.046	***Title***	mm/



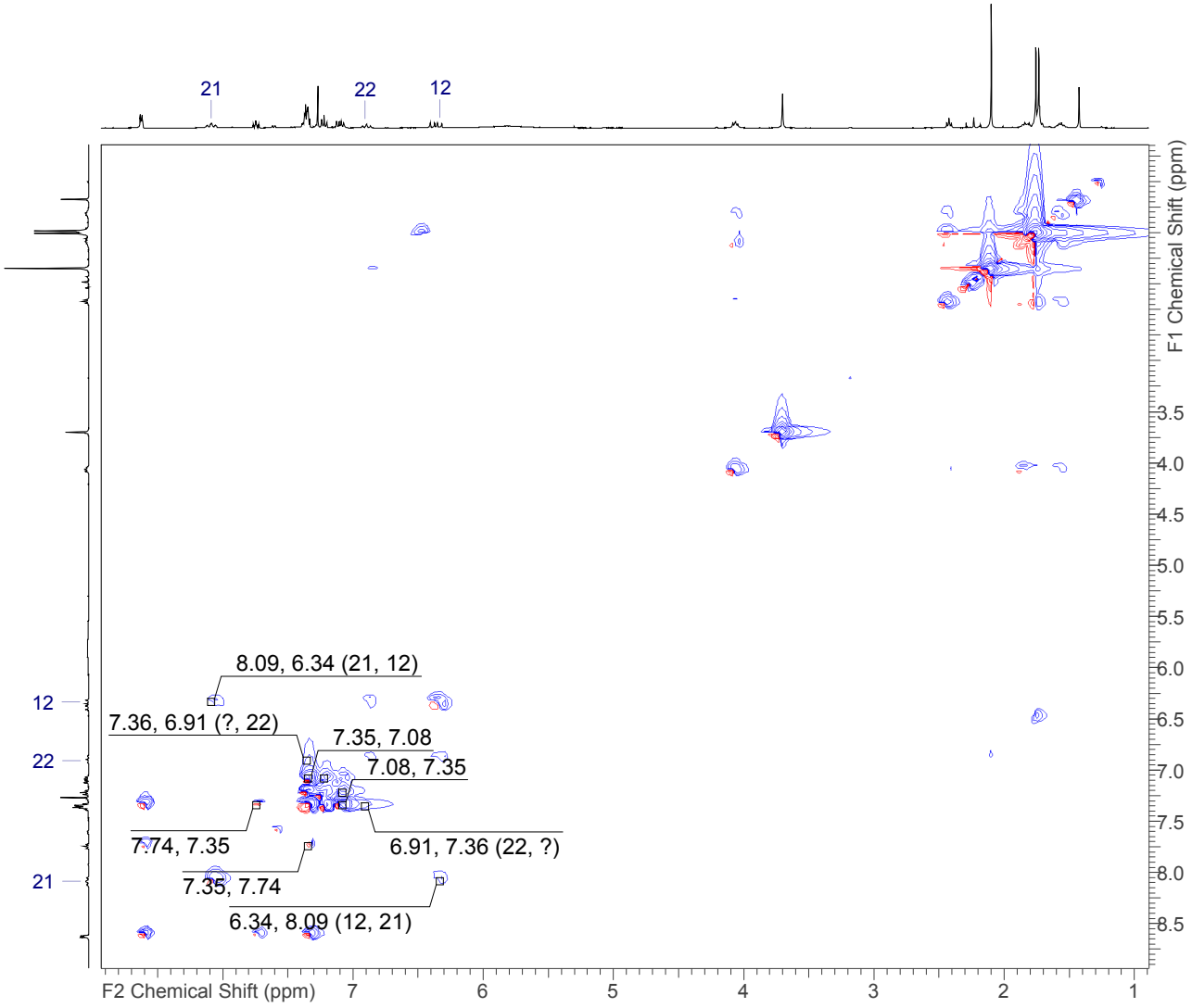


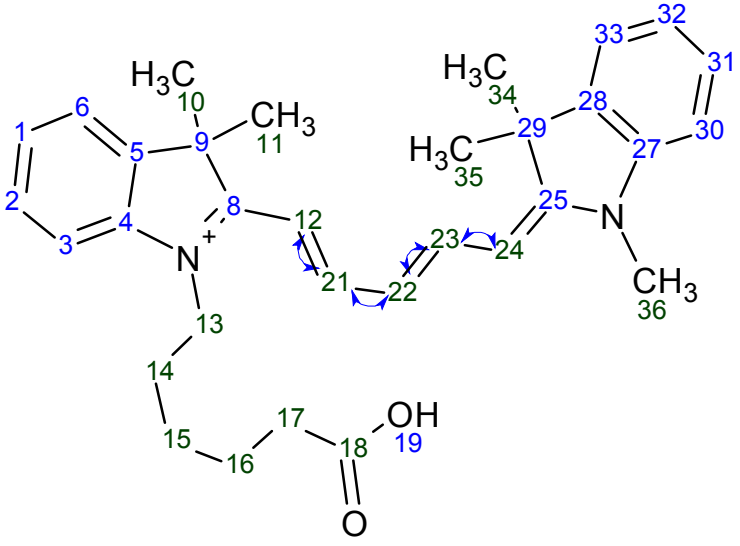
Acquisition Time (sec)	(0.2703, 0.0676)	***Comment***	5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218
Date	08 Aug 2014 19:23:00		
File Name	C:\Users\Mike Moazami\Desktop\au0814mm1\13\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)		
Nucleus	(1H, 1H)	***Number of Transients***	2
Origin	spect	***Original Points Count***	(1024, 256)
Owner	nmr	***Points Count***	(1024, 1024)
Pulse Sequence	cosygpqf	***Solvent***	CHLOROFORM-d
Spectrum Type	COSY	***Sweep Width (Hz)***	(3784.18, 3784.18)
Temperature (degree C)	25.000	***Title***	mm/



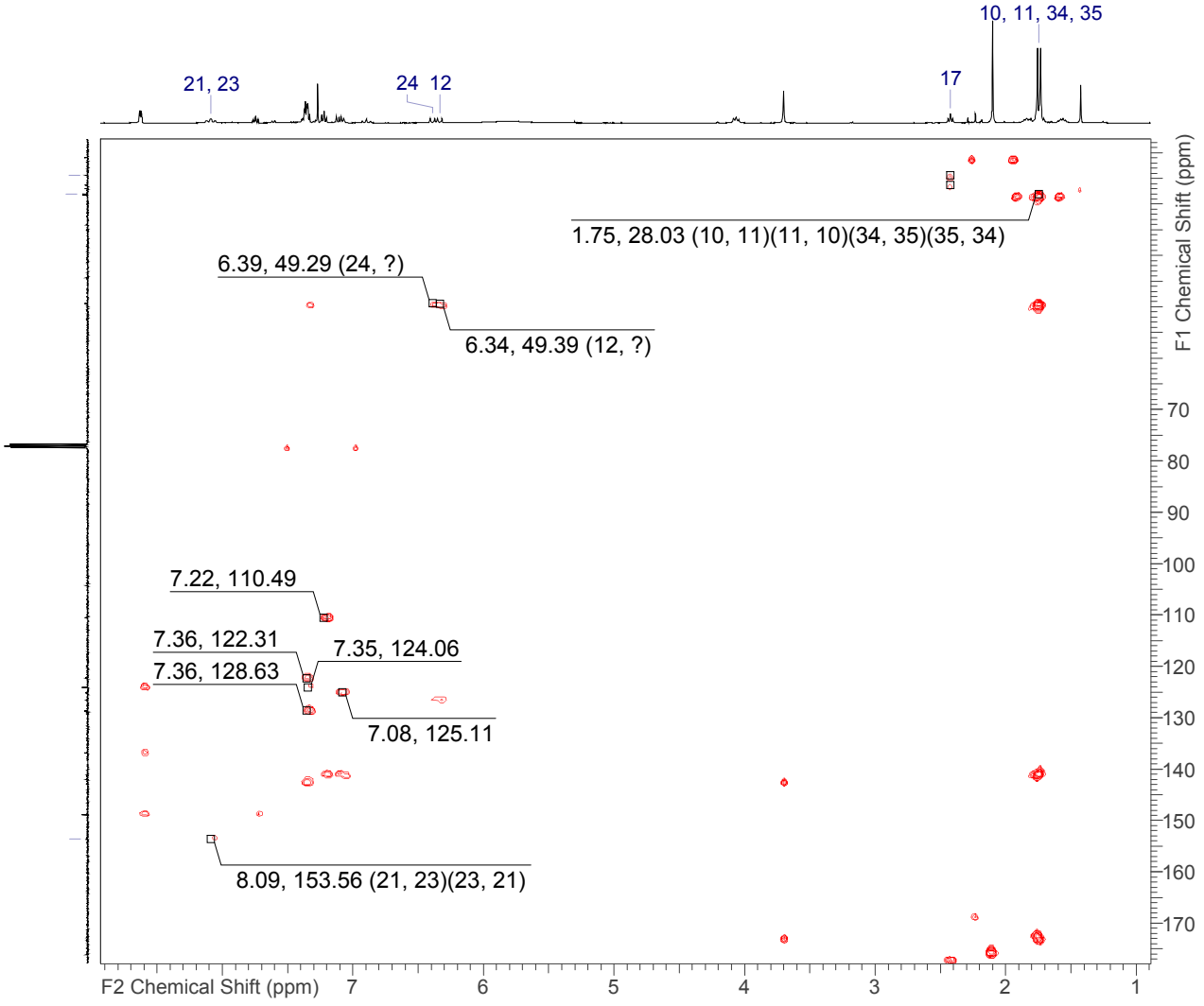


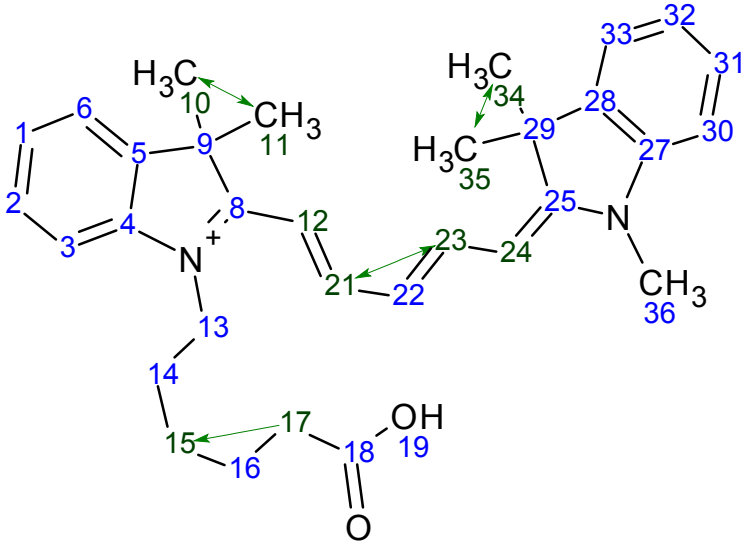
Acquisition Time (sec)	(0.2703, 0.0676)	***Comment***	5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218
Date	08 Aug 2014 19:44:00		
File Name	C:\Users\Mike Moazami\Desktop\au0814mm1\14\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)		
Nucleus	(1H, 1H)	***Number of Transients***	2
Origin	spect	***Original Points Count***	(1024, 256)
Owner	nmr	***Points Count***	(1024, 1024)
Pulse Sequence	mlevetgp	***Solvent***	CHLOROFORM-d
Spectrum Type	TOCSY	***Sweep Width (Hz)***	(3784.18, 3784.18)
Temperature (degree C)	25.005	***Title***	mm/





Acquisition Time (sec)	(0.2703, 0.0115)	***Comment***	5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218
Date	08 Aug 2014 20:47:00		
File Name	C:\Users\Mike Moazami\Desktop\au0814mm1\17\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)		
Nucleus	(1H, 13C)	***Number of Transients***	4
Origin	spect	***Original Points Count***	(1024, 256)
Owner	nmr	***Points Count***	(2048, 1024)
Pulse Sequence	hmbcgplpndqf	***Solvent***	CHLOROFORM-d
Spectrum Type	HMBC	***Sweep Width (Hz)***	(3786.03, 22299.63)
Temperature (degree C)	25.008	***Title***	mm/



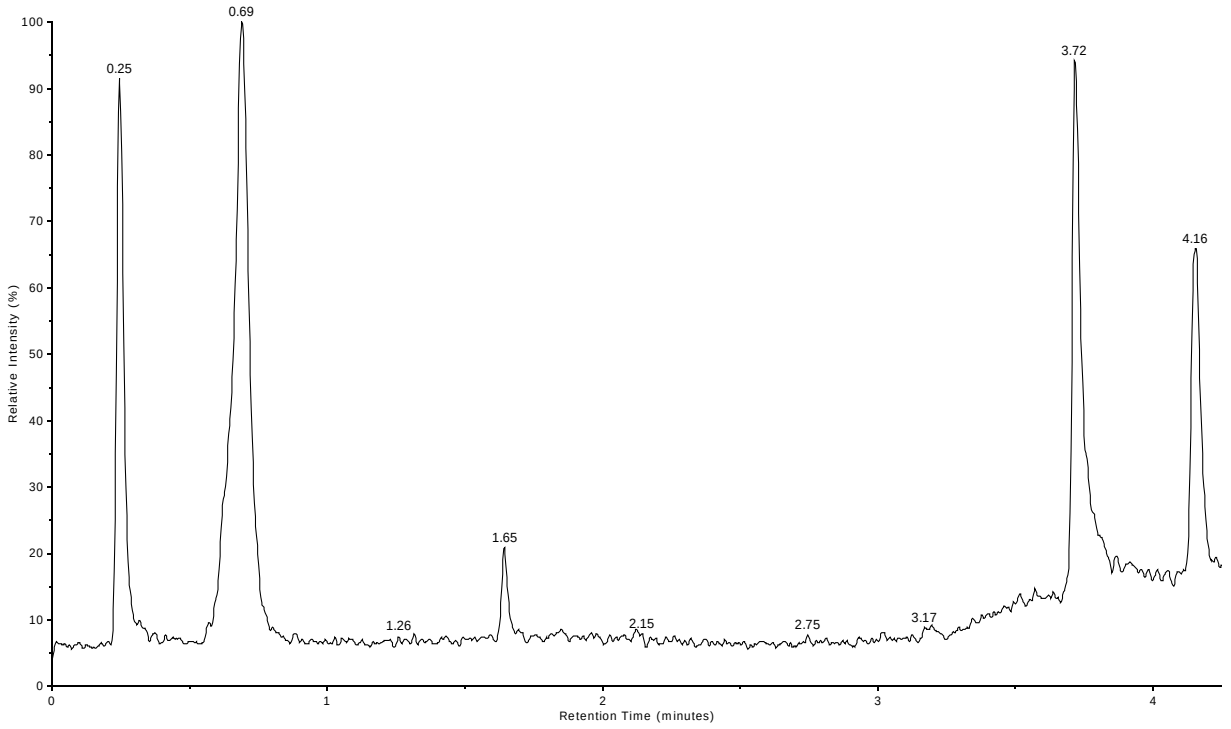


A.8 1,1,2,3-tetramethyl-1H-benzo[e]indol-3-ium iodide

Top 5 Peak Report

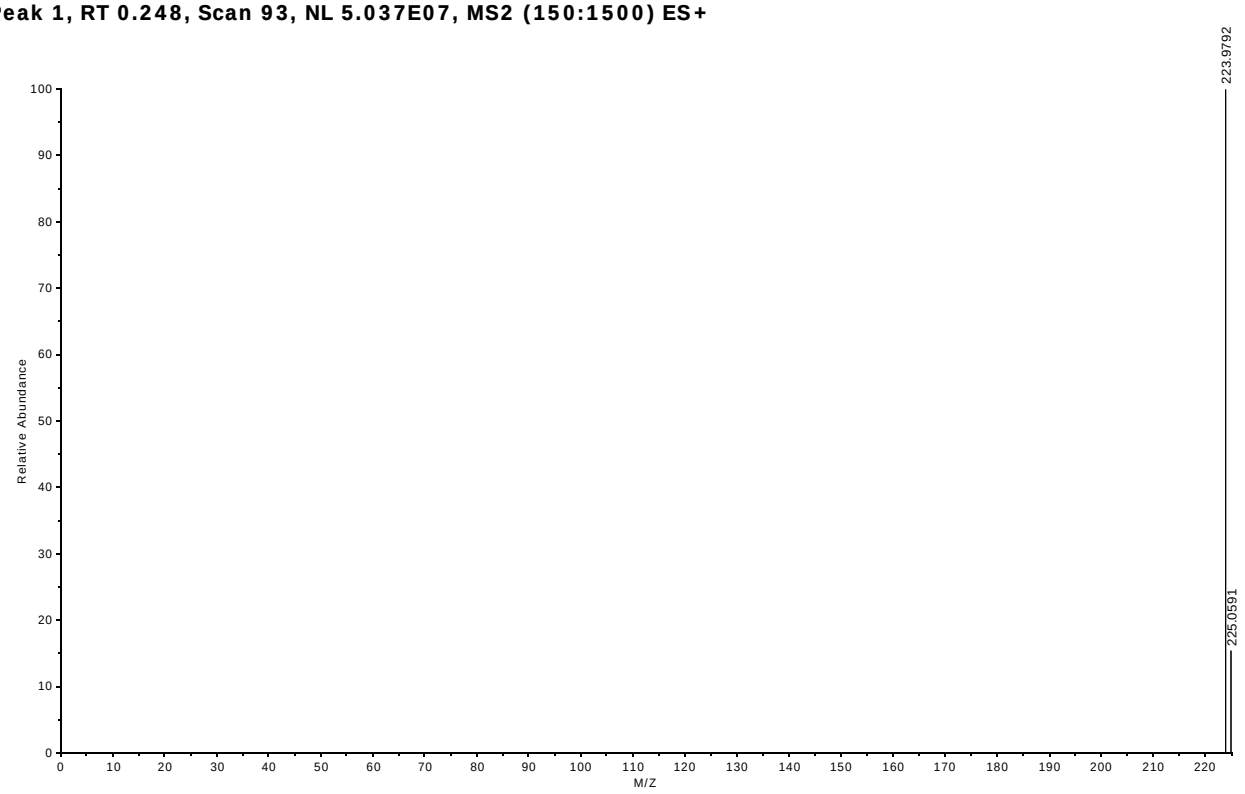
Group: Watts, J
Submitter: Michael Moazami
Project: RP LC C18 < 2 cmpds
Sample ID: X.5 Methyl
Experiment: BLUE ESIPOS C18 5 min
Filename: X_5_Methyl_Michael_Moazami__Watts_J_4267.p
Instrument: Blue RP UHPLC-MS (B30:1023)
Acquisition Date: 27/01/2015 15:53:01

TIC, NL 5.758E07, MS2 (150:1500) ES+

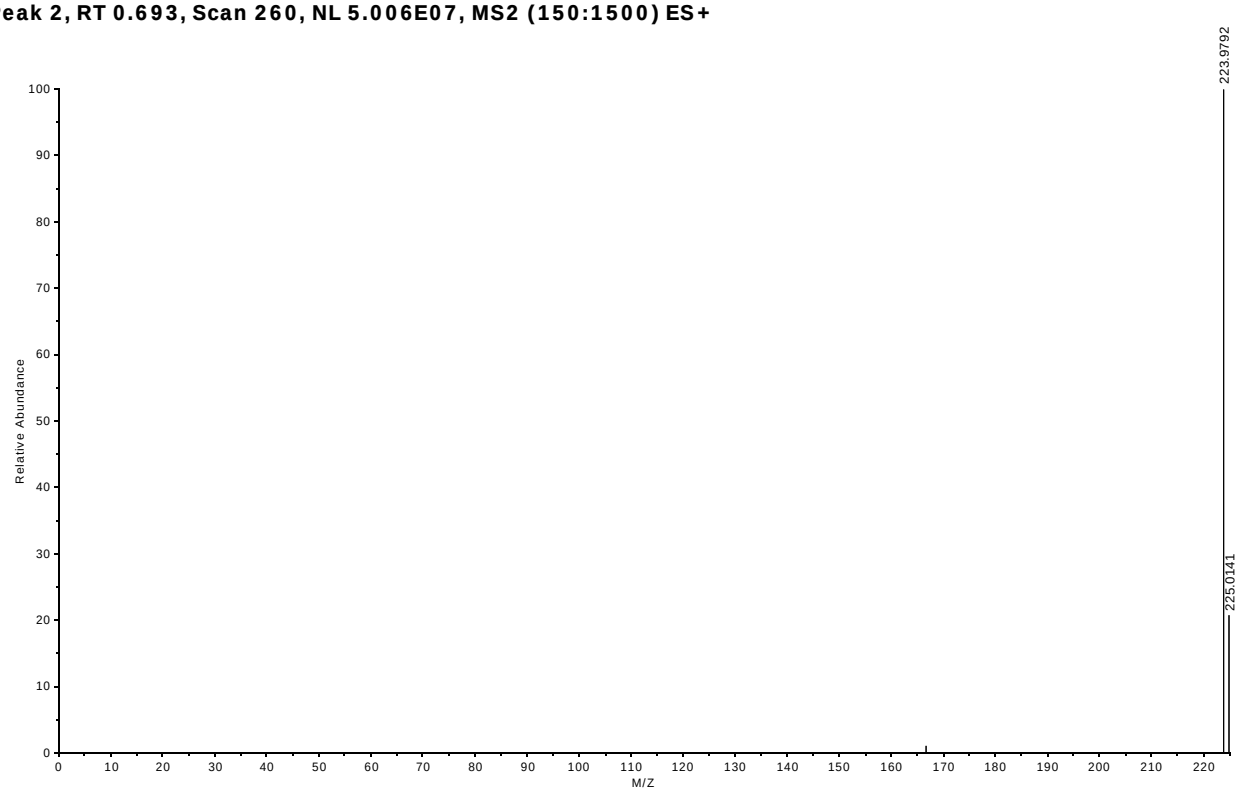


	RT Mins	Height	Height %	Area	Area %
1	0.25	48297842	91.86	92539865	41.61
2	0.69	52579288	100.00	222412935	100.00
3	1.65	7867115	14.96	13974779	6.28
4	3.72	43119164	82.01	117123732	52.66
5	4.16	27452222	52.21	67600277	30.39

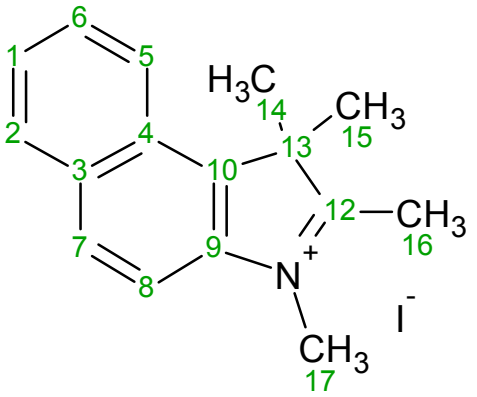
Peak 1, RT 0.248, Scan 93, NL 5.037E07, MS2 (150:1500) ES+



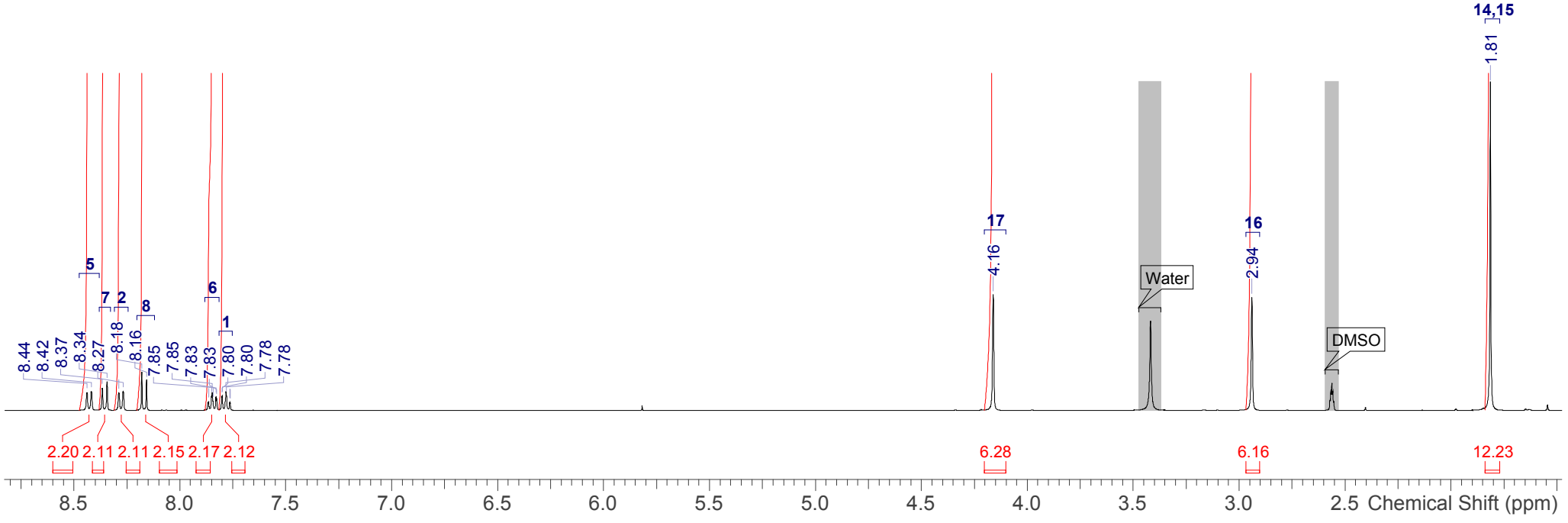
Peak 2, RT 0.693, Scan 260, NL 5.006E07, MS2 (150:1500) ES+



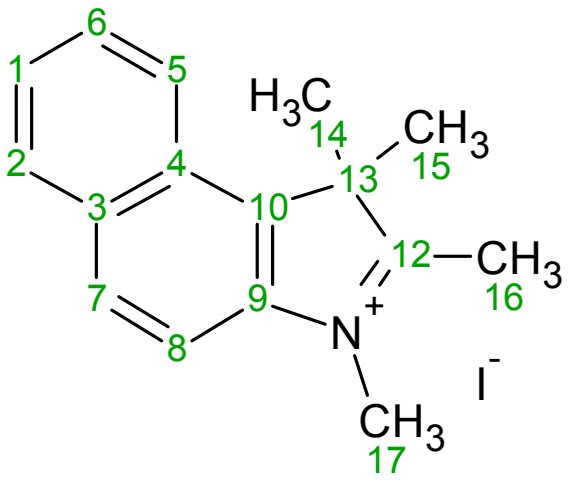
Acquisition Time (sec)	4.0894	***Comment***	mm/7316/32		
Date	01 Aug 2014 19:29:36				
Date Stamp	01 Aug 2014 19:29:36				
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\au0114mm4 benzo[e]indole N-methiodide salt\10\PDATA\1\1r				
Frequency (MHz)	400.1300	***Nucleus***	1H	***Number of Transients***	16
Origin	spect	***Original Points Count***	32768	***Owner***	nmr
Points Count	131072	***Pulse Sequence***	zg30	***Receiver Gain***	72.67
SW(cyclical) (Hz)	8012.82	***Solvent***	DMSO-d6	***Spectrum Offset (Hz)***	2492.3271
Spectrum Type	standard	***Sweep Width (Hz)***	8012.76	***Temperature (degree C)***	24.997



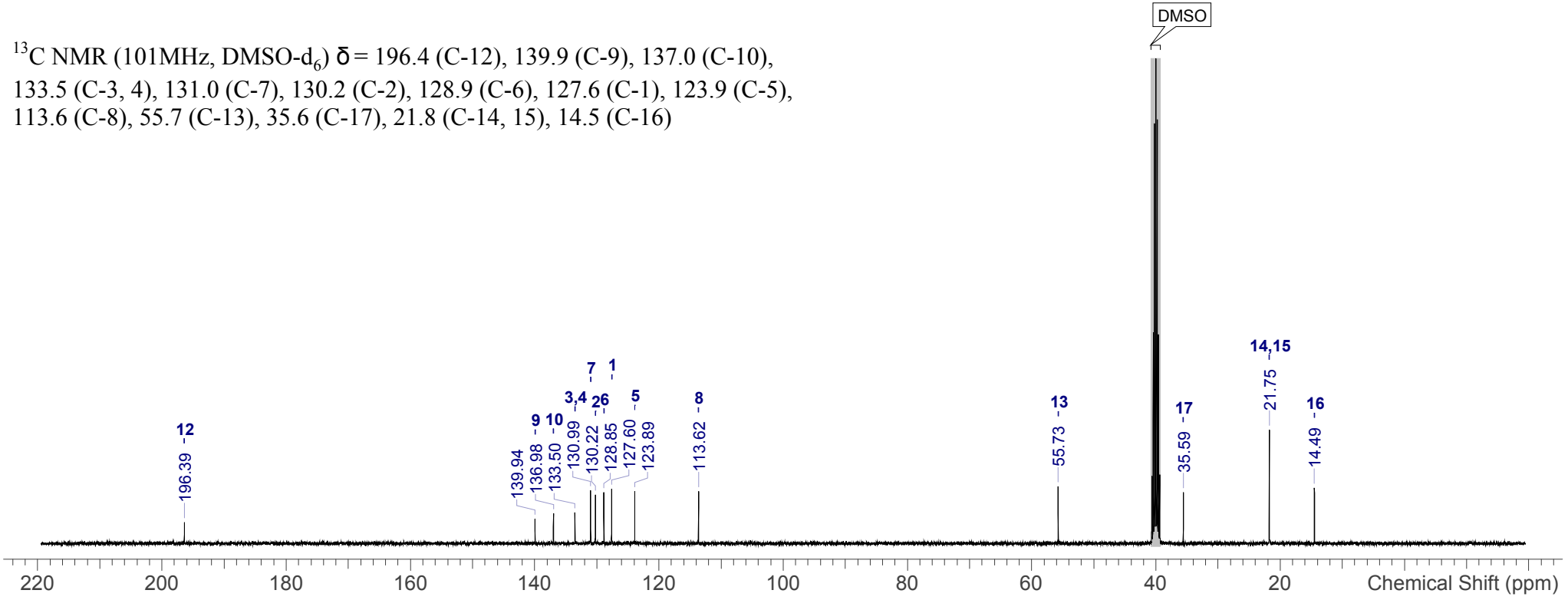
^{1}H NMR (400MHz, DMSO-d_6) δ = 8.43 (1H, d, J = 8.0 Hz, H-5), 8.35 (1H, d, J = 8.9 Hz, H-7), 8.28 (1H, d, J = 7.9 Hz, H-2), 8.17 (1H, d, J = 8.9 Hz, H-8), 7.85 (2H, ddd, J = 8.2 Hz, J = 7.0 Hz, J = 1.3 Hz, H-6), 7.78 (1H, ddd, J = 8.4 Hz, J = 7.0 Hz, J = 1.6 Hz, H-1), 4.16 (3H, s, H-17), 2.94 (3H, s, H-16), 1.81 (6H, s, H-14, 15)



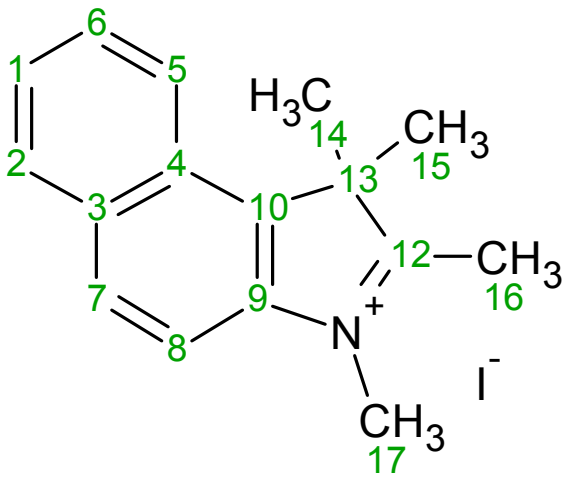
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/32
Date	01 Aug 2014 20:00:19		
Date Stamp	01 Aug 2014 20:00:19		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\au0114mm4 benzo[e]indole N-methiodide salt\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	10060.3320
Spectrum Type	standard	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	24.987		



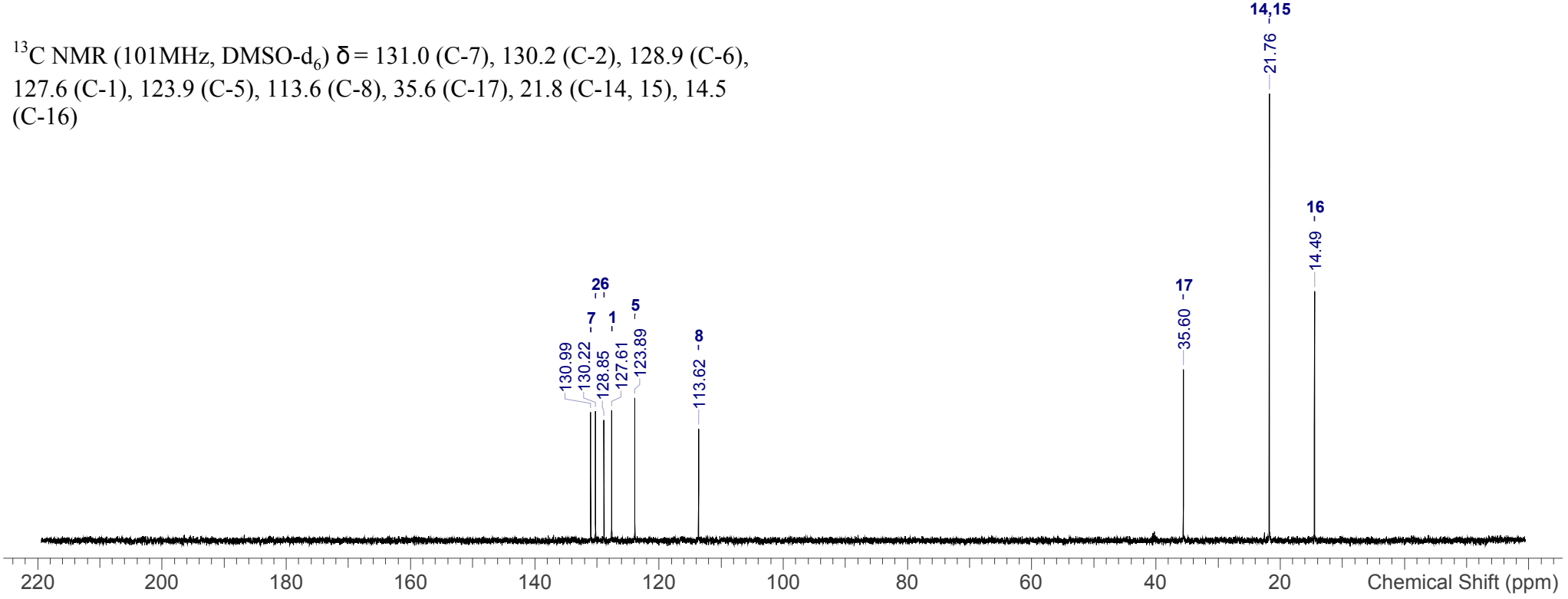
^{13}C NMR (101MHz, DMSO-d_6) δ = 196.4 (C-12), 139.9 (C-9), 137.0 (C-10), 133.5 (C-3, 4), 131.0 (C-7), 130.2 (C-2), 128.9 (C-6), 127.6 (C-1), 123.9 (C-5), 113.6 (C-8), 55.7 (C-13), 35.6 (C-17), 21.8 (C-14, 15), 14.5 (C-16)



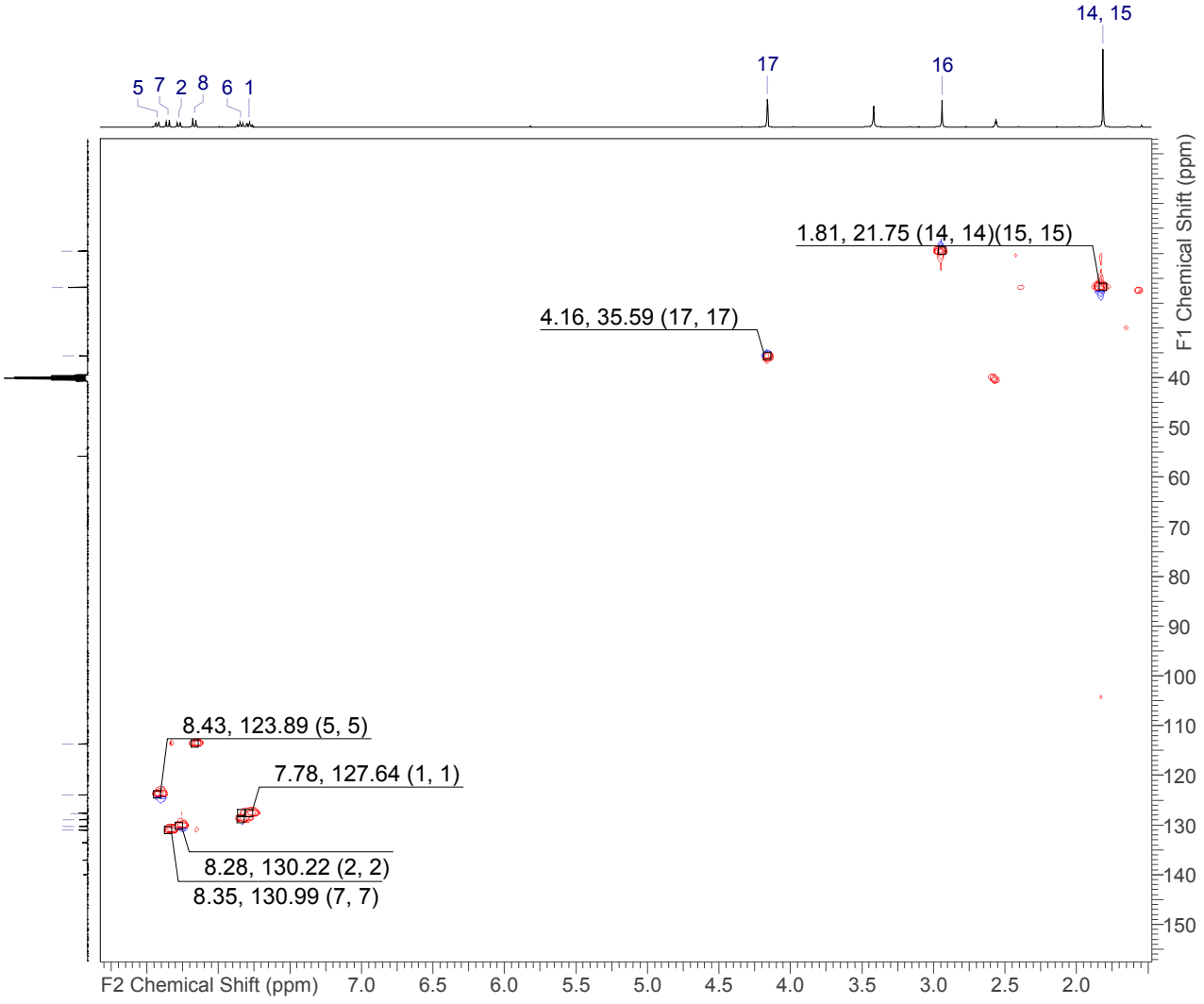
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/32
Date	01 Aug 2014 20:32:18		
Date Stamp	01 Aug 2014 20:32:18		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\au0114mm4 benzo[e]indole N-methiodide salt\13\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp45
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	10061.0684
Spectrum Type	DEPT45	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	25.001		

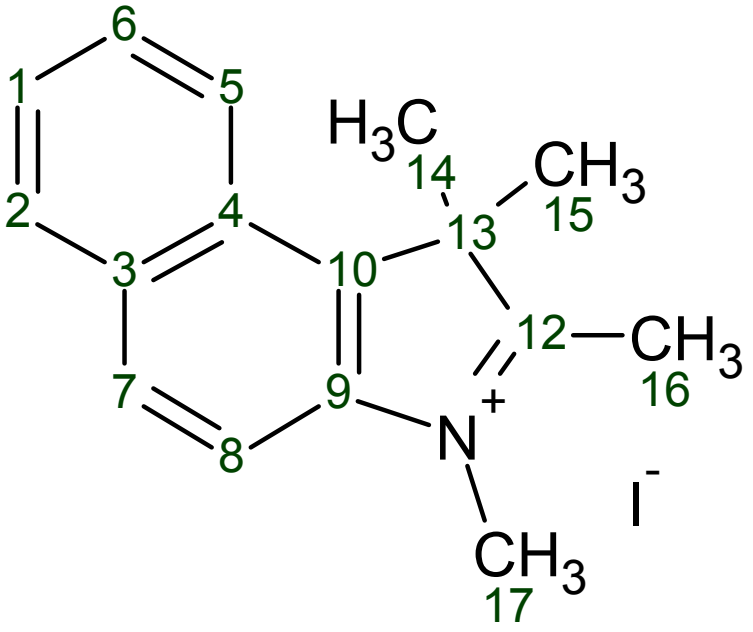


^{13}C NMR (101MHz, DMSO-d_6) δ = 131.0 (C-7), 130.2 (C-2), 128.9 (C-6), 127.6 (C-1), 123.9 (C-5), 113.6 (C-8), 35.6 (C-17), 21.8 (C-14, 15), 14.5 (C-16)

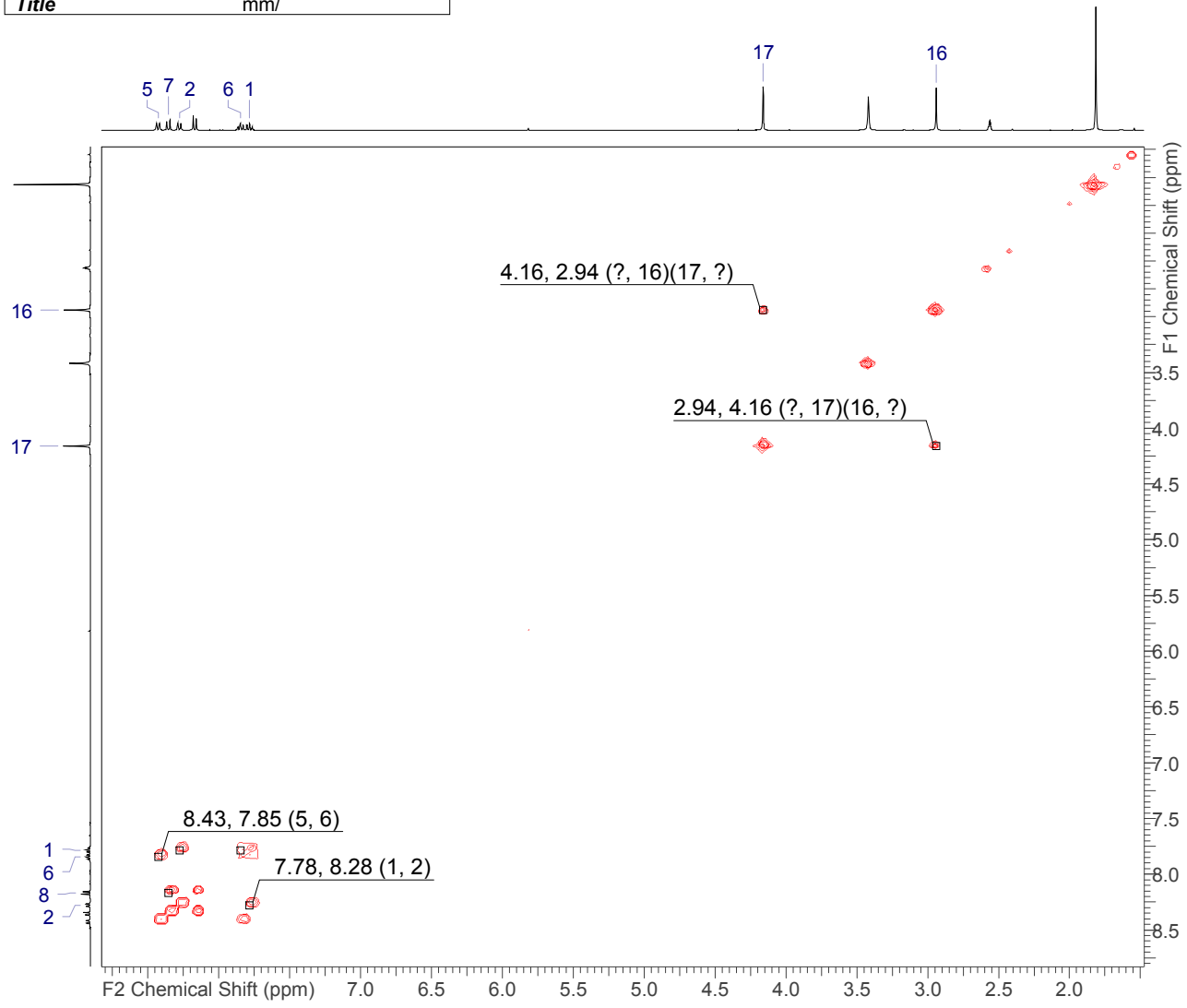


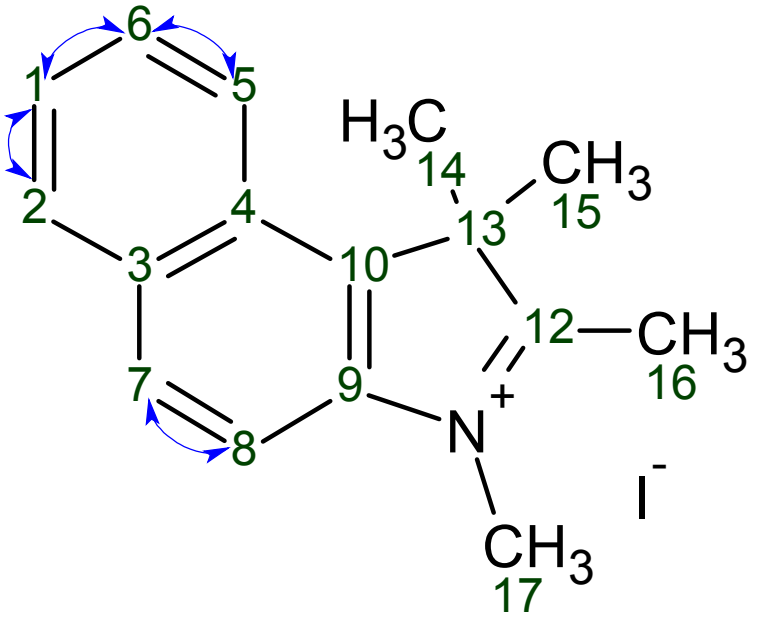
Acquisition Time (sec)	(0.1495, 0.0154)	***Comment***	5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218
Date	01 Aug 2014 20:47:00		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\au0114mm4 benzo[e]indole N-methiodide salt\14\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)	***Nucleus***	(1H, 13C)
Number of Transients	2	***Origin***	spect
Original Points Count	(512, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	hsqcetgp
Solvent	DMSO-d6	***Spectrum Type***	HSQC
Sweep Width (Hz)	(3421.31, 16650.39)	***Temperature (degree C)***	25.044
Title	mm/		





Acquisition Time (sec)	(0.2990, 0.0748)	***Comment***	5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218
Date	01 Aug 2014 20:16:00		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\au0114mm4 benzo[e]indole N-methiodide salt\12\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)	***Nucleus***	(1H, 1H)
Number of Transients	2	***Origin***	spect
Original Points Count	(1024, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	cosygpqf
Solvent	DMSO-d6	***Spectrum Type***	COSY
Sweep Width (Hz)	(3421.31, 3421.31)	***Temperature (degree C)***	24.980
Title	mm/		



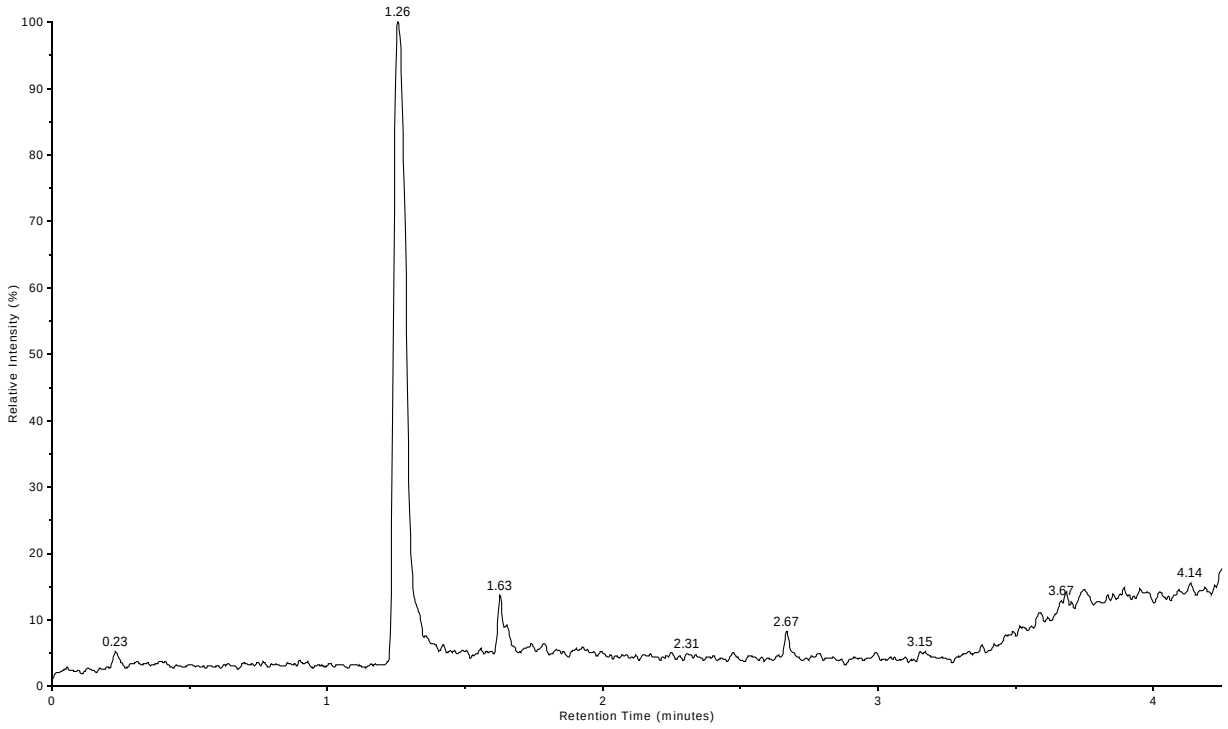


A.9 3-(5-carboxypentyl)-1,1,2-trimethyl-1H-benzo[e]indol-3-ium iodide

Top 5 Peak Report

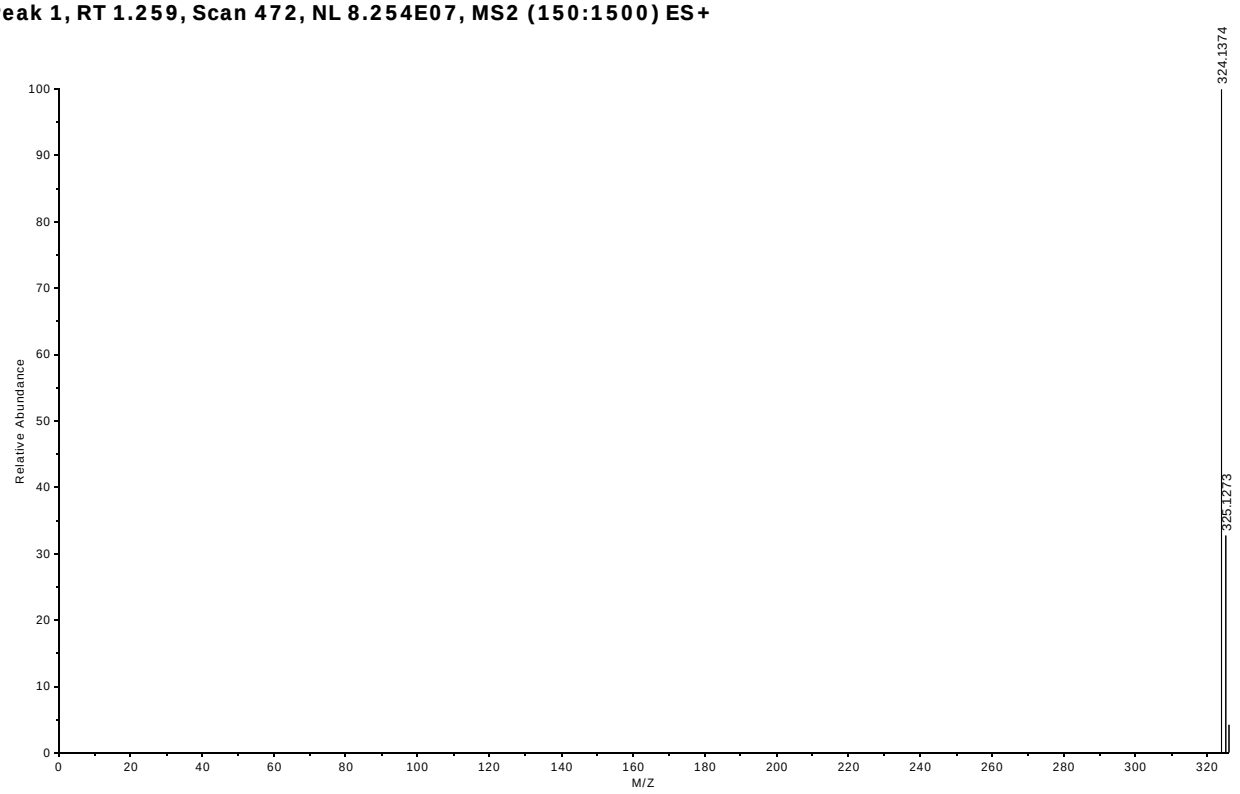
Group: Watts, J
Submitter: Michael Moazami
Project: RP-LC MW 150-1500
Sample ID: mm/7316/36
Experiment: B ESIPOS (simple mix) C18 Acid
Filename: Watts_J_mm731636_MM_362.pdf
Instrument: Blue RP UHPLC-MS (B30:1023)
Acquisition Date: 07/08/2014 16:49:00

TIC, NL 1.171E08, MS2 (150:1500) ES+

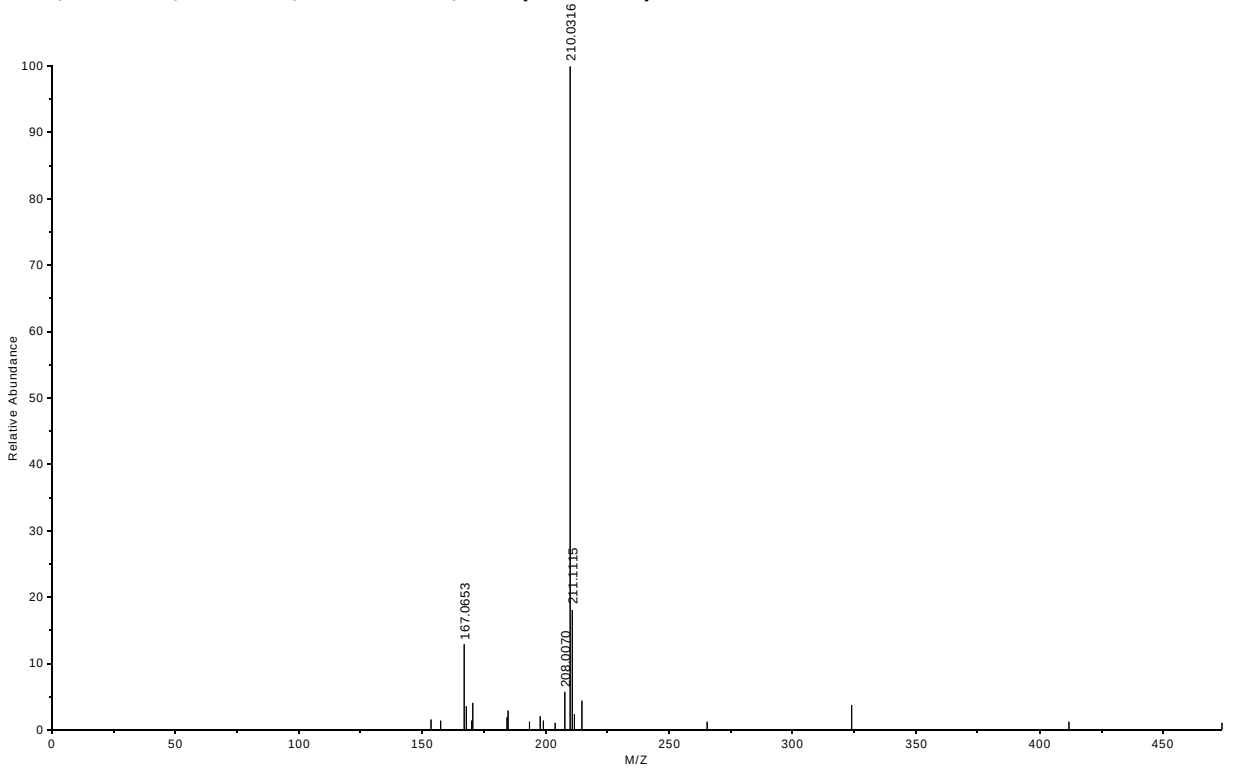


	RT Mins	Height	Height %	Area	Area %
1	1.26	110991667	100.00	360681527	100.00
2	1.63	8081099	7.28	12547584	3.48

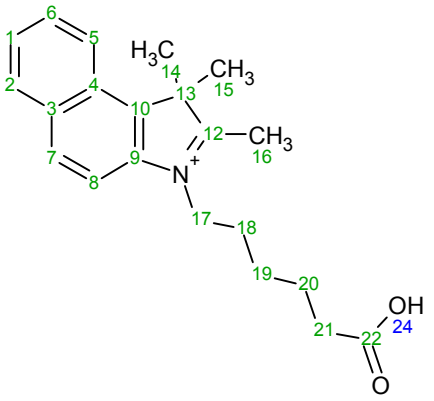
Peak 1, RT 1.259, Scan 472, NL 8.254E07, MS2 (150:1500) ES+



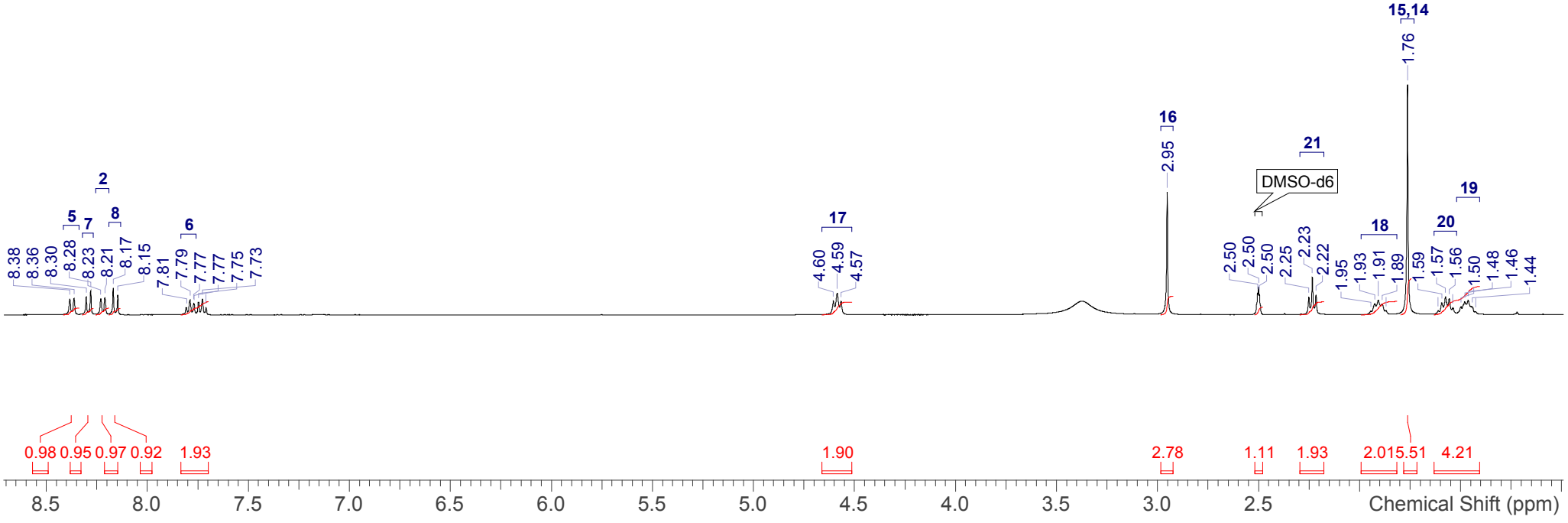
Peak 2, RT 1.629, Scan 611, NL 9.472E06, MS2 (150:1500) ES+



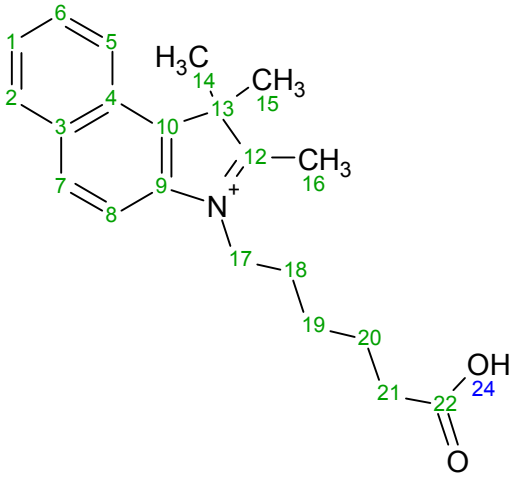
Acquisition Time (sec)	4.0894	***Comment***	mm/7316/36	***Date***	07 Aug 2014 16:25:00
Date Stamp	07 Aug 2014 16:25:00				
File Name	C:\Users\Mike Moazami\Desktop\au0714mm1\10\PDATA\1\1r				
Frequency (MHz)	400.1300	***Nucleus***	1H	***Number of Transients***	16
Original Points Count	32768	***Owner***	nmr	***Points Count***	65536
Pulse Sequence	zg30	***Receiver Gain***	32.00	***SW(cyclical) (Hz)***	8012.82
Solvent	CHLOROFORM-d			***Spectrum Offset (Hz)***	2467.6997
Spectrum Type	standard	***Sweep Width (Hz)***	8012.70	***Temperature (degree C)***	25.000



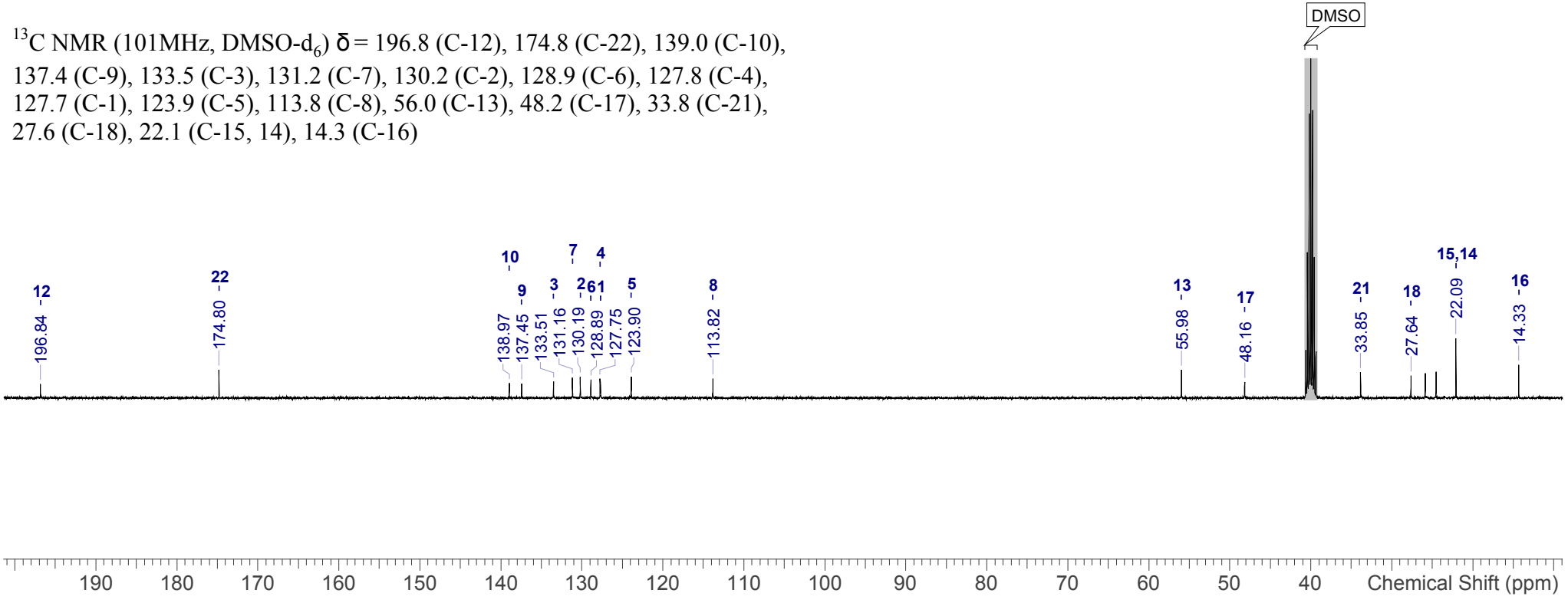
^{1}H NMR (400MHz, CHLOROFORM-d) δ = 8.37 (1H, d, J = 8.3 Hz, H-5), 8.29 (1H, d, J = 8.9 Hz, H-7), 8.22 (1H, d, J = 7.9 Hz, H-2), 8.16 (1H, d, J = 8.9 Hz, H-8), 7.76 - 7.83 (1H, m, H-6), 7.70 - 7.77 (1H, m, M07), 4.59 (2H, br t, J = 7.6 Hz, H-17), 2.95 (3H, s, H-16), 2.48 - 2.52 (1H, m, M13), 2.23 (2H, t, J = 7.2 Hz, H-21), 1.91 (2H, quin, J = 7.6 Hz, H-18), 1.76 (6H, s, H-15, 14), 1.52 - 1.63 (2H, m, H-20), 1.41 - 1.52 (2H, m, H-19)



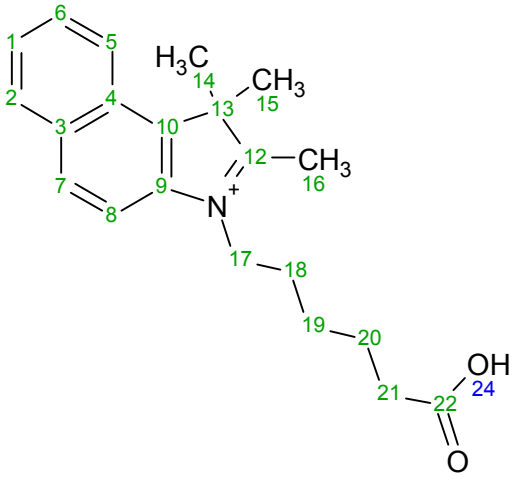
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/36
Date	07 Aug 2014 16:06:33		
Date Stamp	07 Aug 2014 16:06:33		
File Name	C:\Users\Mike Moazami\Desktop\au0714mm1\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	10060.3320
Spectrum Type	standard	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	24.993		



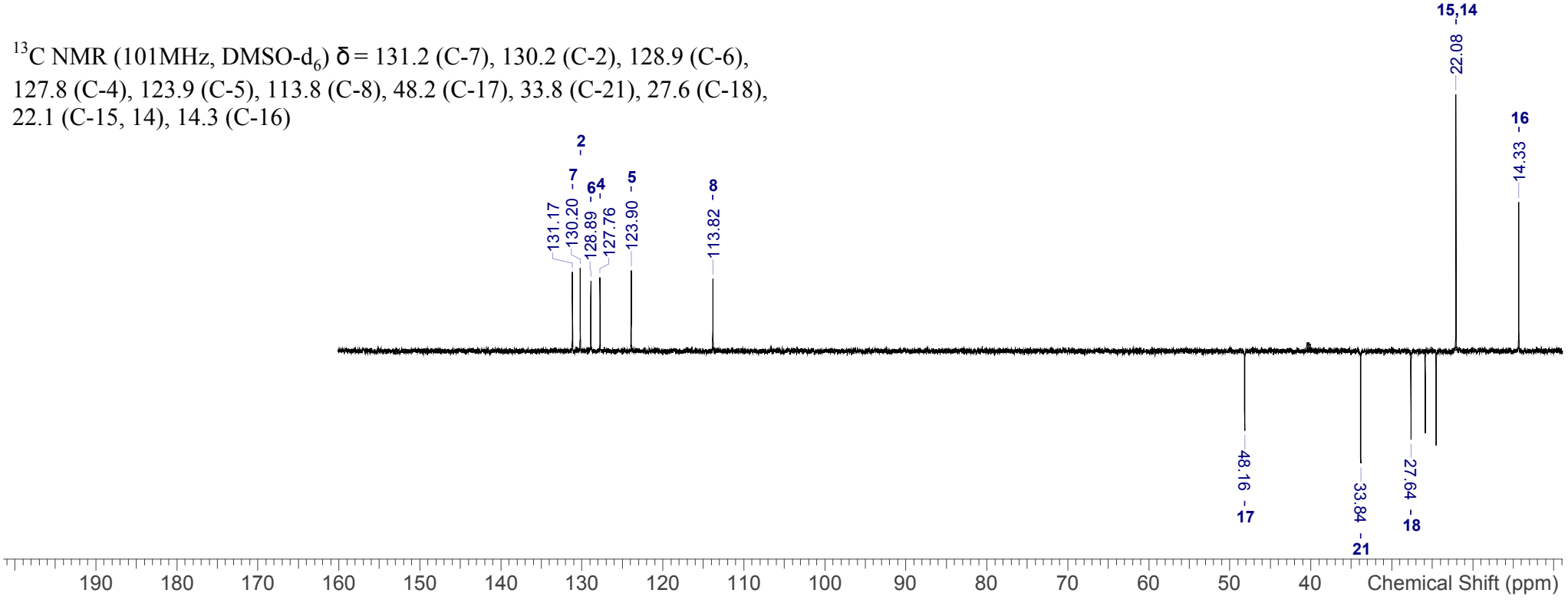
^{13}C NMR (101MHz, DMSO-d_6) δ = 196.8 (C-12), 174.8 (C-22), 139.0 (C-10), 137.4 (C-9), 133.5 (C-3), 131.2 (C-7), 130.2 (C-2), 128.9 (C-6), 127.8 (C-4), 127.7 (C-1), 123.9 (C-5), 113.8 (C-8), 56.0 (C-13), 48.2 (C-17), 33.8 (C-21), 27.6 (C-18), 22.1 (C-15, 14), 14.3 (C-16)



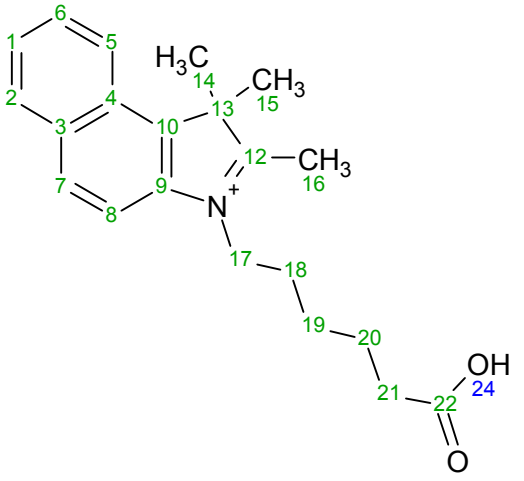
Acquisition Time (sec)	2.0316	***Comment***	mm/7316/36
Date	07 Aug 2014 16:25:09		
Date Stamp	07 Aug 2014 16:25:09		
File Name	C:\Users\Mike Moazami\Desktop\au0714mm1\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	8048.0767
Spectrum Type	DEPT135	***Sweep Width (Hz)***	16128.54
Temperature (degree C)	25.006		



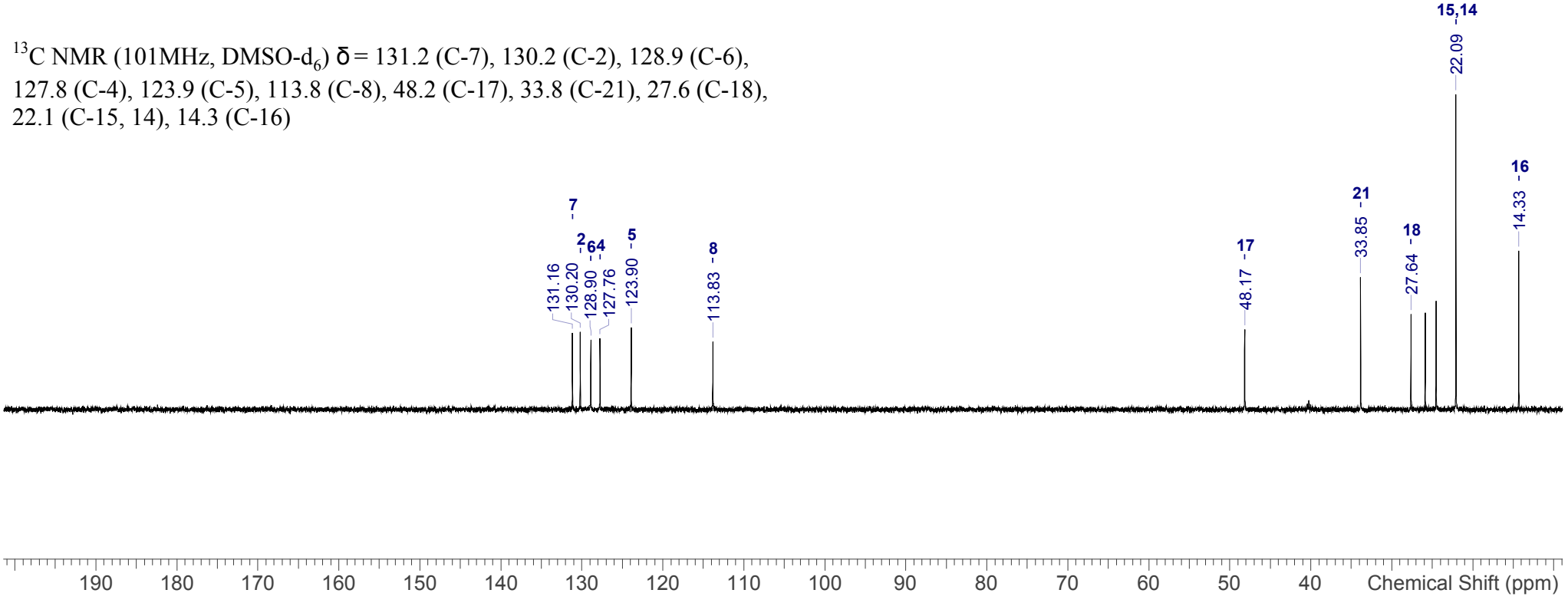
^{13}C NMR (101MHz, DMSO-d_6) δ = 131.2 (C-7), 130.2 (C-2), 128.9 (C-6), 127.8 (C-4), 123.9 (C-5), 113.8 (C-8), 48.2 (C-17), 33.8 (C-21), 27.6 (C-18), 22.1 (C-15, 14), 14.3 (C-16)



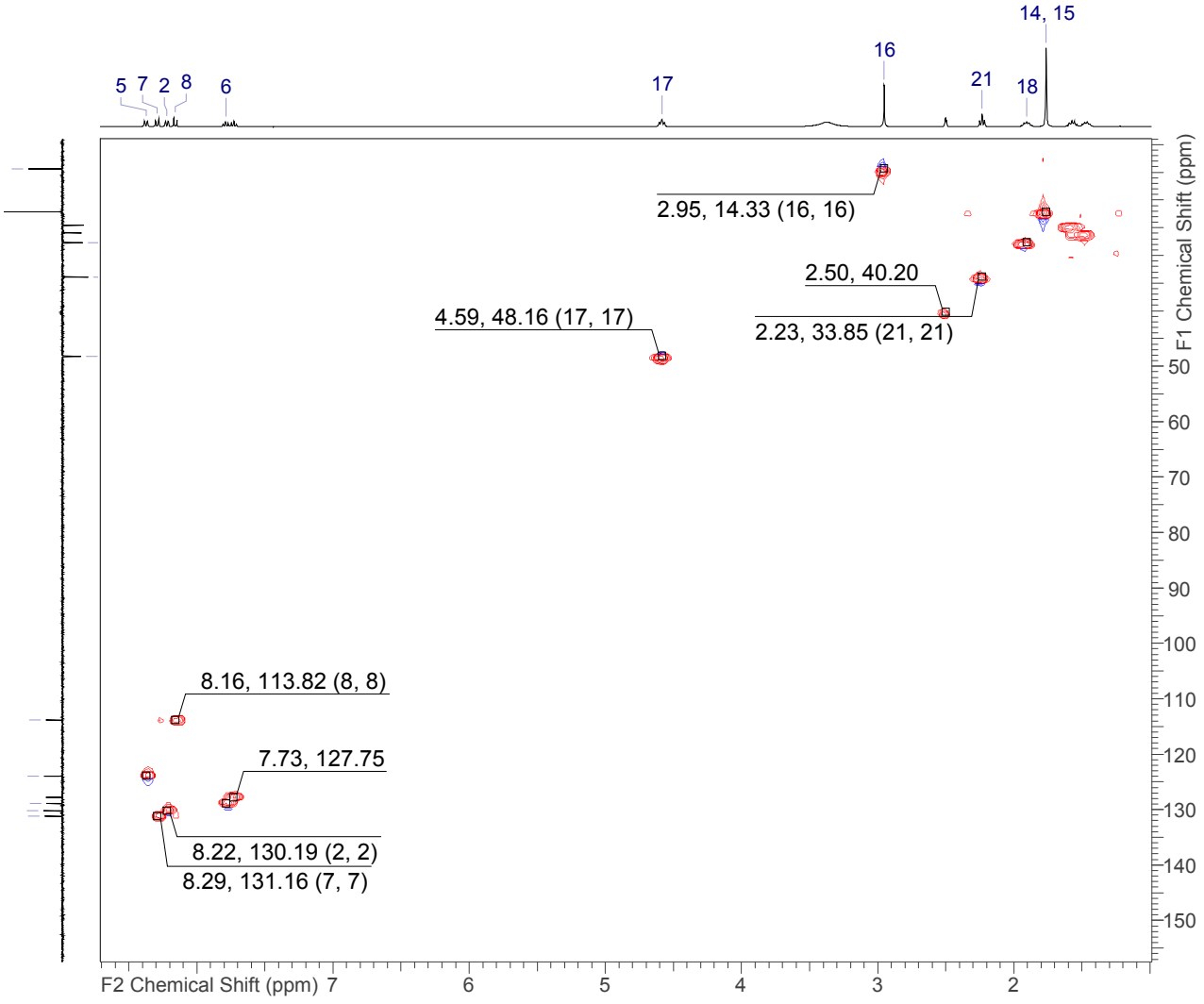
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/36
Date	07 Aug 2014 16:57:02		
Date Stamp	07 Aug 2014 16:57:02		
File Name	C:\Users\Mike Moazami\Desktop\au0714mm1\14\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp45
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	10061.0684
Spectrum Type	DEPT45	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	25.002		

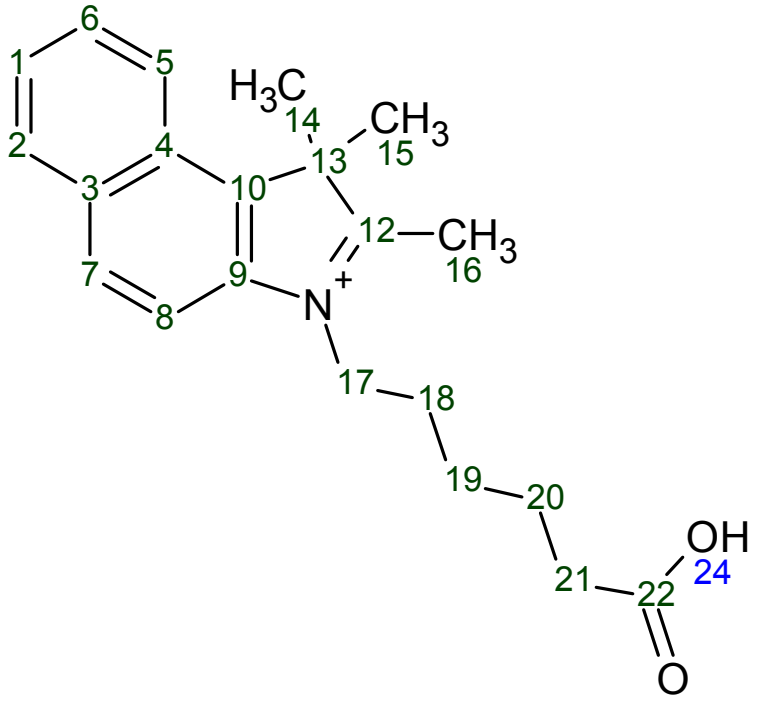


^{13}C NMR (101MHz, DMSO-d_6) δ = 131.2 (C-7), 130.2 (C-2), 128.9 (C-6), 127.8 (C-4), 123.9 (C-5), 113.8 (C-8), 48.2 (C-17), 33.8 (C-21), 27.6 (C-18), 22.1 (C-15, 14), 14.3 (C-16)

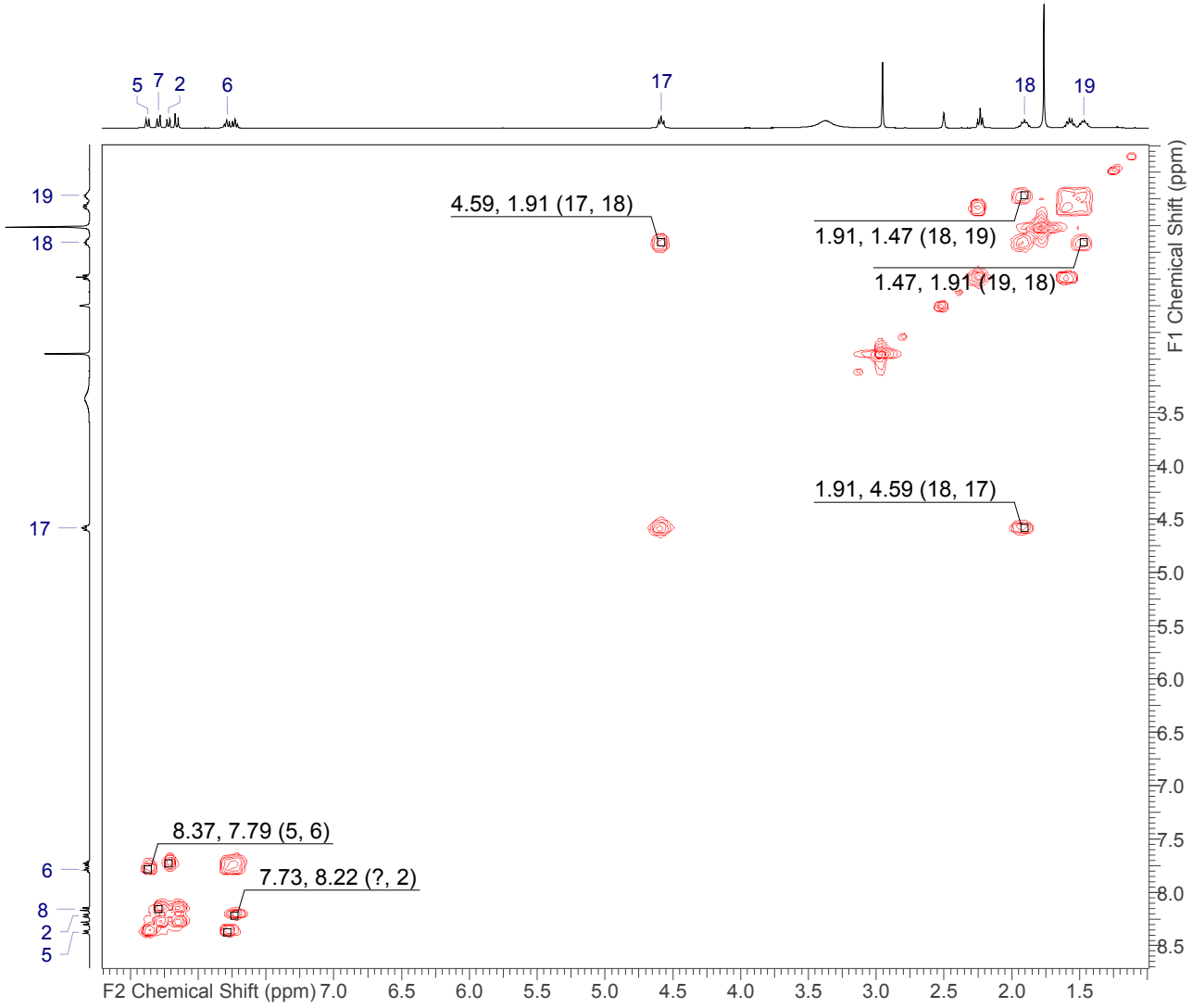


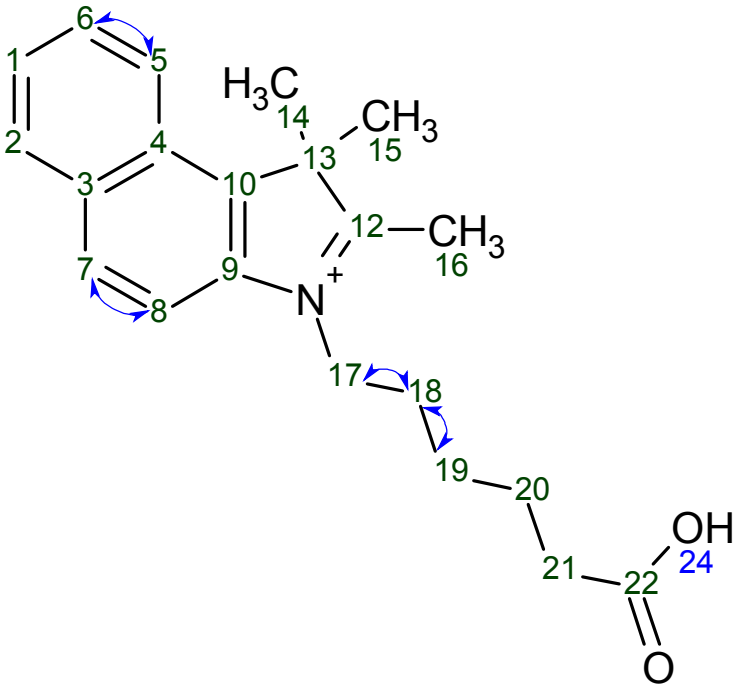
Acquisition Time (sec)	(0.0911, 0.0154)	***Comment***	5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218
Date	07 Aug 2014 17:12:00		
File Name	C:\Users\Mike Moazami\Desktop\au0714mm1\15\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)		
Nucleus	(1H, 13C)	***Number of Transients***	2
Origin	spect	***Original Points Count***	(512, 256)
Owner	nmr	***Points Count***	(1024, 1024)
Pulse Sequence	hsqcetgp	***Solvent***	DMSO-d6
Spectrum Type	HSQC	***Sweep Width (Hz)***	(5612.49, 16650.39)
Temperature (degree C)	25.028	***Title***	mm/





Acquisition Time (sec)	(0.1823, 0.0456)	***Comment***	5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218
Date	07 Aug 2014 16:41:00		
File Name	C:\Users\Mike Moazami\Desktop\au0714mm1\13\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)		
Nucleus	(1H, 1H)	***Number of Transients***	2
Origin	spect	***Original Points Count***	(1024, 256)
Owner	nmr	***Points Count***	(1024, 1024)
Pulse Sequence	cosygpqf	***Solvent***	DMSO-d6
Spectrum Type	COSY	***Sweep Width (Hz)***	(5612.49, 5612.49)
Temperature (degree C)	25.002	***Title***	mm/



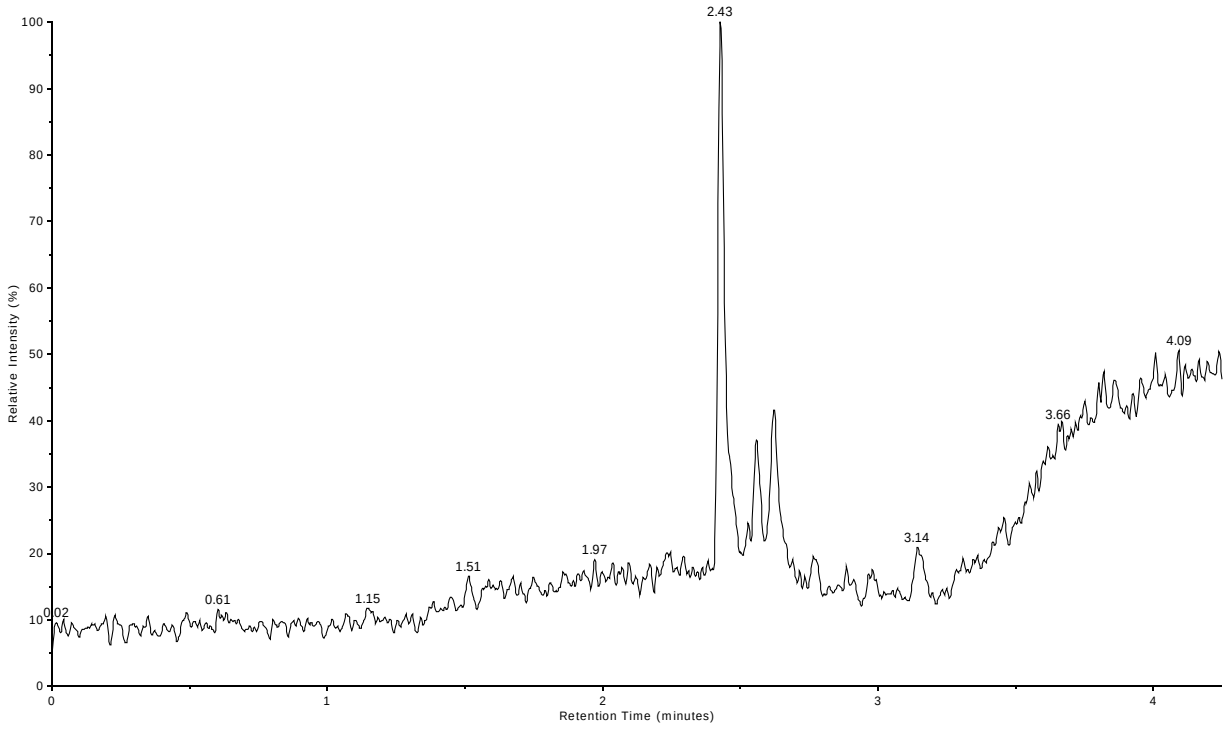


A.10 3-(5-carboxypentyl)-1,1-dimethyl-2-((1E,3E,5E)-5-(1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1H-benzo[e]indol-3-ium iodide

Top 5 Peak Report

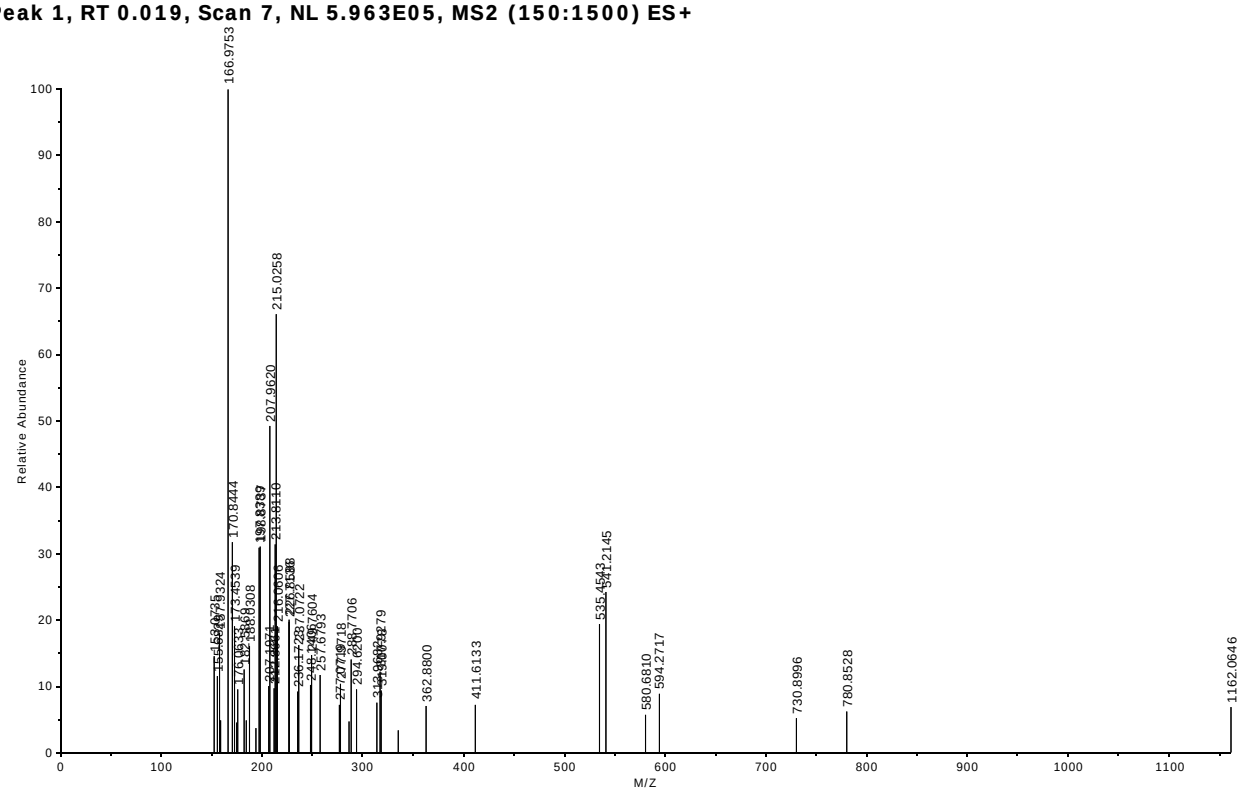
Group: Watts, J
Submitter: Michael Moazami
Project: RP-LC MW 150-1500
Sample ID: mm/7316/40 Cy5.5
Experiment: B ESIPOS (simple mix) C18 Acid
Filename: Watts_J_mm731640_Cy5_5_MM_394.pdf
Instrument: Blue RP UHPLC-MS (B30:1023)
Acquisition Date: 19/08/2014 13:35:51

TIC, NL 3.968E07, MS2 (150:1500) ES+

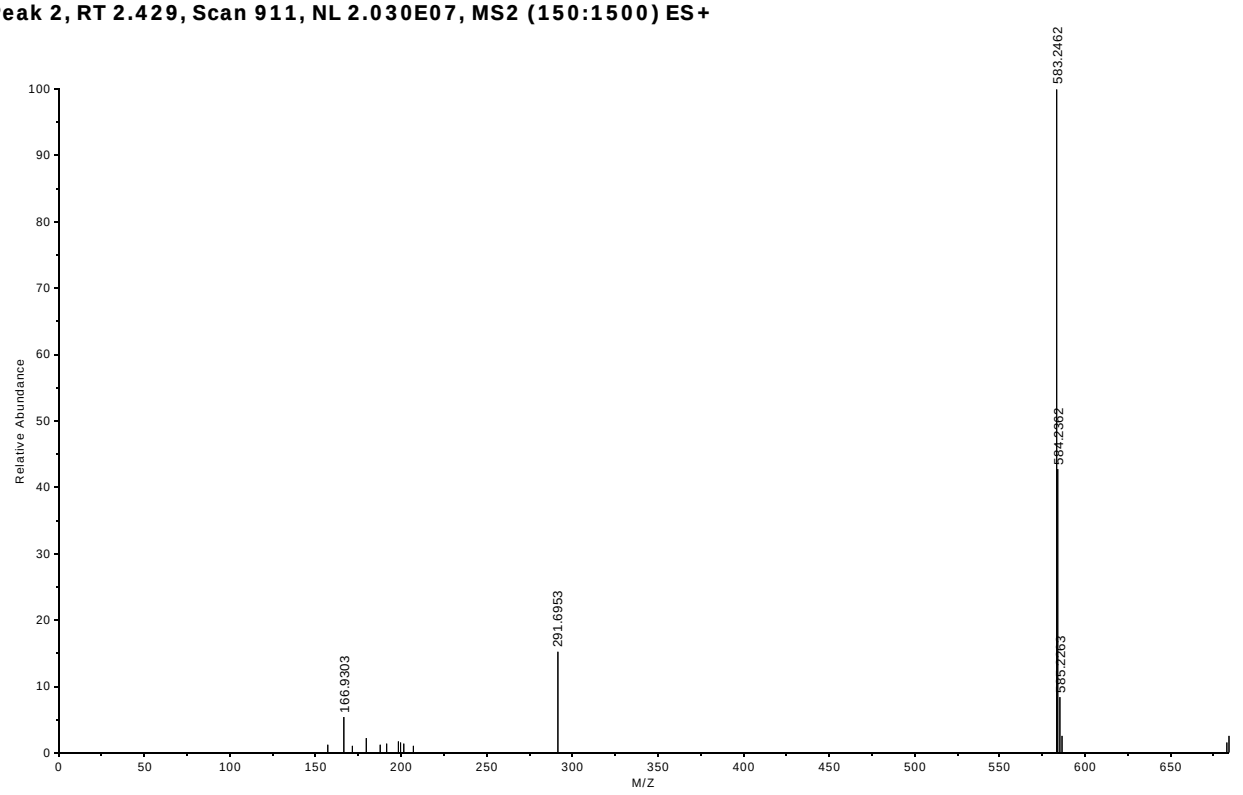


	RT Mins	Height	Height %	Area	Area %
1	0.02	2539710	7.88	3780362	5.87
2	2.43	32219023	100.00	64362367	100.00
3	2.56	5998730	18.62	8736549	13.57
4	2.62	8633294	26.80	21563517	33.50
5	3.14	3210693	9.97	4374569	6.80

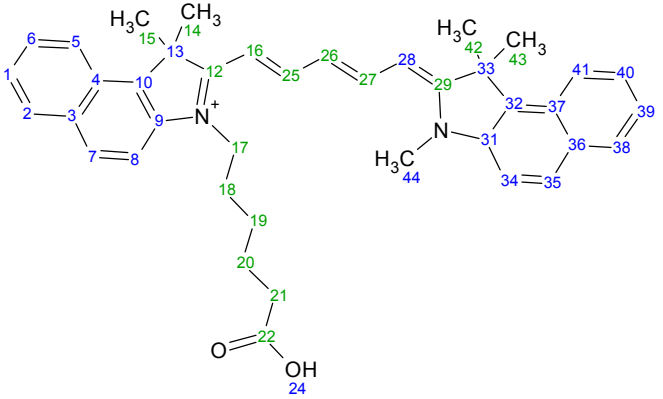
Peak 1, RT 0.019, Scan 7, NL 5.963E05, MS2 (150:1500) ES+



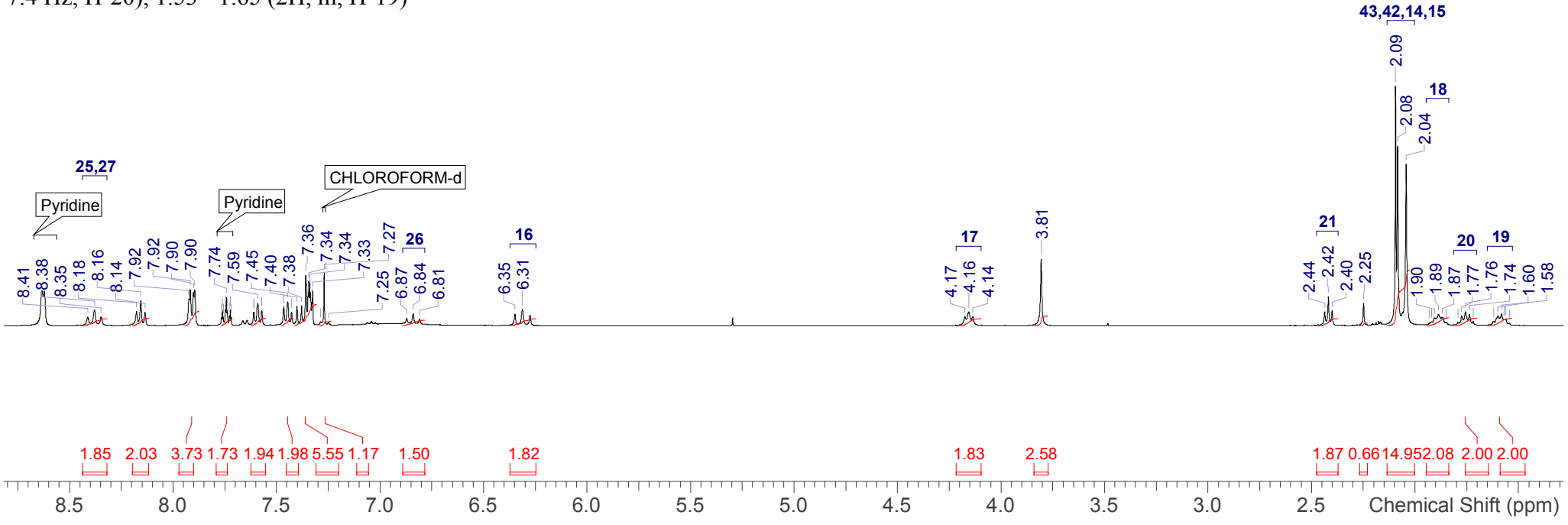
Peak 2, RT 2.429, Scan 911, NL 2.030E07, MS2 (150:1500) ES+



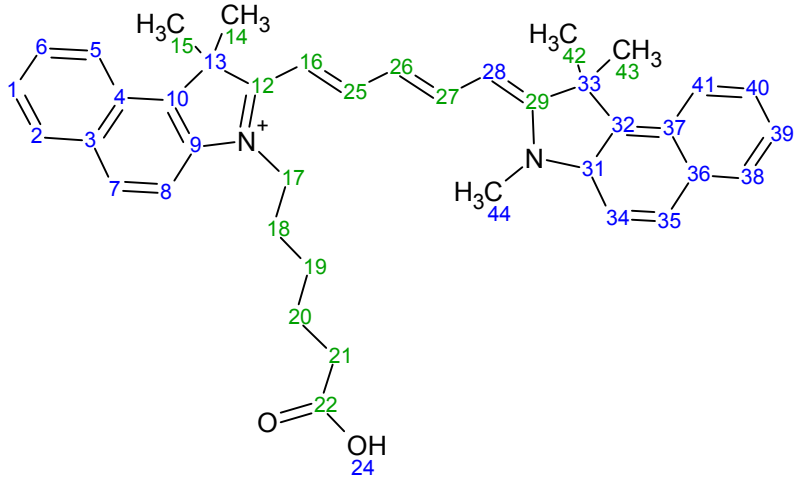
Acquisition Time (sec)	4.0894	***Comment***	mm/7316/40		
Date	19 Aug 2014 21:35:01				
Date Stamp	19 Aug 2014 21:35:01				
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\au1914mm2 Cy5.5 PURE\10\PDATA\1\1r				
Frequency (MHz)	400.1300	***Nucleus***	1H	***Number of Transients***	16
Origin	spect	***Original Points Count***	32768	***Owner***	nmr
Points Count	65536	***Pulse Sequence***	zg30	***Receiver Gain***	64.46
SW(cyclical) (Hz)	8012.82	***Solvent***	CHLOROFORM-d		
Spectrum Offset (Hz)	2465.0588	***Spectrum Type***	standard	***Sweep Width (Hz)***	8012.70
Temperature (degree C)	25.003				



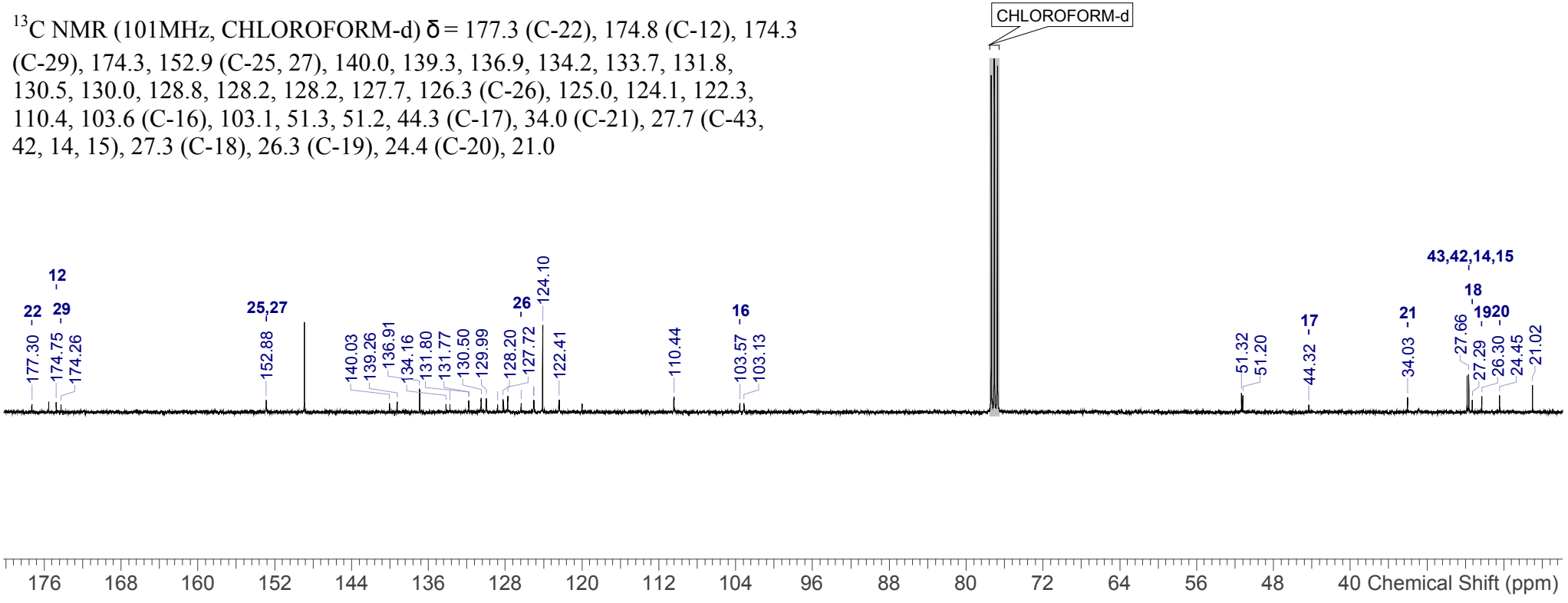
^{1}H NMR (400MHz, CHLOROFORM-d) δ = 8.38 (2H, br t, J = 13.1 Hz, H-25, 27), 8.16 (2H, t, J = 8.3 Hz, M13), 7.91 (4H, dd, J = 8.7 Hz, J = 2.4 Hz, M14), 7.74 (2H, tt, J = 7.7 Hz, J = 1.8 Hz, M19), 7.55 - 7.62 (2H, m, M16), 7.42 - 7.48 (2H, m, M17), 7.39 (3H, d, J = 8.8 Hz, M15), 7.36 - 7.41 (2H, m, M18), 7.31 - 7.37 (3H, m, M11), 7.24 - 7.29 (1H, m, M20), 6.84 (1H, br t, J = 12.4 Hz, H-26), 6.31 (2H, t, J = 14.5 Hz, H-16), 4.16 (2H, br t, J = 7.5 Hz, H-17), 3.81 (3H, s, M07), 2.42 (2H, t, J = 7.2 Hz, H-21), 2.25 (1H, s, M05), 2.00 - 2.13 (15H, m, H-43, 42, 14, 15), 1.84 - 1.95 (2H, m, H-18), 1.76 (2H, quin, J = 7.4 Hz, H-20), 1.53 - 1.65 (2H, m, H-19)



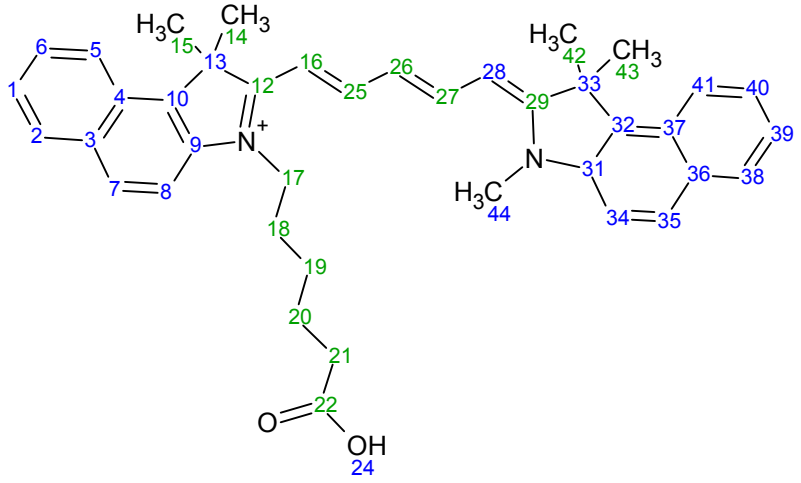
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/40
Date	19 Aug 2014 22:05:22		
Date Stamp	19 Aug 2014 22:05:22		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\au1914mm2 Cy5.5 PURE\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	10060.3320	***Spectrum Type***	standard
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	24.998



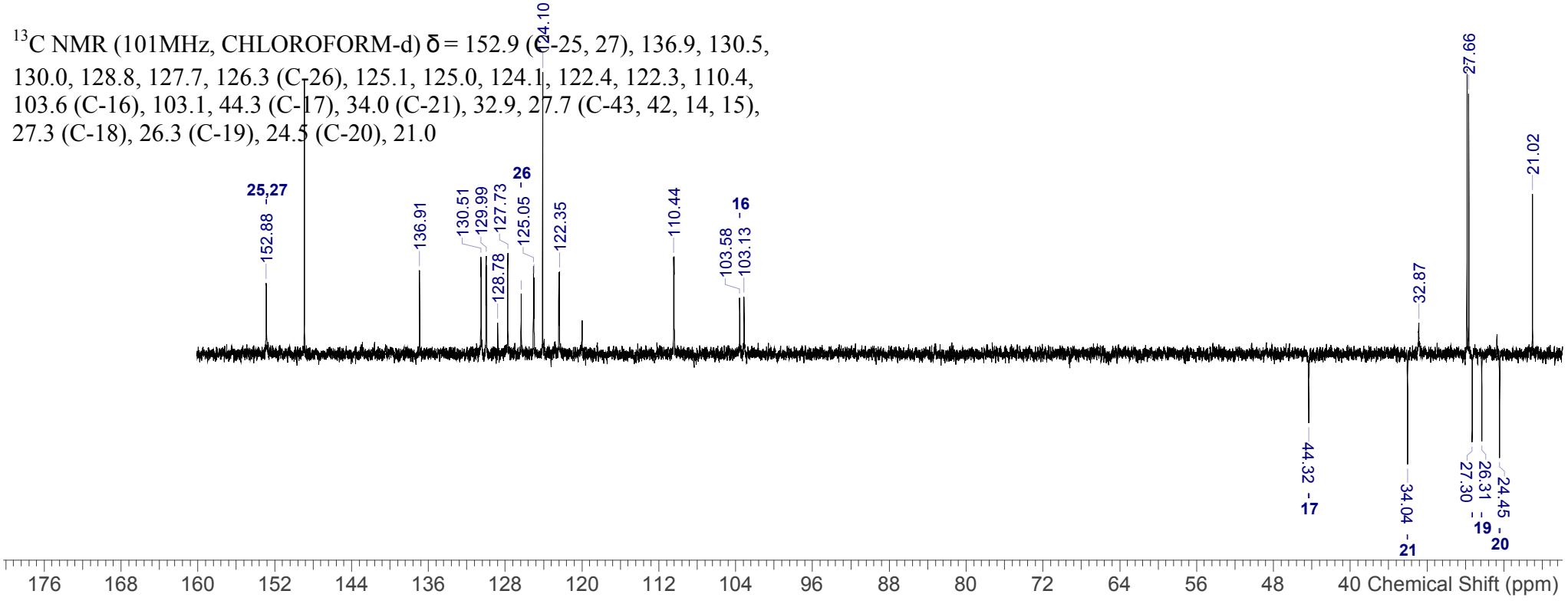
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 177.3 (C-22), 174.8 (C-12), 174.3 (C-29), 174.3, 152.9 (C-25, 27), 140.0, 139.3, 136.9, 134.2, 133.7, 131.8, 130.5, 130.0, 128.8, 128.2, 128.2, 127.7, 126.3 (C-26), 125.0, 124.1, 122.3, 110.4, 103.6 (C-16), 103.1, 51.3, 51.2, 44.3 (C-17), 34.0 (C-21), 27.7 (C-43, 42, 14, 15), 27.3 (C-18), 26.3 (C-19), 24.4 (C-20), 21.0



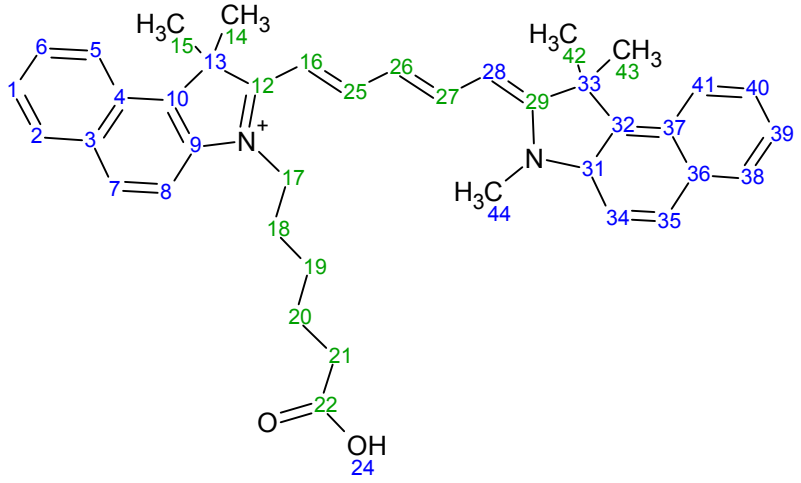
Acquisition Time (sec)	2.0316	***Comment***	mm/7316/40
Date	19 Aug 2014 22:24:01		
Date Stamp	19 Aug 2014 22:24:01		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\au1914mm2 Cy5.5 PURE\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	8048.0767	***Spectrum Type***	DEPT135
Sweep Width (Hz)	16128.54	***Temperature (degree C)***	25.002



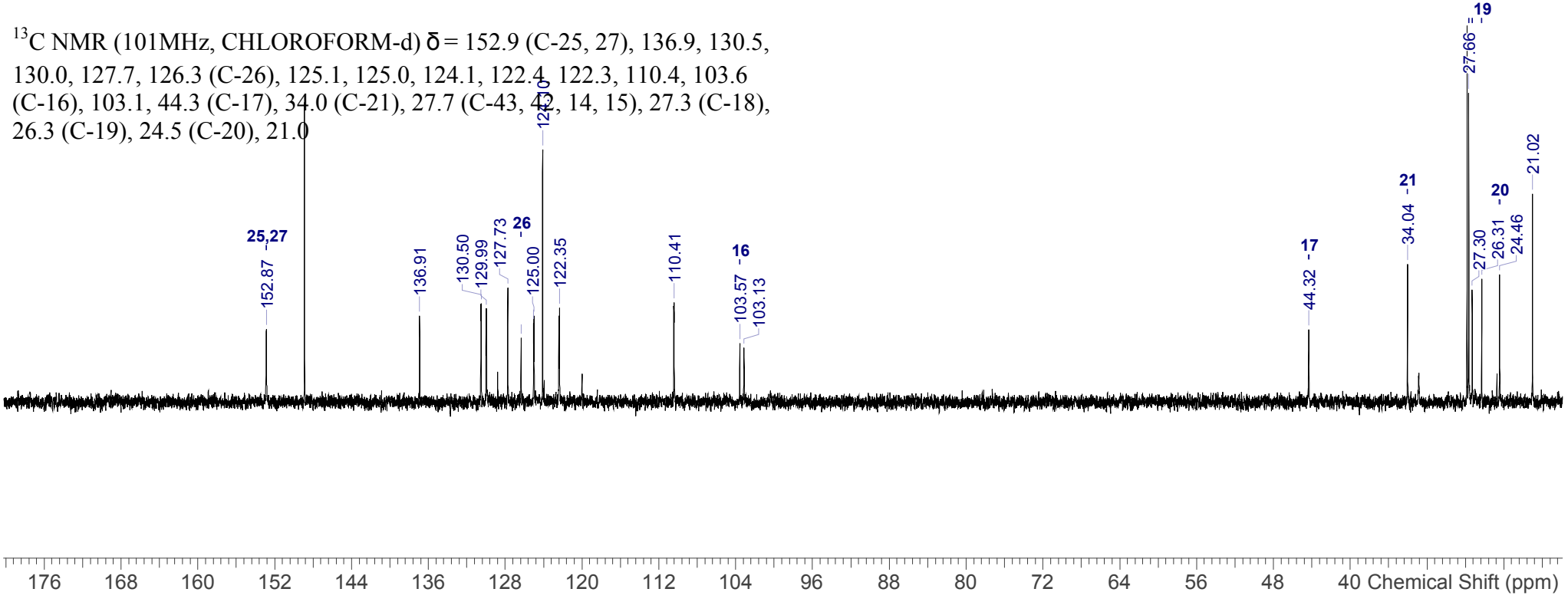
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 152.9 (C-25, 27), 136.9, 130.5, 130.0, 128.8, 127.7, 126.3 (C-26), 125.1, 125.0, 124.1, 122.4, 122.3, 110.4, 103.6 (C-16), 103.1, 44.3 (C-17), 34.0 (C-21), 32.9, 27.7 (C-43, 42, 14, 15), 27.3 (C-18), 26.3 (C-19), 24.5 (C-20), 21.0



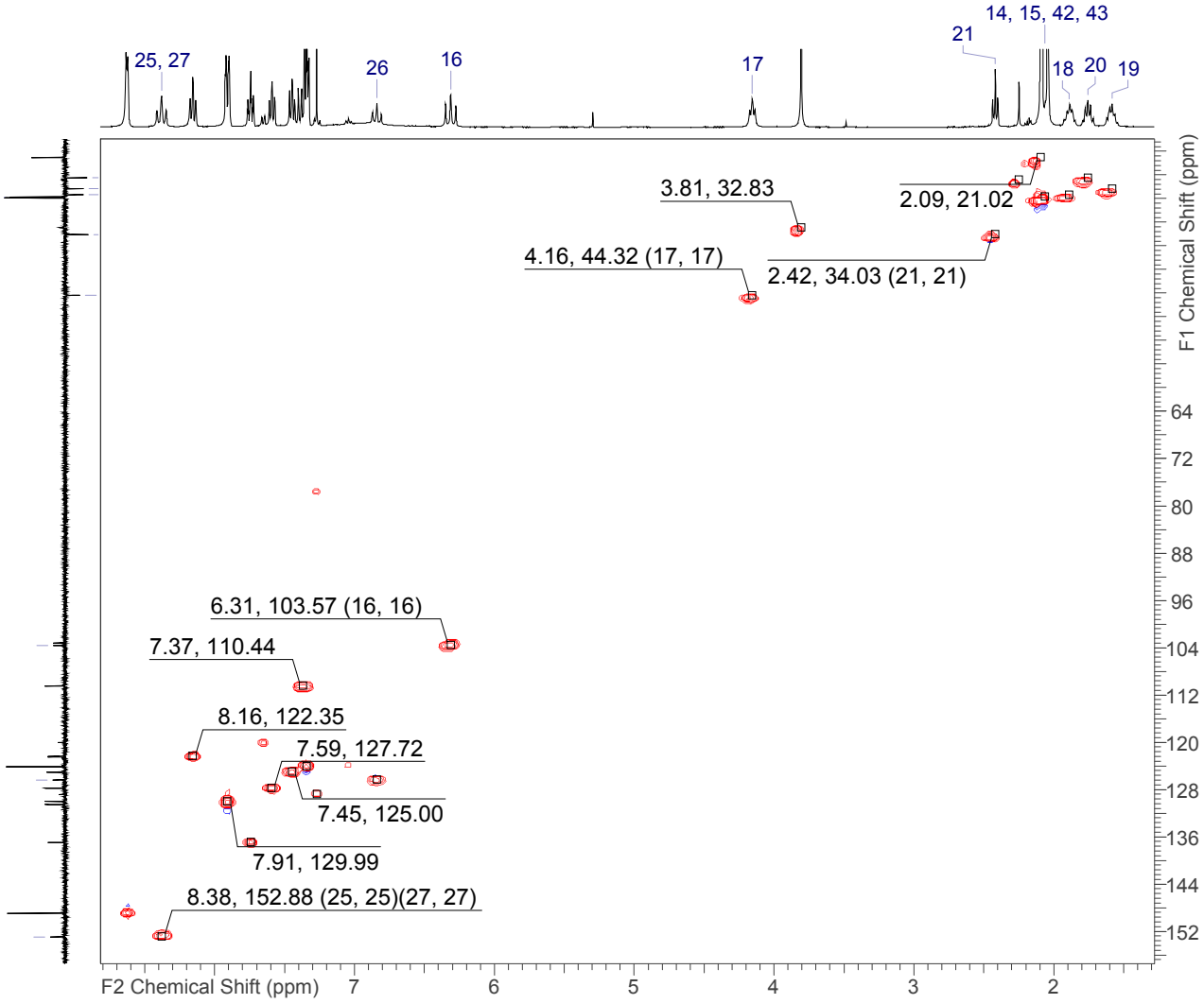
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/40
Date	19 Aug 2014 23:17:32		
Date Stamp	19 Aug 2014 23:17:32		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\au1914mm2 Cy5.5 PURE\15\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp45
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	10061.0684	***Spectrum Type***	DEPT45
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	24.992

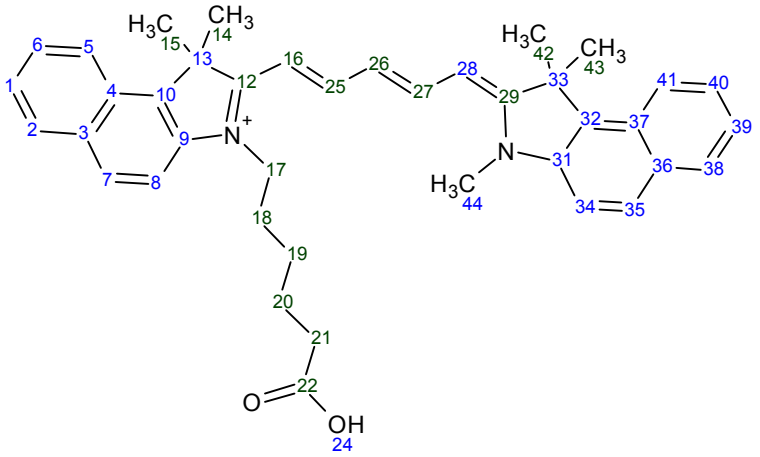


^{13}C NMR (101MHz, CHLOROFORM-d) δ = 152.9 (C-25, 27), 136.9, 130.5, 130.0, 127.7, 126.3 (C-26), 125.1, 125.0, 124.1, 122.4, 122.3, 110.4, 103.6 (C-16), 103.1, 44.3 (C-17), 34.0 (C-21), 27.7 (C-43, 42, 14, 15), 27.3 (C-18), 26.3 (C-19), 24.5 (C-20), 21.0

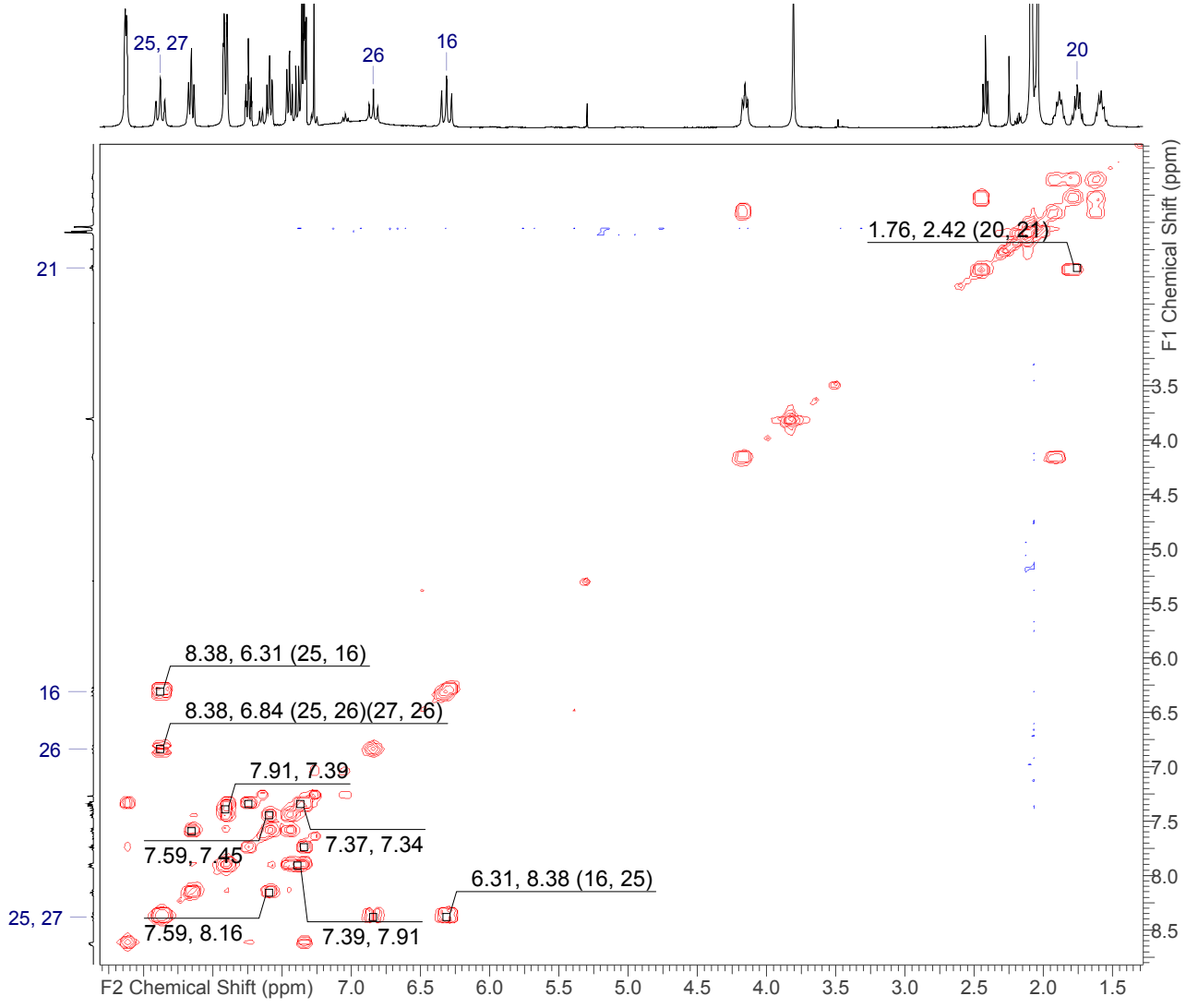


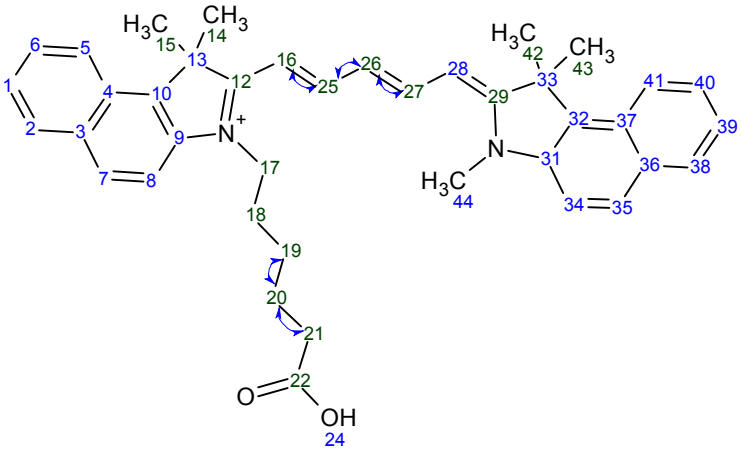
Acquisition Time (sec)	(0.1290, 0.0154)	***Comment***	5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218
Date	19 Aug 2014 23:32:00		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\au1914mm2 Cy5.5 PURE\16\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)	***Nucleus***	(1H, 13C)
Number of Transients	2	***Origin***	spect
Original Points Count	(512, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	hsqcetgp
Solvent	CHLOROFORM-d	***Spectrum Type***	HSQC
Sweep Width (Hz)	(3964.38, 16650.39)	***Temperature (degree C)***	25.041
Title	mm/		



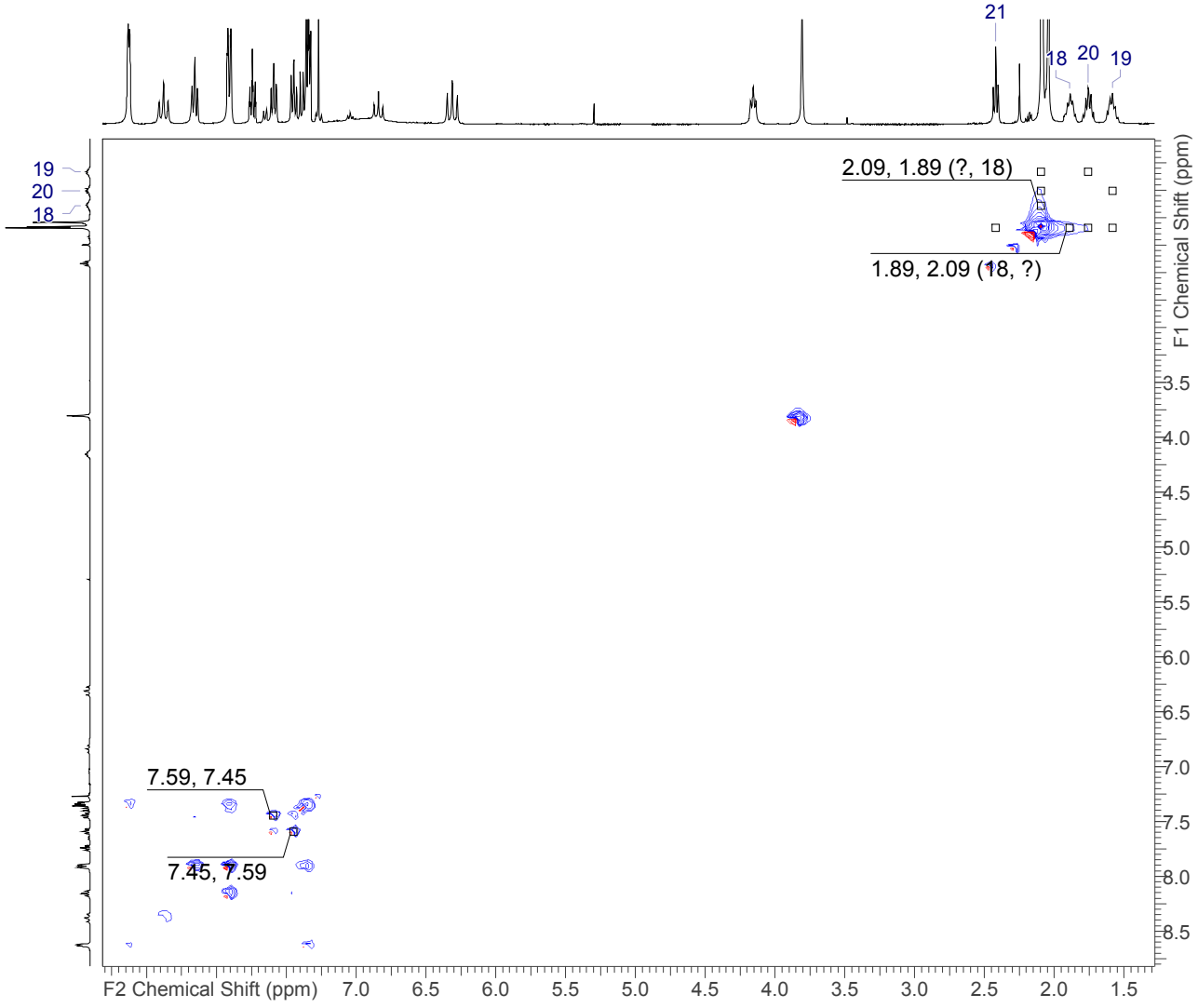


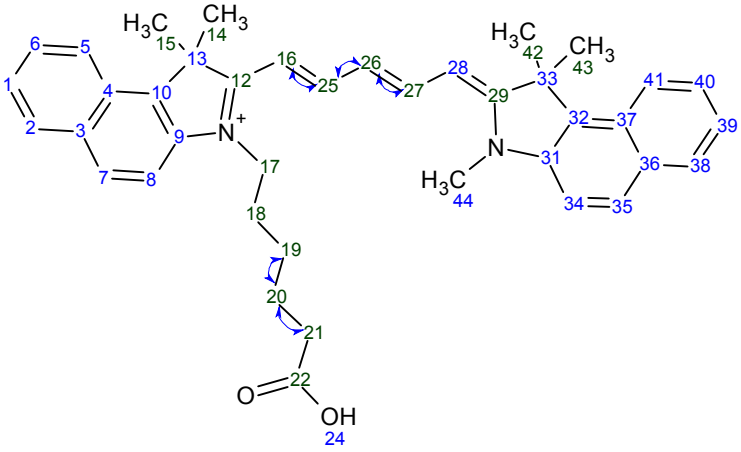
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Date	19 Aug 2014 22:40:00		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\au1914mm2 Cy5.5 PURE\13\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)	***Nucleus***	(1H, 1H)
Number of Transients	2	***Origin***	spect
Original Points Count	(1024, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	cosygpqf
Solvent	CHLOROFORM-d	***Spectrum Type***	COSY
Sweep Width (Hz)	(3996.09, 3996.09)	***Temperature (degree C)***	25.001
Title	mm/		



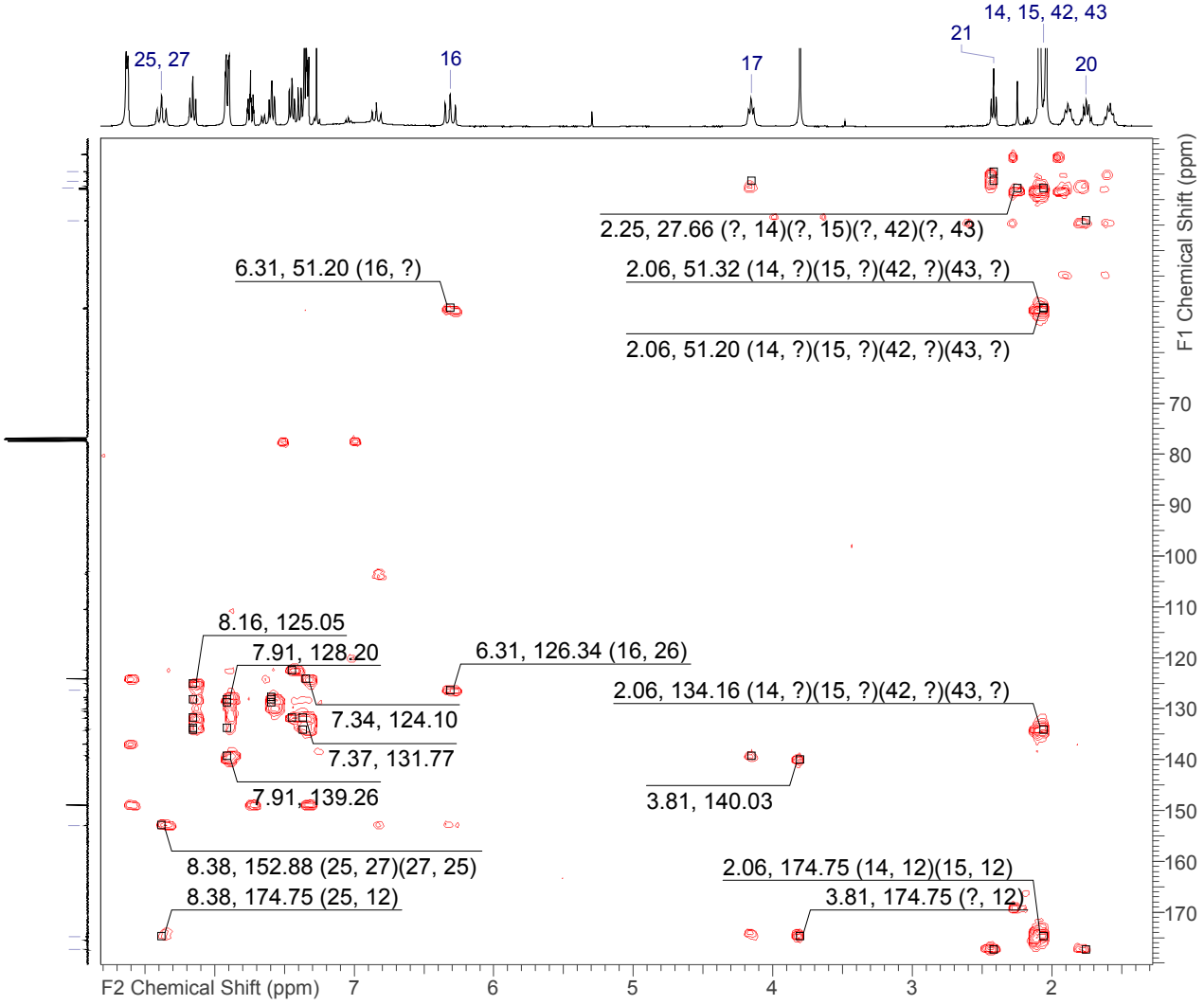


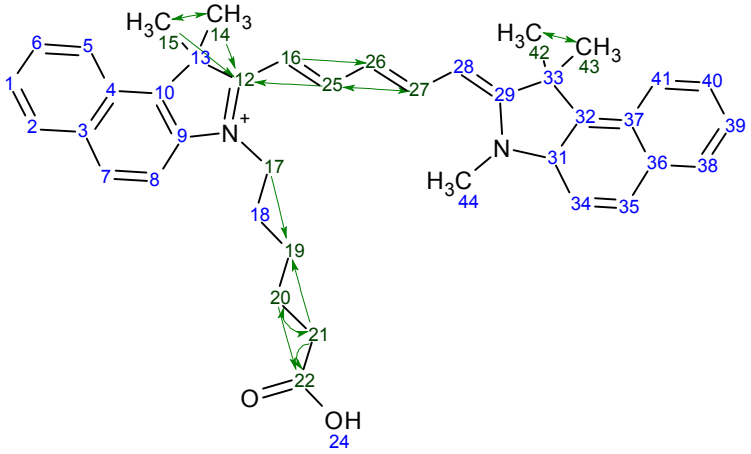
Acquisition Time (sec)	(0.2580, 0.0645)	***Comment***	5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218
Date	19 Aug 2014 23:01:00		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\au1914mm2 Cy5.5 PURE\14\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)	***Nucleus***	(1H, 1H)
Number of Transients	2	***Origin***	spect
Original Points Count	(1024, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	mlevetgp
Solvent	CHLOROFORM-d	***Spectrum Type***	TOCSY
Sweep Width (Hz)	(3964.38, 3964.38)	***Temperature (degree C)***	25.008
Title	mm/		





Acquisition Time (sec)	(0.2580, 0.0115)	***Comment***	5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218
Date	20 Aug 2014 00:04:00		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\au1914mm2 Cy5.5 PURE\17\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)	***Nucleus***	(1H, 13C)
Number of Transients	4	***Origin***	spect
Original Points Count	(1024, 256)	***Owner***	nmr
Points Count	(2048, 1024)	***Pulse Sequence***	hmbcgplpndqf
Solvent	CHLOROFORM-d	***Spectrum Type***	HMBC
Sweep Width (Hz)	(3966.32, 22299.63)	***Temperature (degree C)***	25.010
Title	mm/		





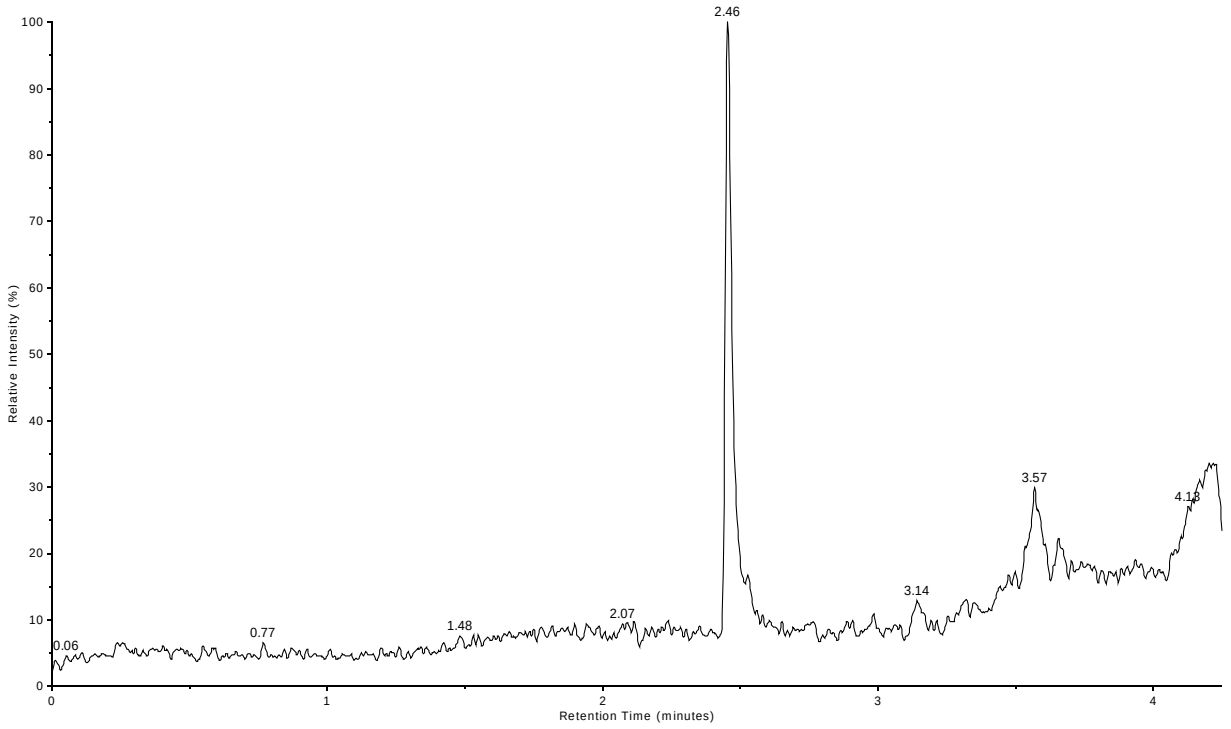
A.11 3-(6-((3-azidopropyl)amino)-6-oxohexyl)-1,1-dimethyl-2-((1E,3E,5E)-5-(1,1,3-trimethyl-1H-benzo[e]indol-2(3H)-ylidene)penta-1,3-dien-1-yl)-1H-benzo[e]indol-3-ium iodide

Top 5 Peak Report

Group: Watts, J
Project: RP-LC MW 150-1500
Experiment: B ESIPOS (simple mix) C18 Acid
Instrument: Blue RP UHPLC-MS (B30:1023)

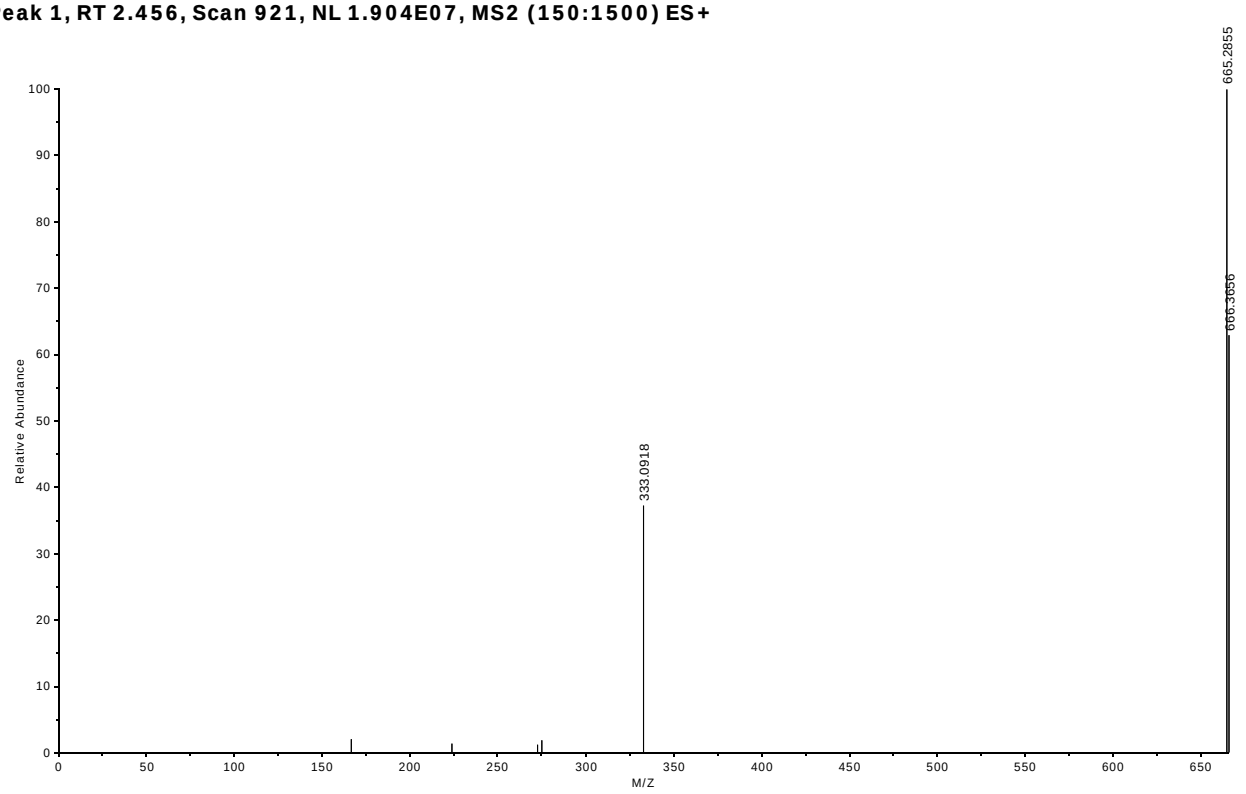
Submitter: Michael Moazami
Sample ID: mm/7316/45 Cy 5.5 azide column
Filename: Watts_J_mm731645_Cy_5_5_azide_column_MM
Acquisition Date: 22/08/2014 14:01:37

TIC, NL 3.827E07, MS2 (150:1500) ES+

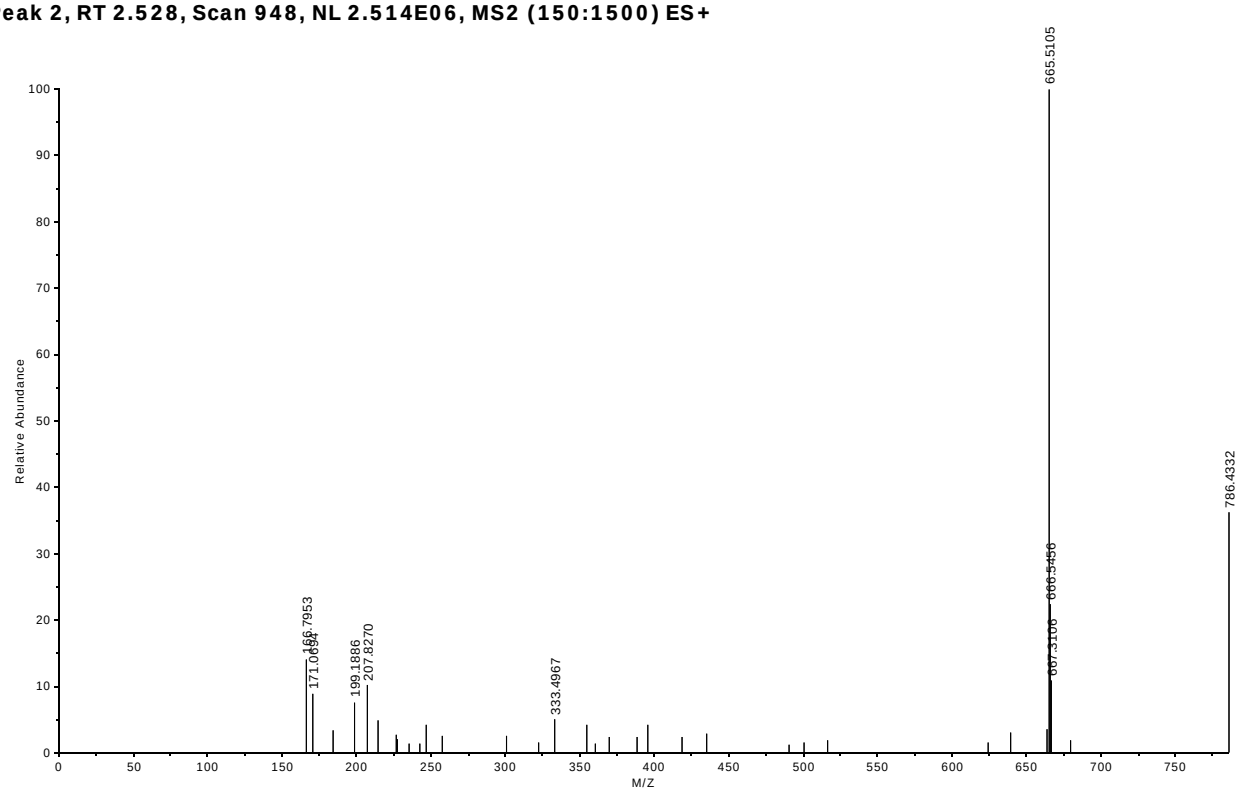


	RT Mins	Height	Height %	Area	Area %
1	2.46	33948599	100.00	66969826	100.00
2	2.53	2206800	6.50	2812907	4.20
3	3.14	2031429	5.98	4014947	6.00
4	3.57	3409555	10.04	4144561	6.19
5	4.13	1832601	5.40	1908325	2.85

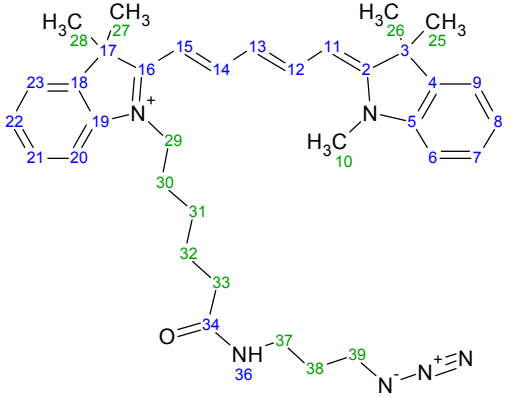
Peak 1, RT 2.456, Scan 921, NL 1.904E07, MS2 (150:1500) ES+



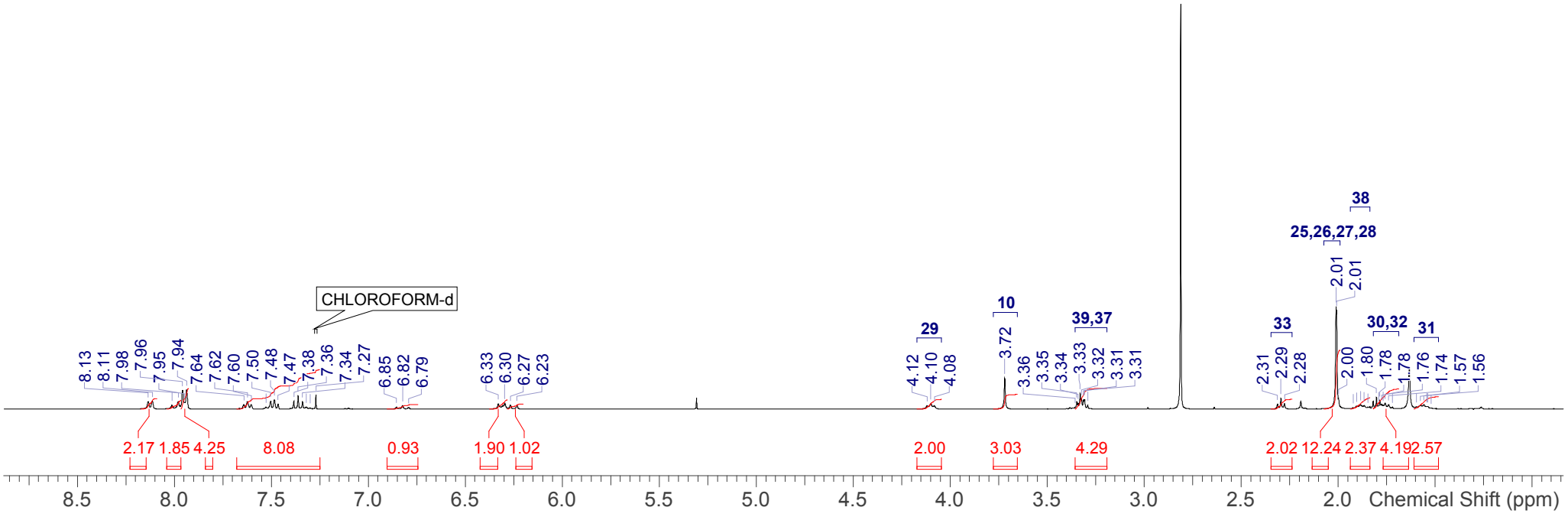
Peak 2, RT 2.528, Scan 948, NL 2.514E06, MS2 (150:1500) ES+



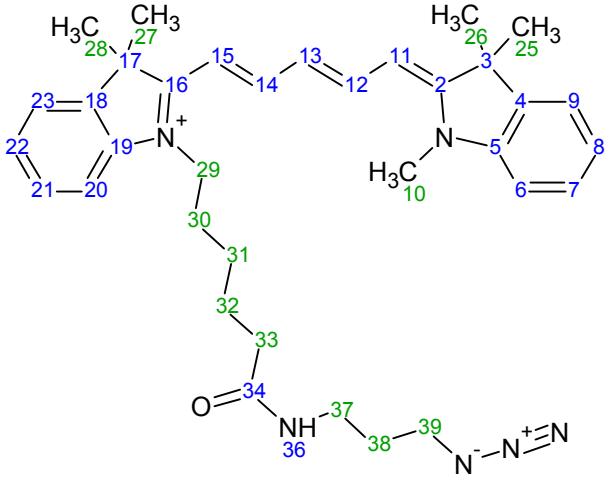
Acquisition Time (sec)	4.0894	***Comment***	mm/7316/45
Date	22 Aug 2014 19:20:37		
Date Stamp	22 Aug 2014 19:20:37		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\au2214mm1 Cy5.5 Azide PURE\au2214mm1 Cy5 Azide PURE\10\PDATA\1\1r		
Frequency (MHz)	400.1300	***Nucleus***	1H
Number of Transients	16	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	65536	***Pulse Sequence***	zg30
Receiver Gain	100.45	***SW(cyclical) (Hz)***	8012.82
Solvent	CHLOROFORM-d	***Spectrum Offset (Hz)***	2465.1934
Spectrum Type	standard	***Sweep Width (Hz)***	8012.70
Temperature (degree C)	25.001		



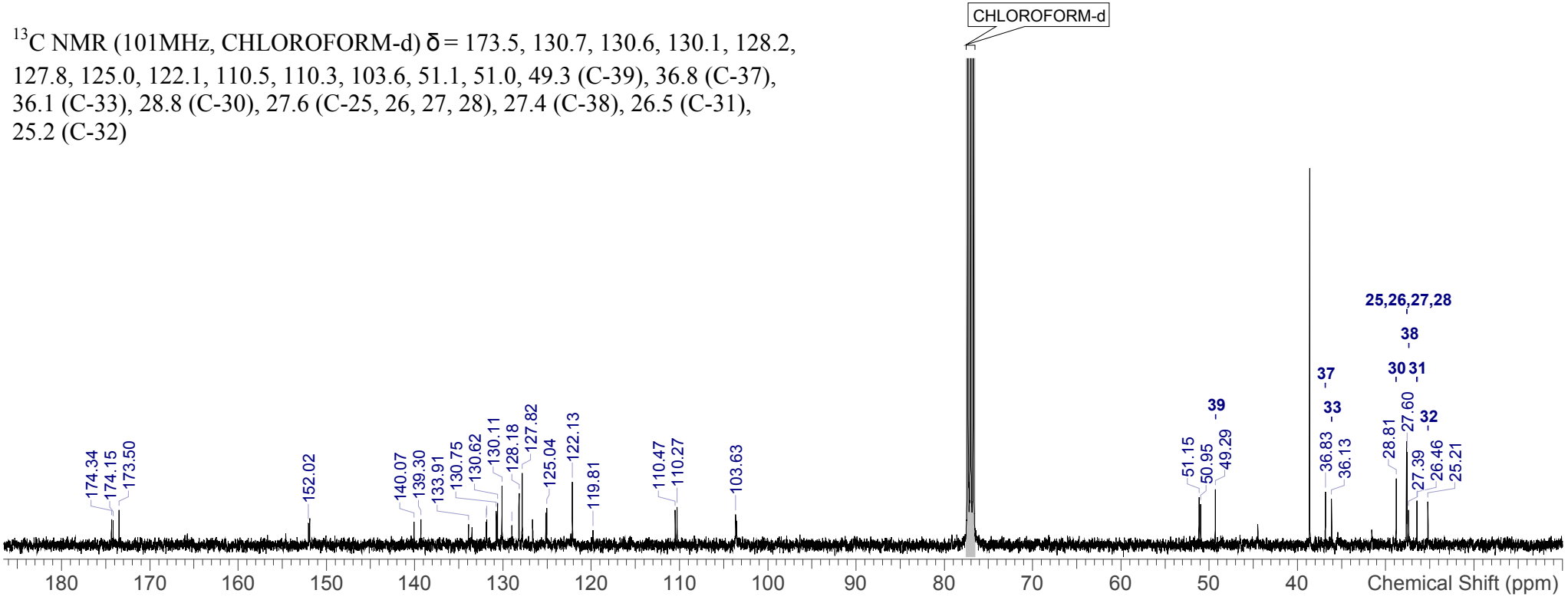
^{1}H NMR (400MHz, CHLOROFORM-d) δ = 8.12 (2H, br d, J = 8.6 Hz, M11), 7.97 - 8.04 (2H, m, M14), 7.93 - 7.96 (4H, m, M15), 7.59 - 7.65 (2H, m, M17), 7.33 - 7.39 (2H, m, M16), 7.25 - 7.68 (2H, m, M12), 6.82 (1H, t, J = 12.5 Hz, M09), 6.31 (2H, br d, J = 13.6 Hz, M13), 6.25 (1H, d, J = 13.7 Hz, M10), 4.10 (2H, br t, J = 7.5 Hz, H-29), 3.72 (3H, s, H-10), 3.19 - 3.36 (4H, m, H-39, 37), 2.29 (2H, t, J = 7.2 Hz, H-33), 1.99 - 2.07 (12H, m, H-25, 26, 27, 28), 1.84 - 1.94 (2H, m, H-38), 1.69 - 1.82 (4H, m, H-30, 32), 1.48 - 1.61 (3H, m, H-31)



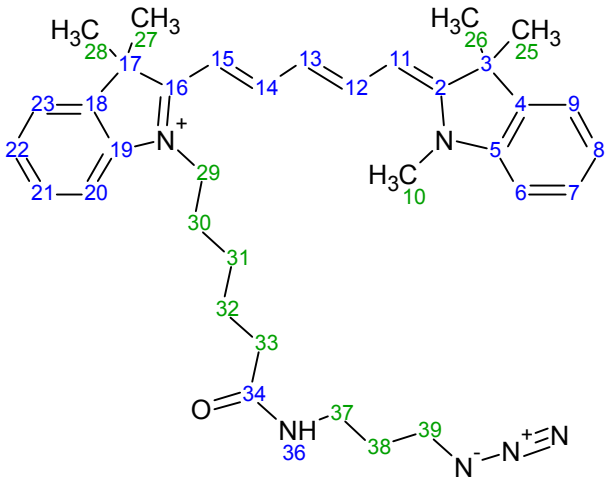
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/45
Date	22 Aug 2014 21:18:42		
Date Stamp	22 Aug 2014 21:18:42		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\au2214mm1 Cy5.5 Azide PURE\au2214mm1 Cy5 Azide PURE\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	2048	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d	***Spectrum Offset (Hz)***	10060.3320
Spectrum Type	standard	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	25.001		



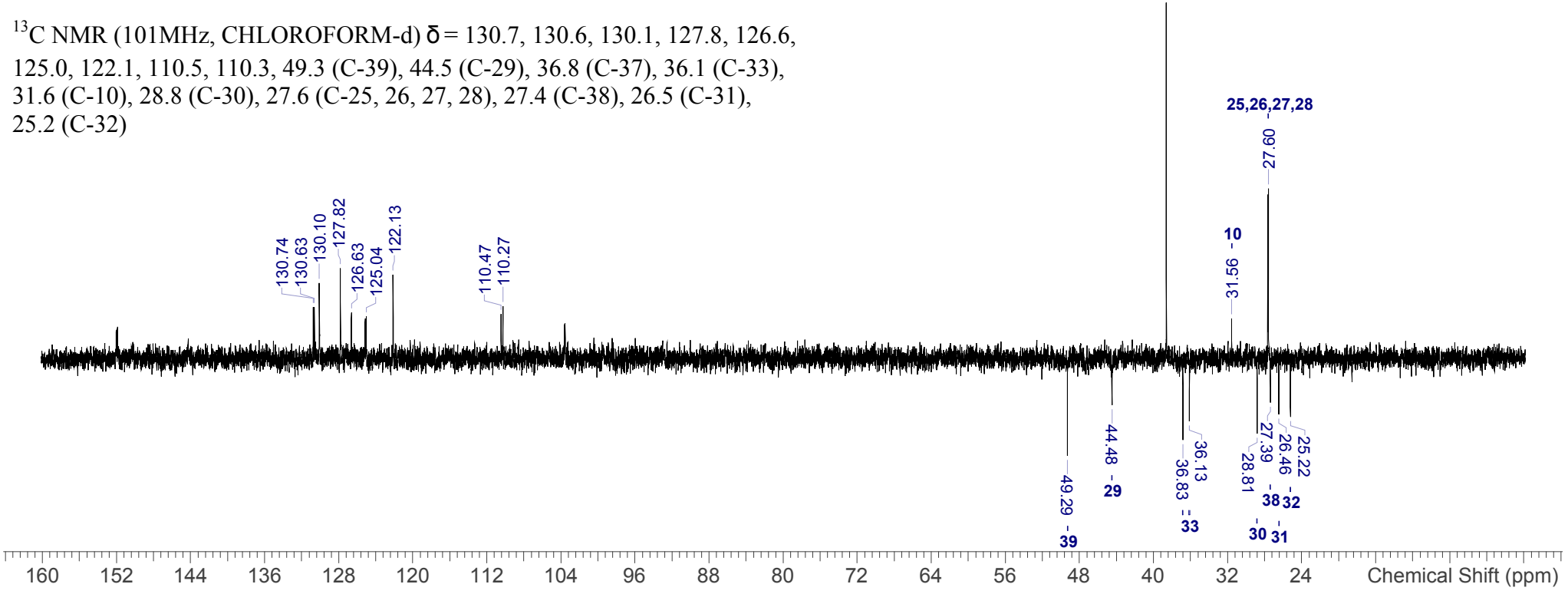
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 173.5, 130.7, 130.6, 130.1, 128.2, 127.8, 125.0, 122.1, 110.5, 110.3, 103.6, 51.1, 51.0, 49.3 (C-39), 36.8 (C-37), 36.1 (C-33), 28.8 (C-30), 27.6 (C-25, 26, 27, 28), 27.4 (C-38), 26.5 (C-31), 25.2 (C-32)



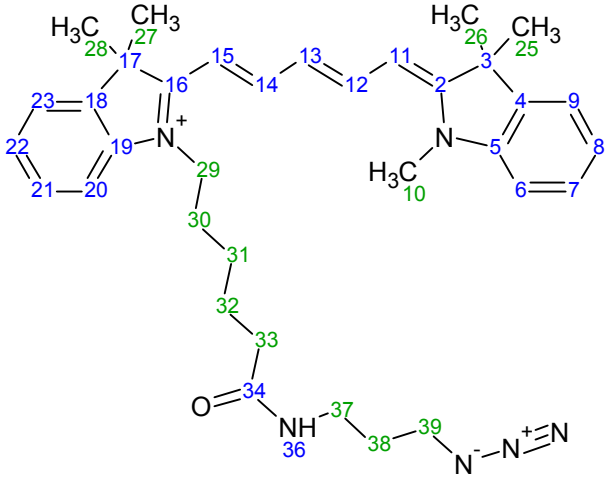
Acquisition Time (sec)	2.0316	***Comment***	mm/7316/45
Date	22 Aug 2014 21:37:19		
Date Stamp	22 Aug 2014 21:37:19		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\au2214mm1 Cy5.5 Azide PURE\au2214mm1 Cy5 Azide PURE\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	CHLOROFORM-d	***Spectrum Offset (Hz)***	8047.9619
Spectrum Type	DEPT135	***Sweep Width (Hz)***	16128.54
Temperature (degree C)	25.001		



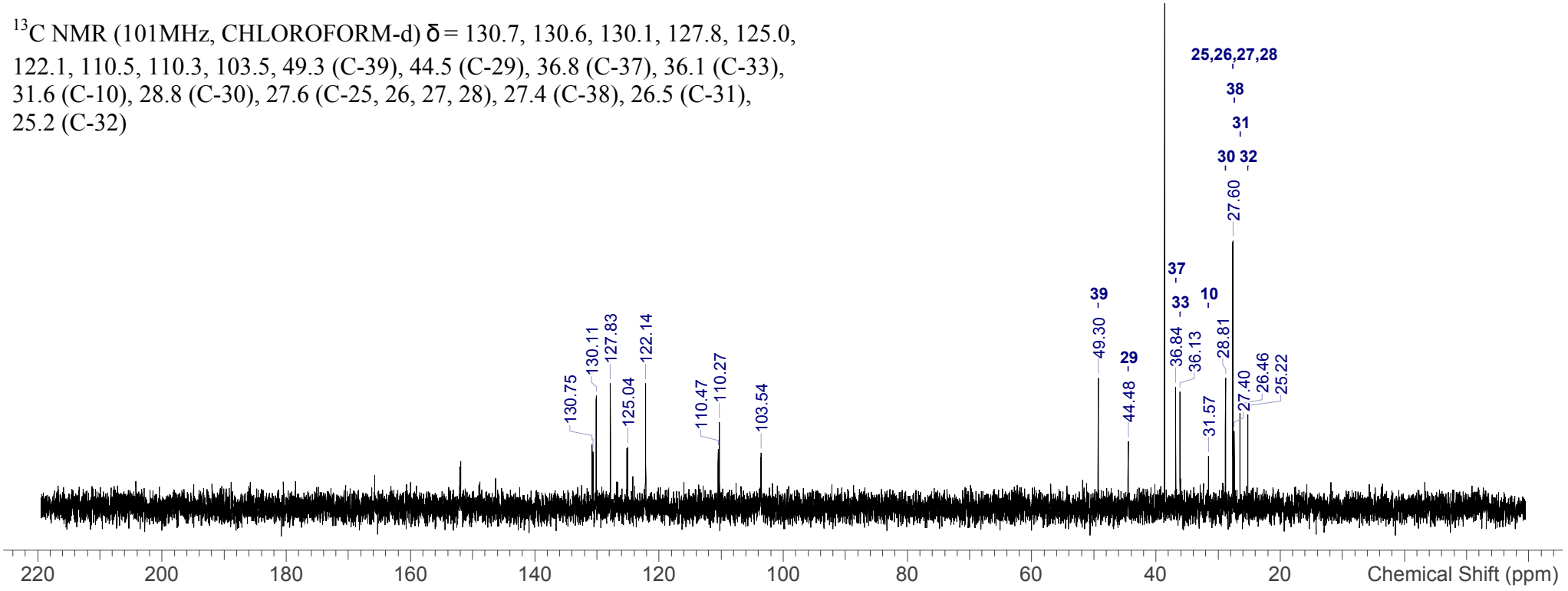
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 130.7, 130.6, 130.1, 127.8, 126.6, 125.0, 122.1, 110.5, 110.3, 49.3 (C-39), 44.5 (C-29), 36.8 (C-37), 36.1 (C-33), 31.6 (C-10), 28.8 (C-30), 27.6 (C-25, 26, 27, 28), 27.4 (C-38), 26.5 (C-31), 25.2 (C-32)



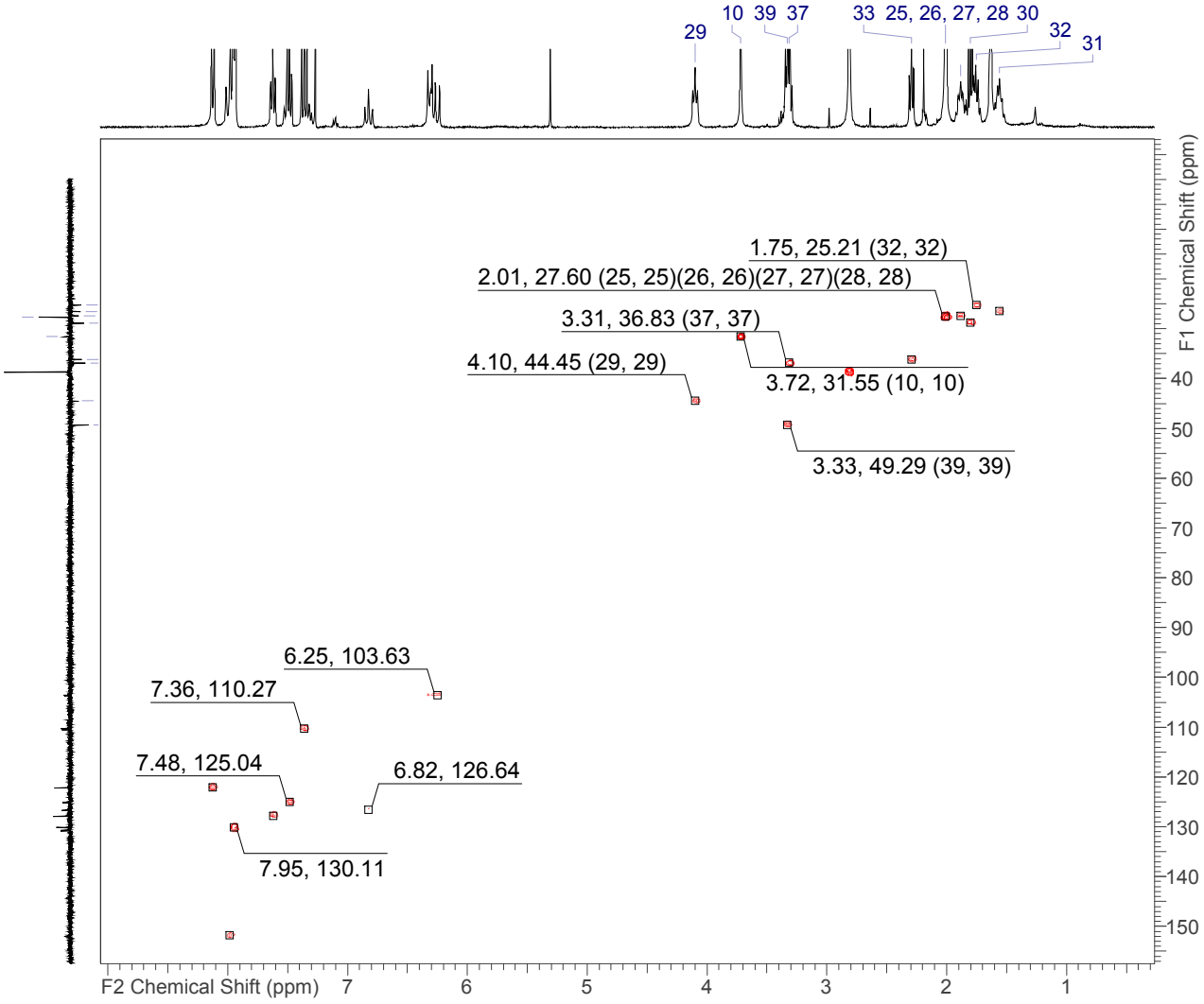
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/45
Date	22 Aug 2014 22:09:14		
Date Stamp	22 Aug 2014 22:09:14		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\au2214mm1 Cy5.5 Azide PURE\au2214mm1 Cy5 Azide PURE\14\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp45
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d	***Spectrum Offset (Hz)***	10061.0684
Spectrum Type	DEPT45	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	25.003		

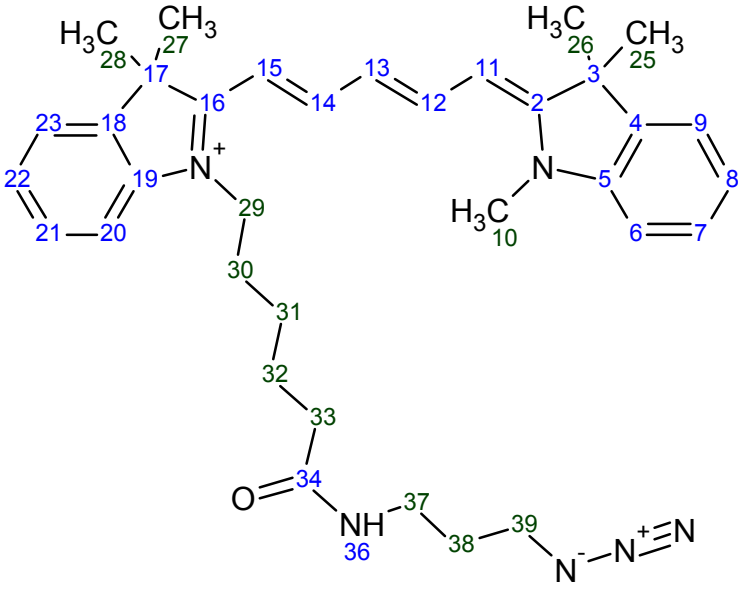


^{13}C NMR (101MHz, CHLOROFORM-d) δ = 130.7, 130.6, 130.1, 127.8, 125.0, 122.1, 110.5, 110.3, 103.5, 49.3 (C-39), 44.5 (C-29), 36.8 (C-37), 36.1 (C-33), 31.6 (C-10), 28.8 (C-30), 27.6 (C-25, 26, 27, 28), 27.4 (C-38), 26.5 (C-31), 25.2 (C-32)

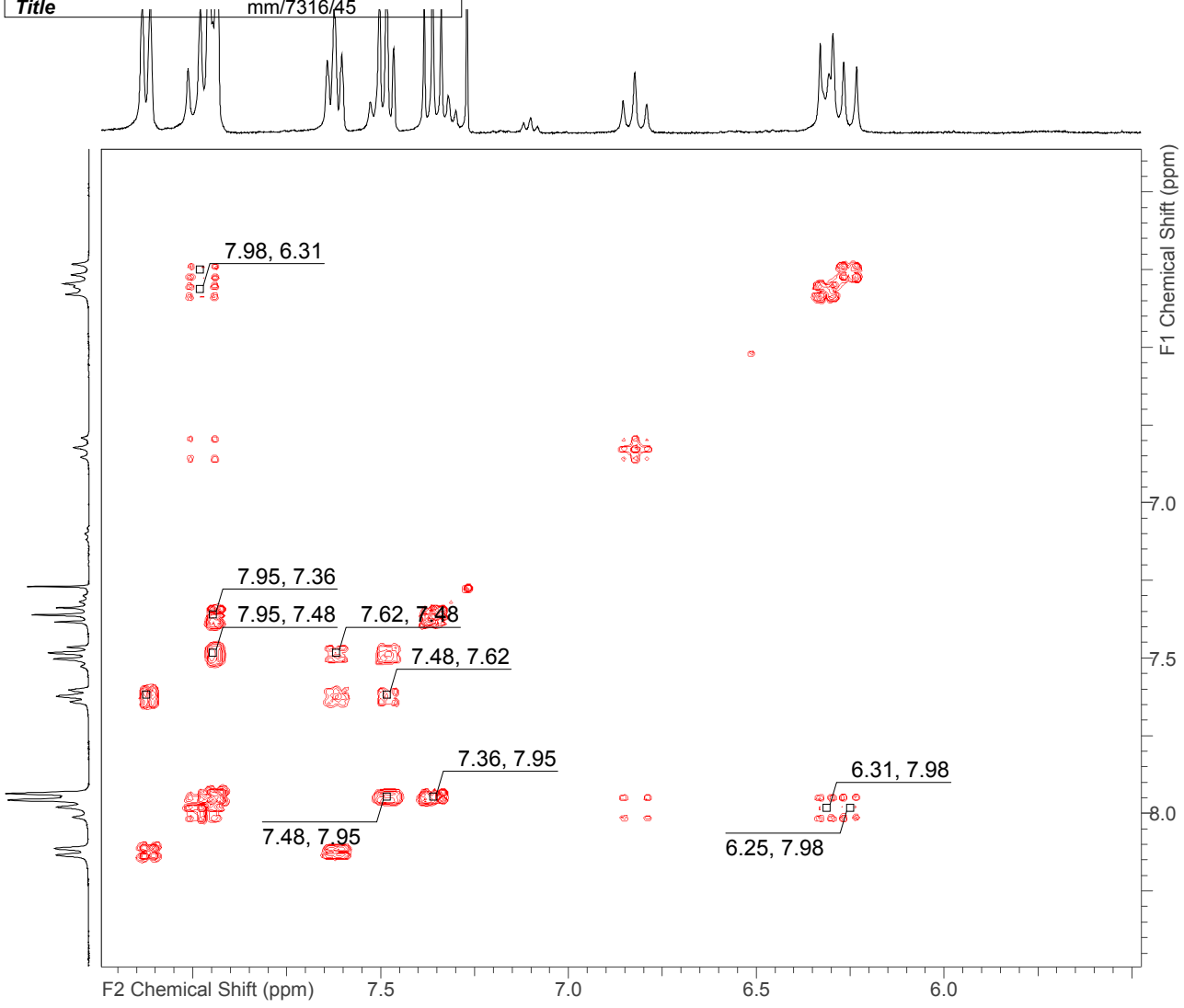


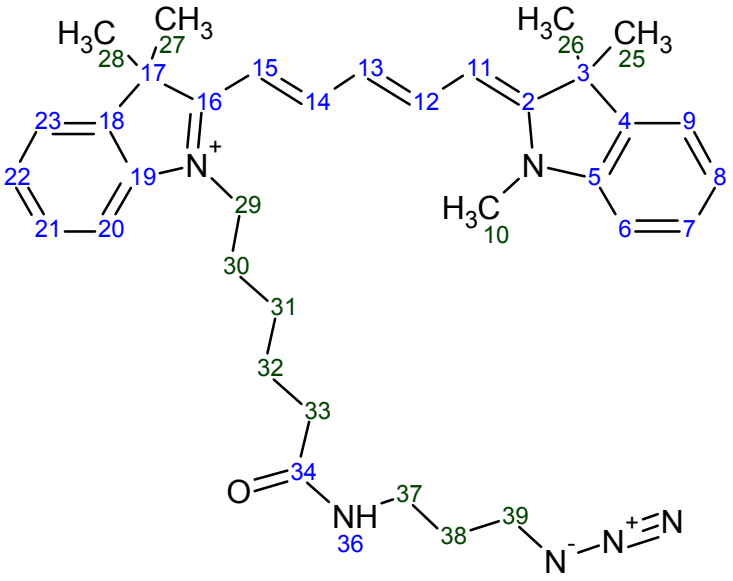
Acquisition Time (sec)	(0.1454, 0.0154)	***Comment***	mm/7316/45
Date	26 Aug 2014 12:50:48		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\au2214mm1 Cy5.5 Azide PURE\au2214mm1 Cy5 Azide PURE\15\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)	***Nucleus***	(1H, 13C)
Number of Transients	2	***Origin***	spect
Original Points Count	(512, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	hsqcetgp
Solvent	CHLOROFORM-d	***Spectrum Type***	HSQC
Sweep Width (Hz)	(3517.69, 16650.39)	***Temperature (degree C)***	25.069
Title	mm/7316/45		





Acquisition Time (sec)	(0.2908, 0.0727)	***Comment***	mm/7316/45
Date	26 Aug 2014 12:50:46		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\au2214mm1 Cy5.5 Azide PURE\au2214mm1 Cy5 Azide PURE\13\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)	***Nucleus***	(1H, 1H)
Number of Transients	2	***Origin***	spect
Original Points Count	(1024, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	cosygpqf
Solvent	CHLOROFORM-d	***Spectrum Type***	COSY
Sweep Width (Hz)	(3517.69, 3517.69)	***Temperature (degree C)***	25.000
Title	mm/7316/45		



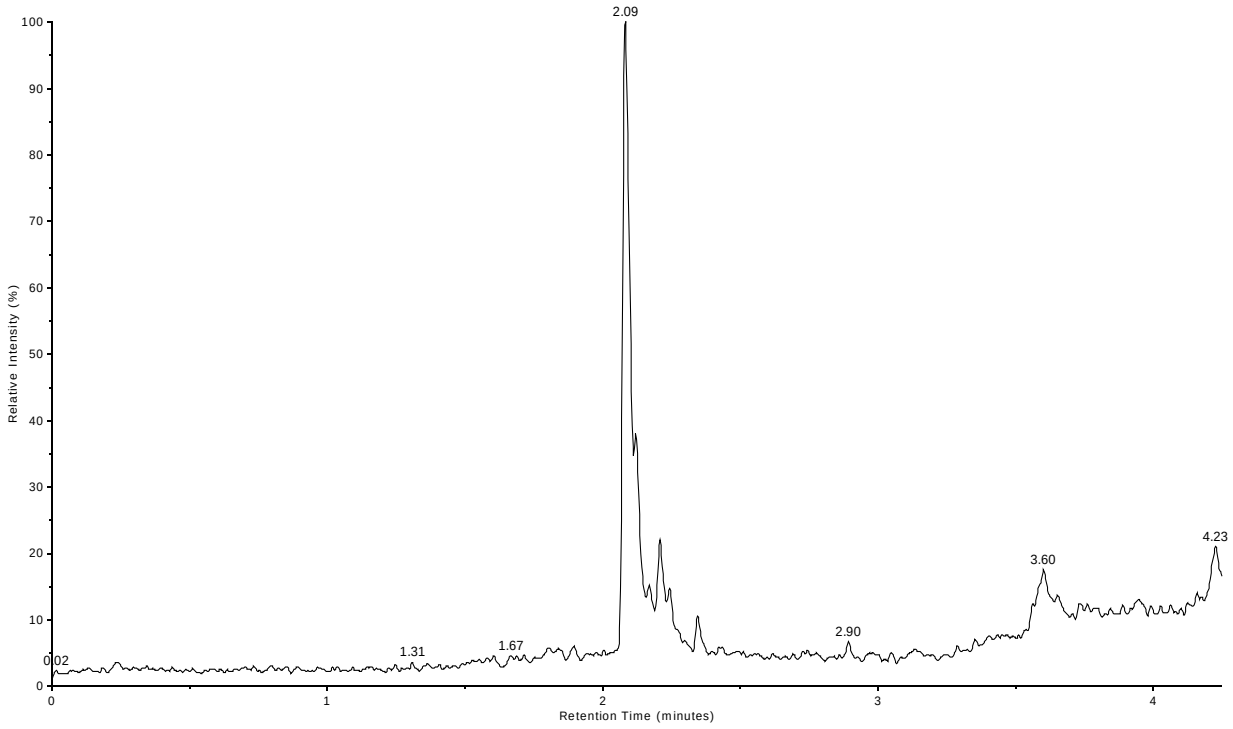


A.12 1-(6-((3-azidopropyl)amino)-6-oxohexyl)-2-((1E,3E)-3-(1,3,3-trimethylindolin-2-ylidene)prop-1-en-1-yl)-3H-indol-1-ium bromide

Top 5 Peak Report

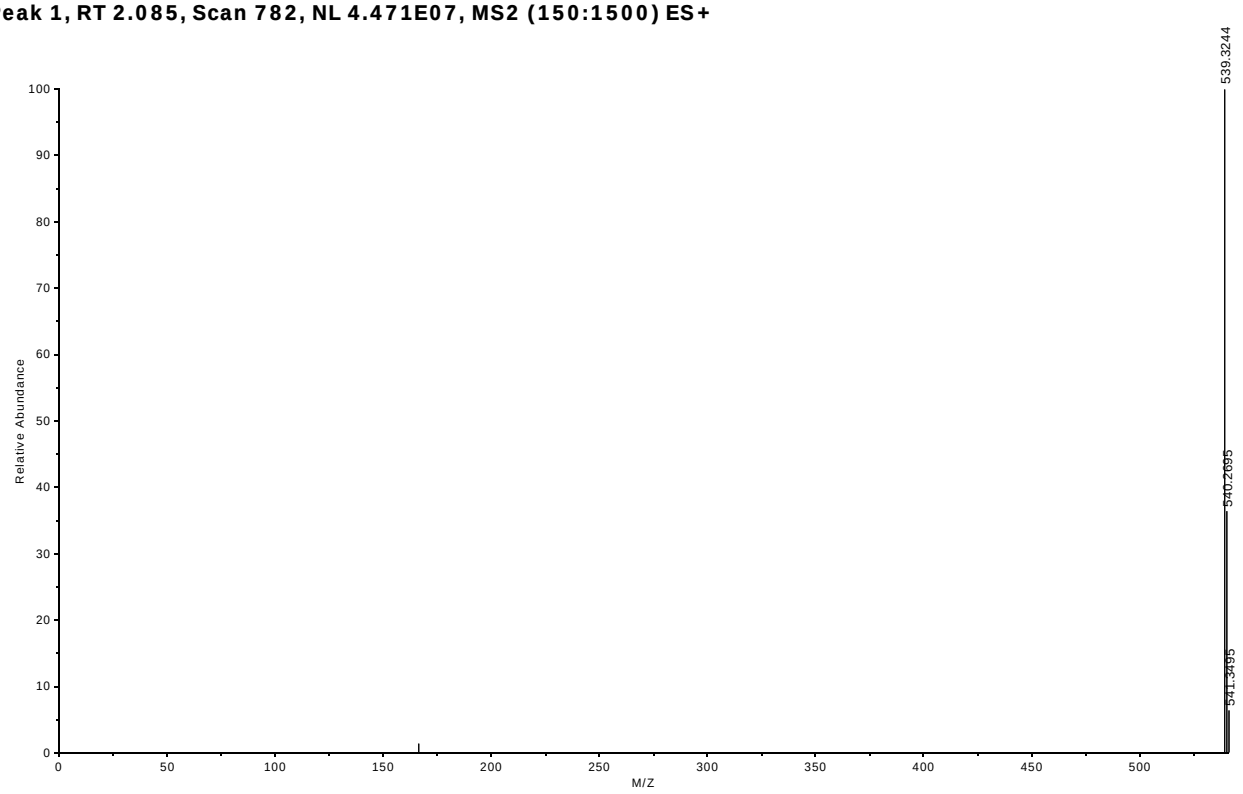
Group: Watts, J
Submitter: Michael Moazami
Project: RP-LC MW 150-1500
Sample ID: mm/7316/46 Cy3 azide column
Experiment: B ESIPOS (simple mix) C18 Acid
Filename: Watts_J_mm731646_Cy3_azide_column_MM_41
Instrument: Blue RP UHPLC-MS (B30:1023)
Acquisition Date: 26/08/2014 12:20:43

TIC, NL 7.907E07, MS2 (150:1500) ES+

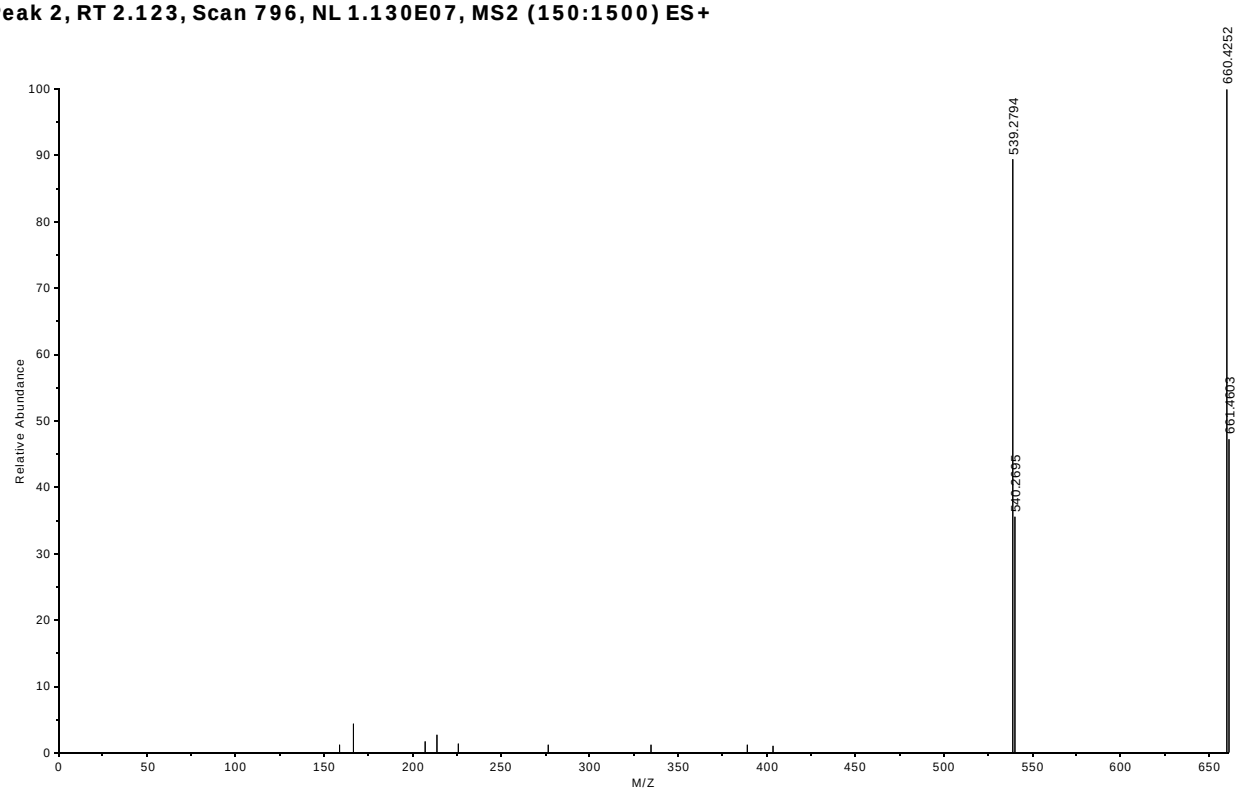


	RT Mins	Height	Height %	Area	Area %
1	2.09	63364064	100.00	137188639	100.00
2	2.12	19532824	30.83	25435334	18.54
3	2.21	7898224	12.46	10639997	7.76
4	2.25	6460459	10.20	10365501	7.56
5	4.23	5014146	7.91	13403260	9.77

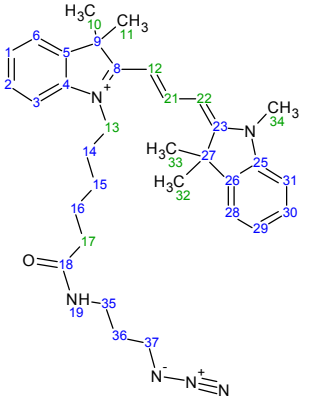
Peak 1, RT 2.085, Scan 782, NL 4.471E07, MS2 (150:1500) ES+



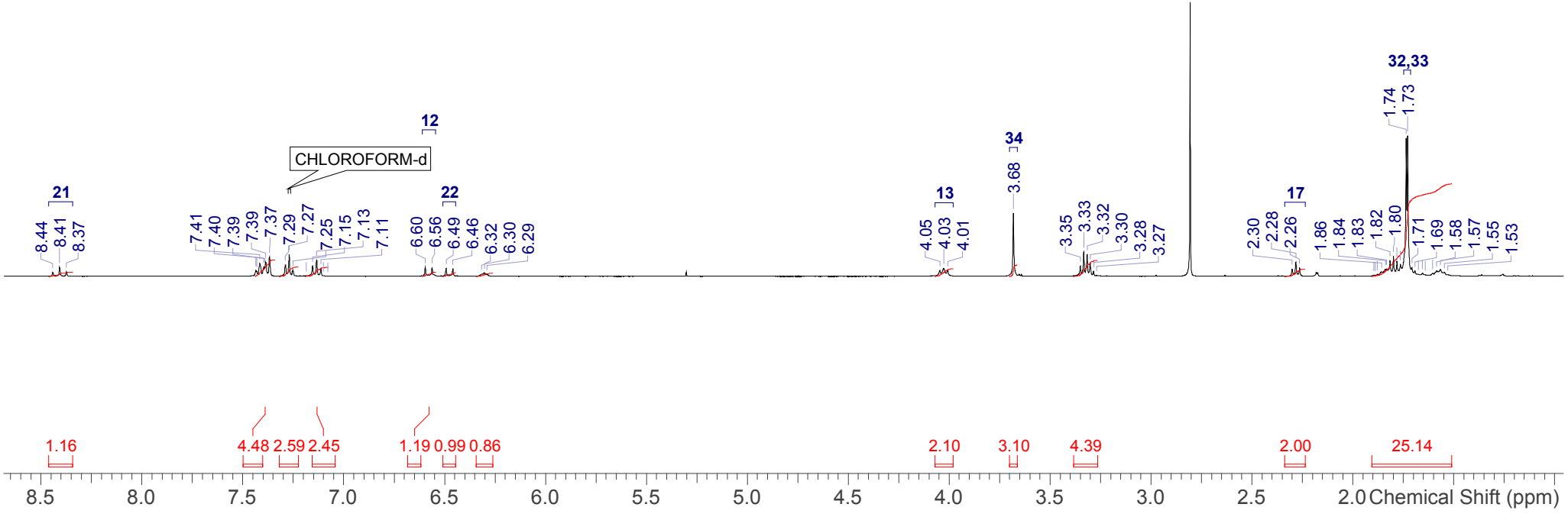
Peak 2, RT 2.123, Scan 796, NL 1.130E07, MS2 (150:1500) ES+



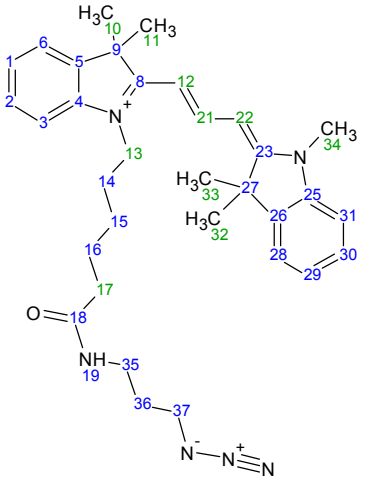
Acquisition Time (sec)	4.0894	***Comment***	mm/7316/46
Date	22 Aug 2014 22:48:10		
Date Stamp	22 Aug 2014 22:48:10		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\au2214mm2 Cy3 Azide PURE\10\PDATA\1\1r		
Frequency (MHz)	400.1300	***Nucleus***	1H
Number of Transients	16	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	262144	***Pulse Sequence***	zg30
Receiver Gain	64.46	***SW(cyclical) (Hz)***	8012.82
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	2465.0029	***Spectrum Type***	standard
Sweep Width (Hz)	8012.79	***Temperature (degree C)***	25.003



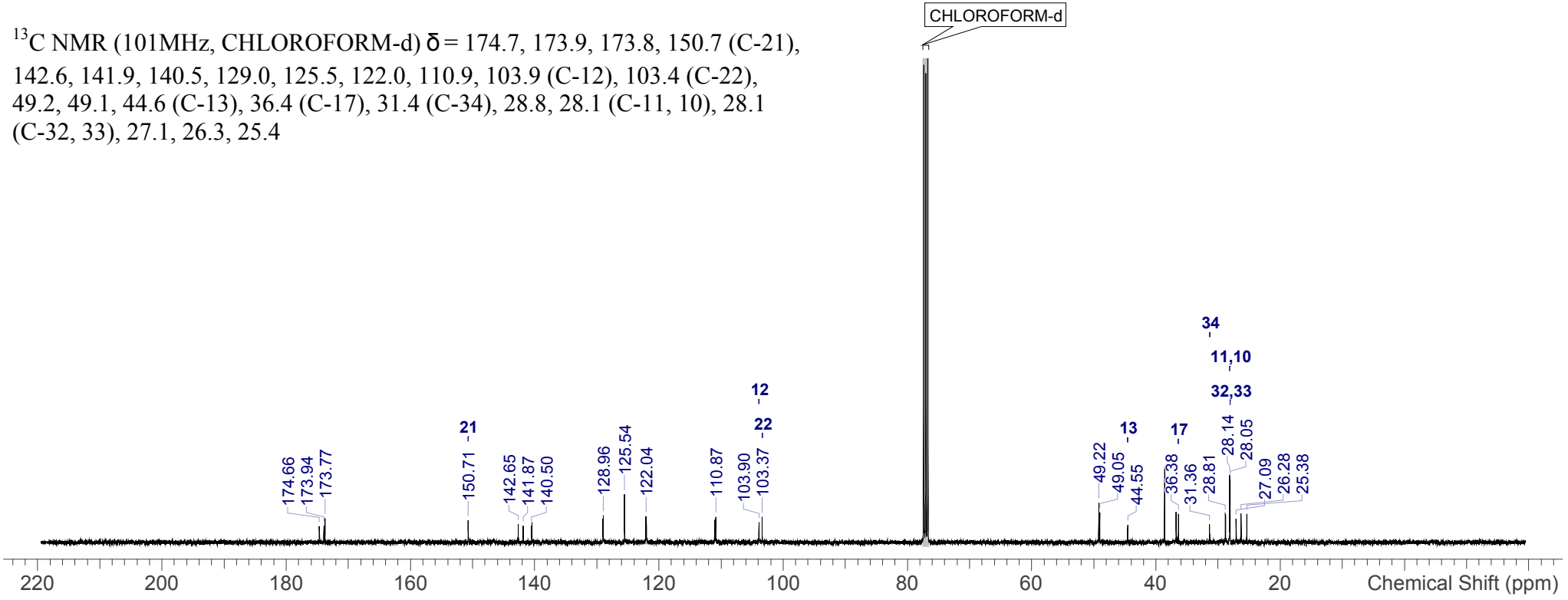
^{1}H NMR (400MHz, CHLOROFORM-d) δ = 8.41 (1H, t, J = 13.5 Hz, H-21), 7.34 - 7.44 (4H, m, M12), 7.23 - 7.32 (3H, m, M13), 7.08 - 7.19 (2H, m, M11), 6.58 (1H, d, J = 13.5 Hz, H-12), 6.48 (1H, d, J = 13.5 Hz, H-22), 6.30 (1H, br t, J = 5.5 Hz, M08), 3.98 - 4.07 (2H, m, H-13), 3.68 (3H, s, H-34), 3.27 - 3.38 (4H, m, M05), 2.28 (2H, t, J = 7.3 Hz, H-17), 1.72 - 1.75 (15H, m, H-32, 33), 1.51 - 1.91 (8H, m, M06)



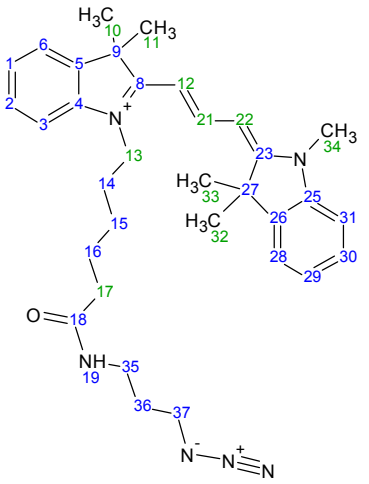
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/46
Date	22 Aug 2014 23:18:46		
Date Stamp	22 Aug 2014 23:18:46		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\au2214mm2 Cy3 Azide PURE\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	10060.3320	***Spectrum Type***	standard
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	25.000



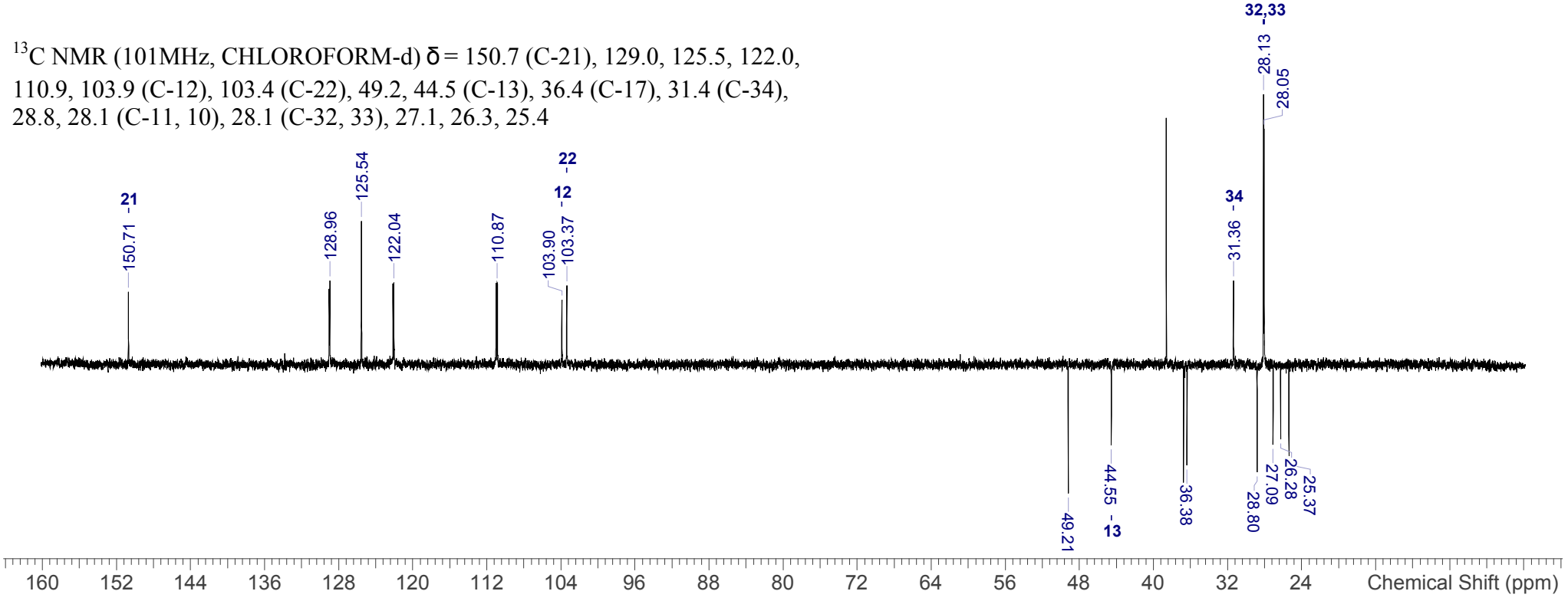
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 174.7, 173.9, 173.8, 150.7 (C-21), 142.6, 141.9, 140.5, 129.0, 125.5, 122.0, 110.9, 103.9 (C-12), 103.4 (C-22), 49.2, 49.1, 44.6 (C-13), 36.4 (C-17), 31.4 (C-34), 28.8, 28.1 (C-11, 10), 28.1 (C-32, 33), 27.1, 26.3, 25.4



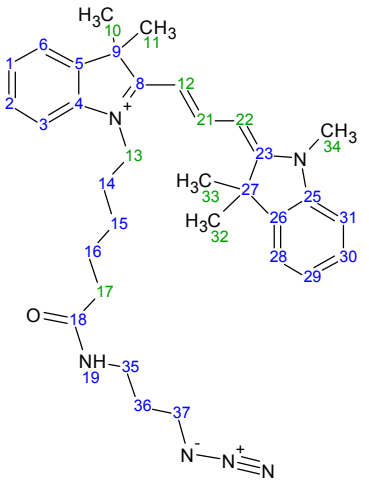
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Date	22 Aug 2014 23:37:23		
Date Stamp	22 Aug 2014 23:37:23		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\au2214mm2 Cy3 Azide PURE\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	CHLOROFORM-d	***Spectrum Offset (Hz)***	8047.9146
Spectrum Type	DEPT135	***Sweep Width (Hz)***	16128.54
Temperature (degree C)	25.000		



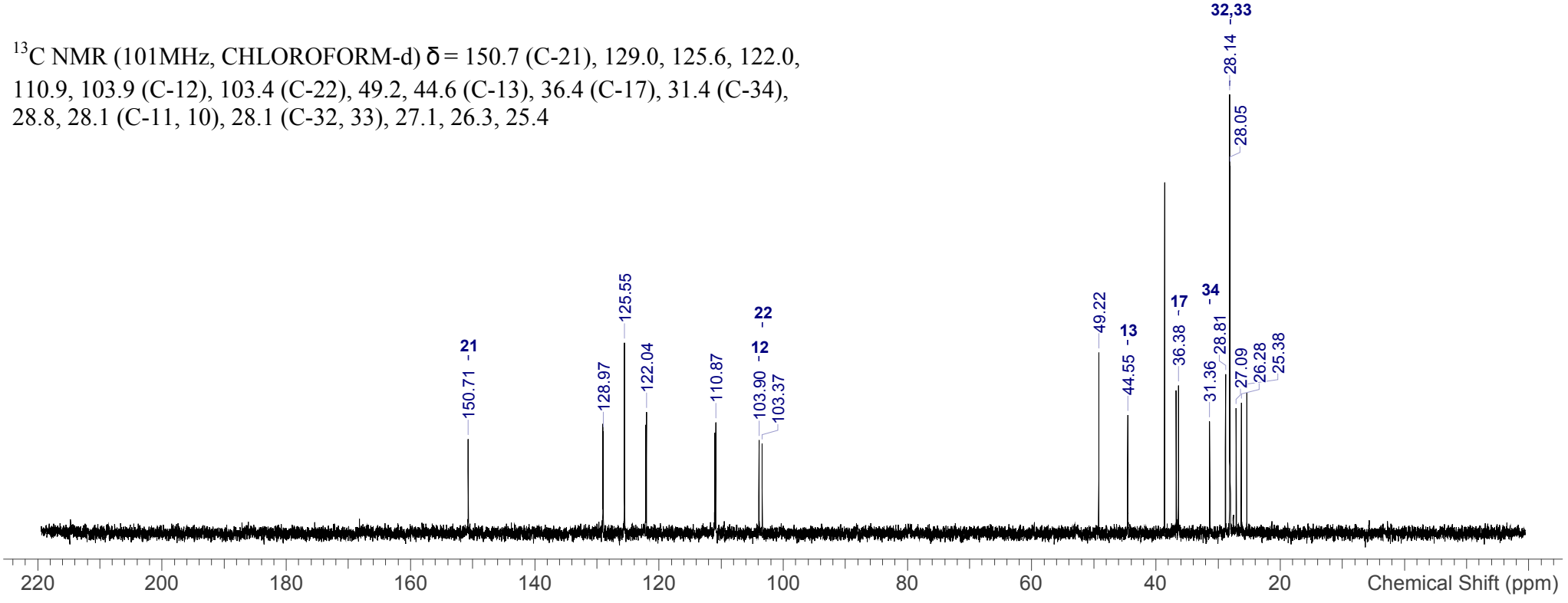
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 150.7 (C-21), 129.0, 125.5, 122.0, 110.9, 103.9 (C-12), 103.4 (C-22), 49.2, 44.5 (C-13), 36.4 (C-17), 31.4 (C-34), 28.8, 28.1 (C-11, 10), 28.1 (C-32, 33), 27.1, 26.3, 25.4



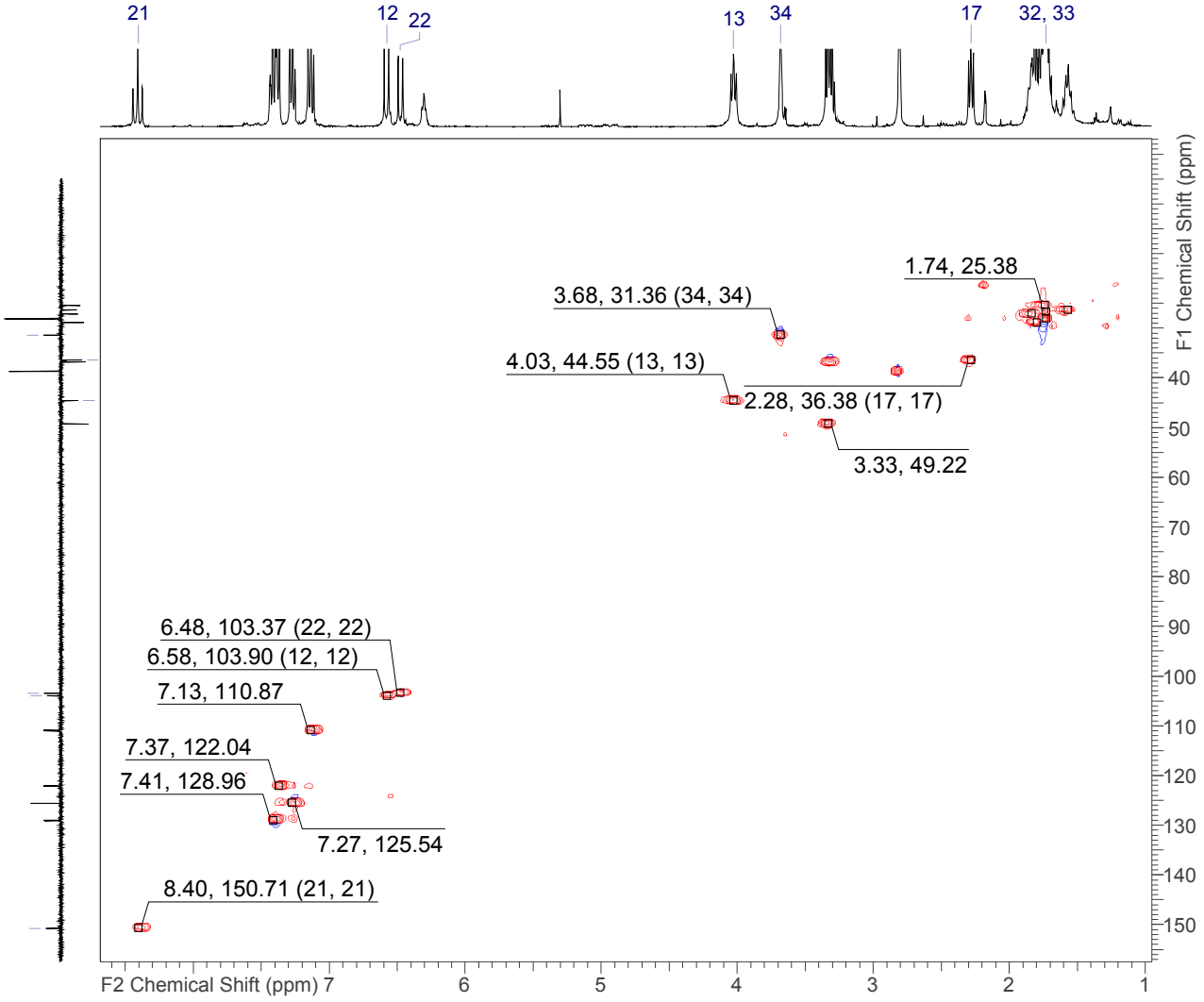
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/46
Date	23 Aug 2014 00:09:19		
Date Stamp	23 Aug 2014 00:09:19		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\au2214mm2 Cy3 Azide PURE\14\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp45
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	10061.0684	***Spectrum Type***	DEPT45
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	25.003

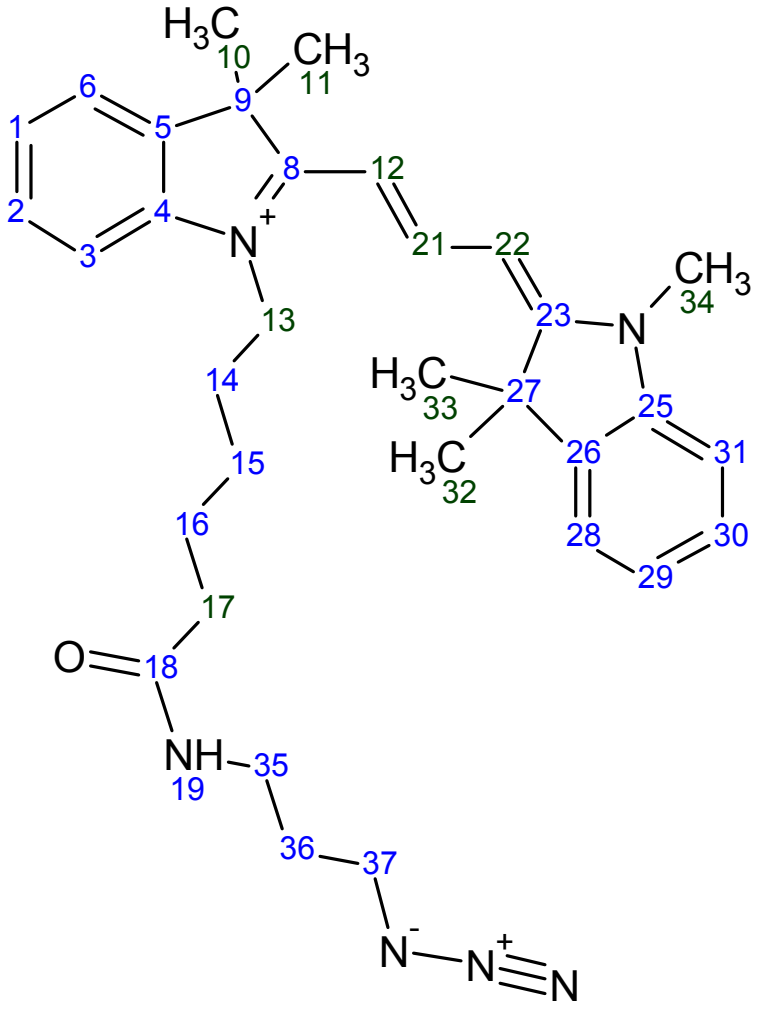


^{13}C NMR (101MHz, CHLOROFORM-d) δ = 150.7 (C-21), 129.0, 125.6, 122.0, 110.9, 103.9 (C-12), 103.4 (C-22), 49.2, 44.6 (C-13), 36.4 (C-17), 31.4 (C-34), 28.8, 28.1 (C-11, 10), 28.1 (C-32, 33), 27.1, 26.3, 25.4

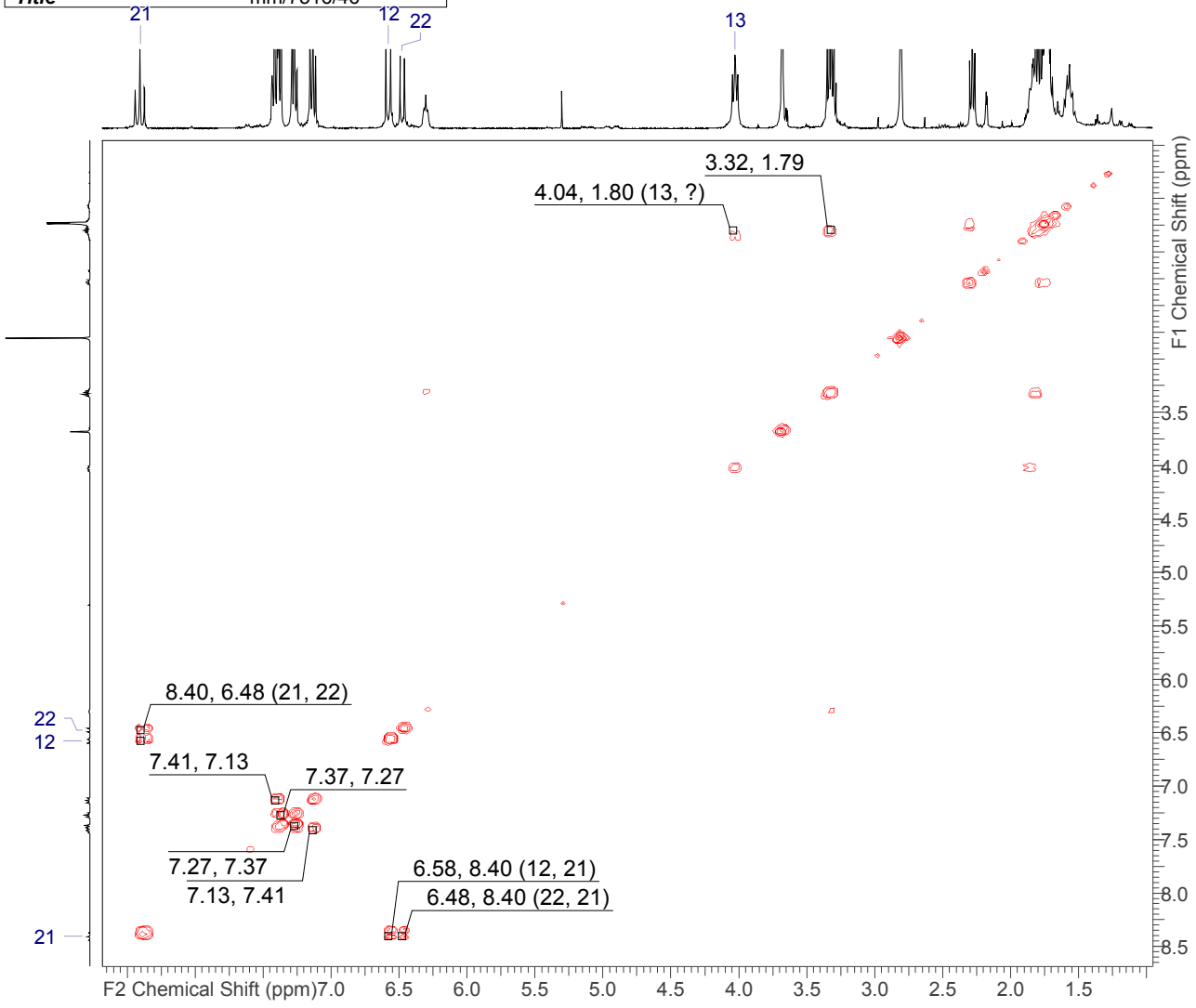


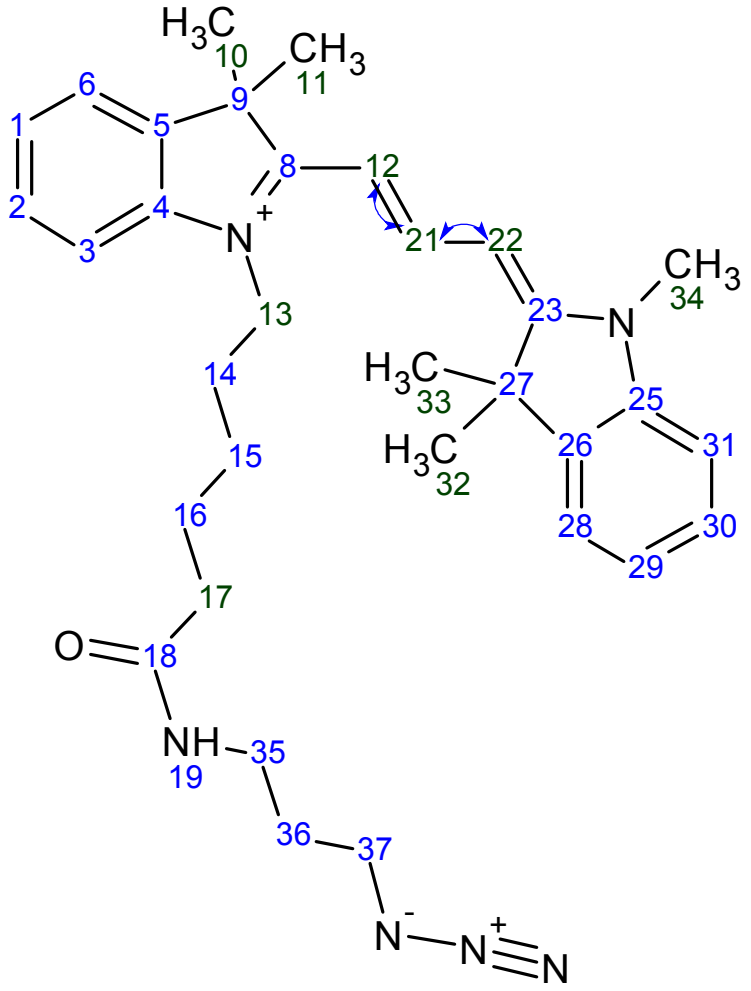
Acquisition Time (sec)	(0.1382, 0.0154)	***Comment***	mm/7316/46
Date	22 Aug 2014 23:24:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\au2214mm2 Cy3 Azide PURE\15\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)	***Nucleus***	(1H, 13C)
Number of Transients	2	***Origin***	spect
Original Points Count	(512, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	hsqcetgp
Solvent	CHLOROFORM-d	***Spectrum Type***	HSQC
Sweep Width (Hz)	(3700.09, 16650.39)	***Temperature (degree C)***	25.063
Title	mm/7316/46		





Acquisition Time (sec)	(0.2765, 0.0691)	***Comment***	mm/7316/46
Date	22 Aug 2014 22:53:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\au2214mm2 Cy3 Azide PURE\13\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)	***Nucleus***	(1H, 1H)
Number of Transients	2	***Origin***	spect
Original Points Count	(1024, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	cosygpqf
Solvent	CHLOROFORM-d	***Spectrum Type***	COSY
Sweep Width (Hz)	(3700.09, 3700.09)	***Temperature (degree C)***	25.000
Title	mm/7316/46		



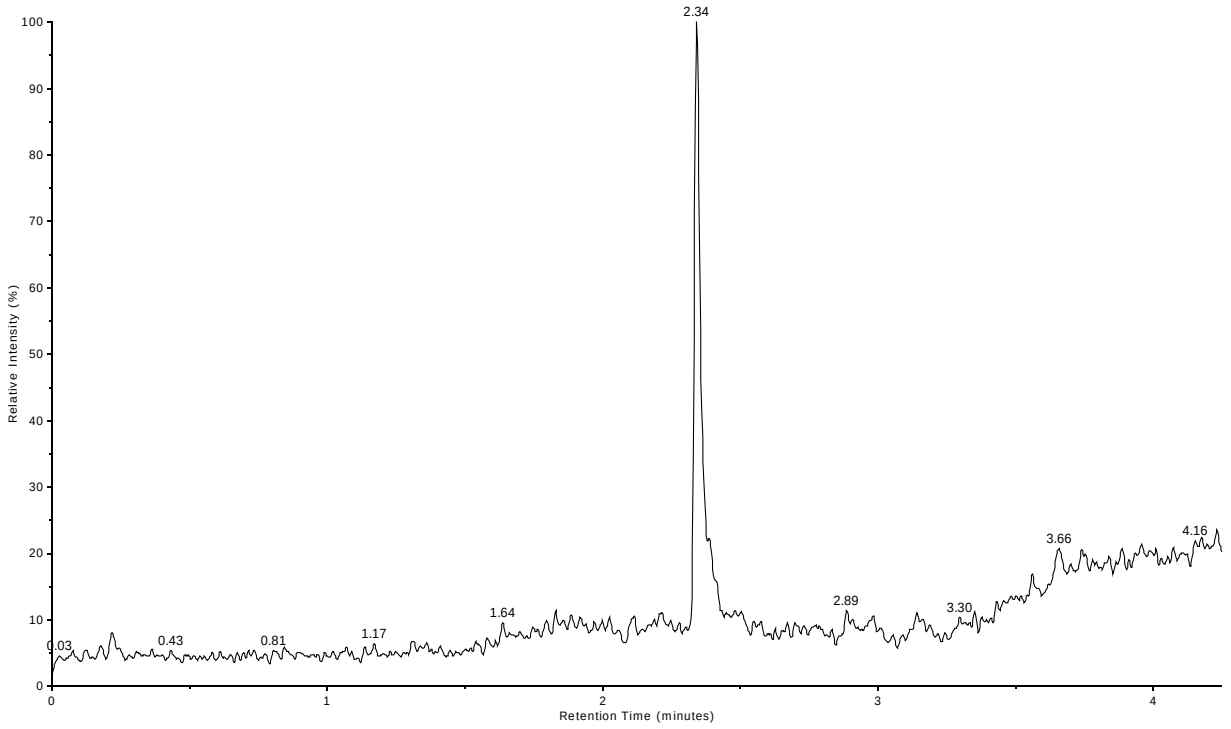


A.13 2-((1E,3Z)-3-(3-(5-carboxypentyl)-1,1-dimethyl-1H-benzo[e]indol-2(3H)-ylidene)prop-1-en-1-yl)-1,1,3-trimethyl-1H-benzo[e]indol-3-ium iodide

Top 5 Peak Report

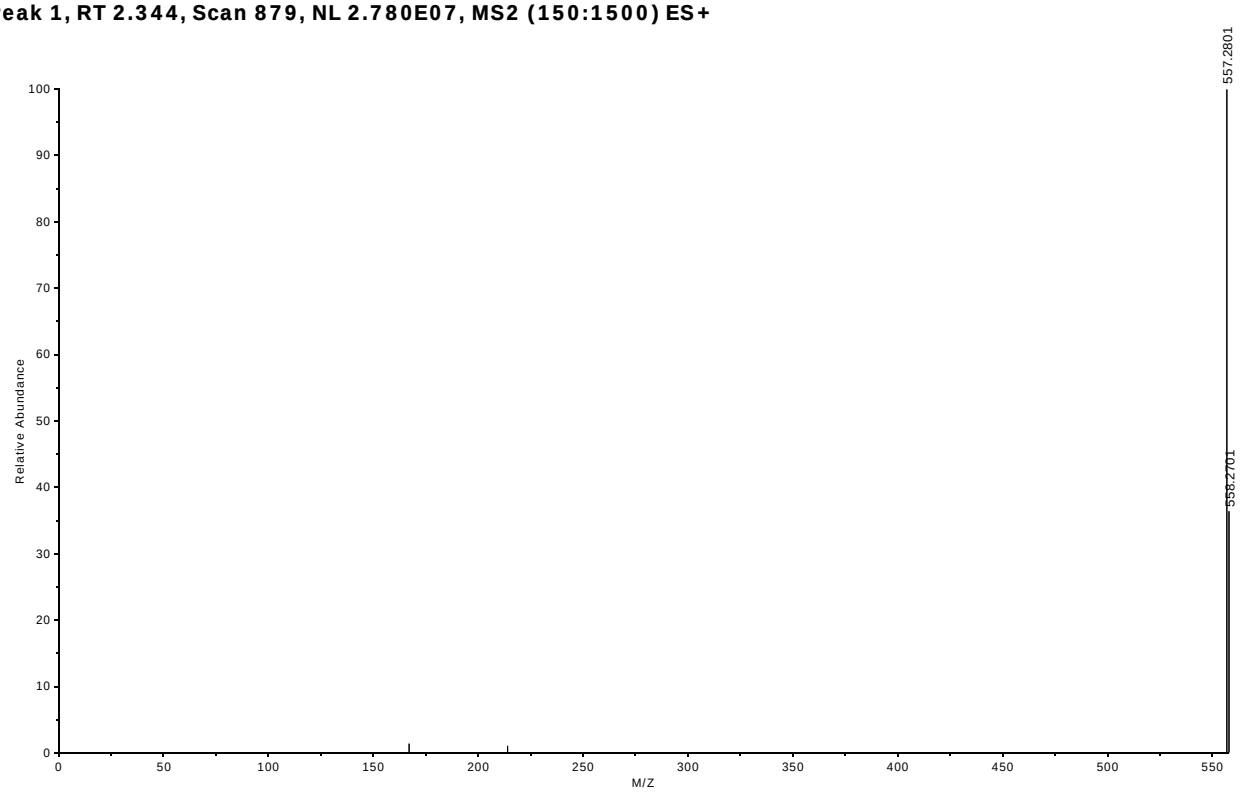
Group: Watts, J
Submitter: Michael Moazami
Project: RP-LC MW 150-1500
Sample ID: mm/7316/48 1st crop Cy3.5
Experiment: B ESIPOS (simple mix) C18 Acid
Filename: Watts_J_mm731648_1st_crop_Cy3_5_MM_4009
Instrument: Blue RP UHPLC-MS (B30:1023)
Acquisition Date: 27/08/2014 15:14:20

TIC, NL 3.787E07, MS2 (150:1500) ES+

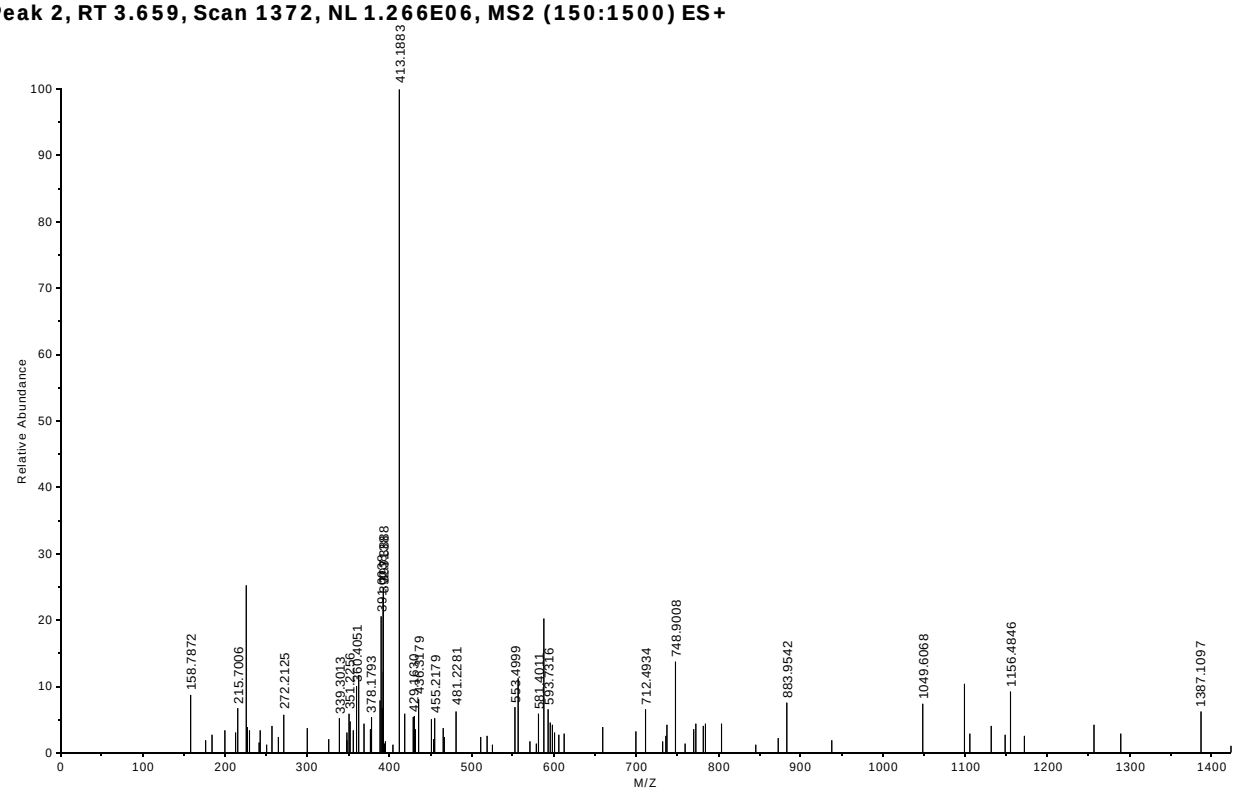


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2	3.66	1762158	5.48	4425042	7.65

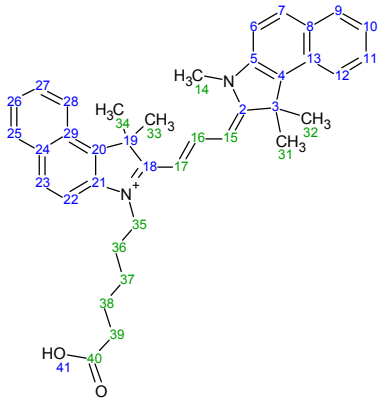
Peak 1, RT 2.344, Scan 879, NL 2.780E07, MS2 (150:1500) ES+



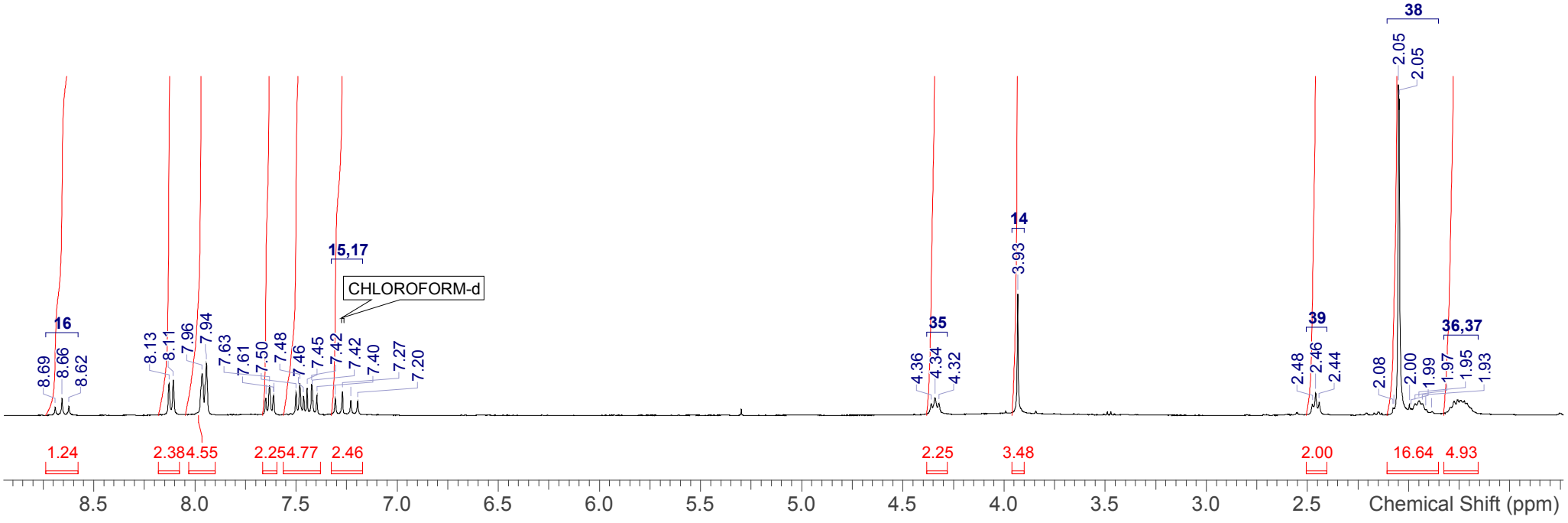
Peak 2, RT 3.659, Scan 1372, NL 1.266E06, MS2 (150:1500) ES+



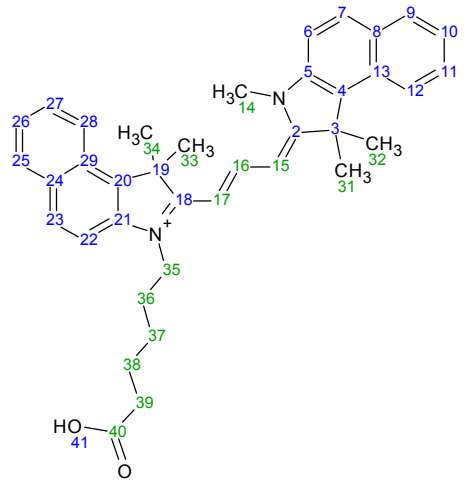
Acquisition Time (sec)	4.0894	***Comment***	mm/7316/48 Cy3.5 Carboxylic acid
Date	27 Aug 2014 21:57:07		
Date Stamp	27 Aug 2014 21:57:07		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\au2714mm1 Cy3.5 Carboxylic acid\10\PDATA\1\1r		
Frequency (MHz)	400.1300	***Nucleus***	1H
Number of Transients	16	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	1048576	***Pulse Sequence***	zg30
Receiver Gain	72.67	***SW(cyclical) (Hz)***	8012.82
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	2464.8901	***Spectrum Type***	standard
Sweep Width (Hz)	8012.81	***Temperature (degree C)***	25.005



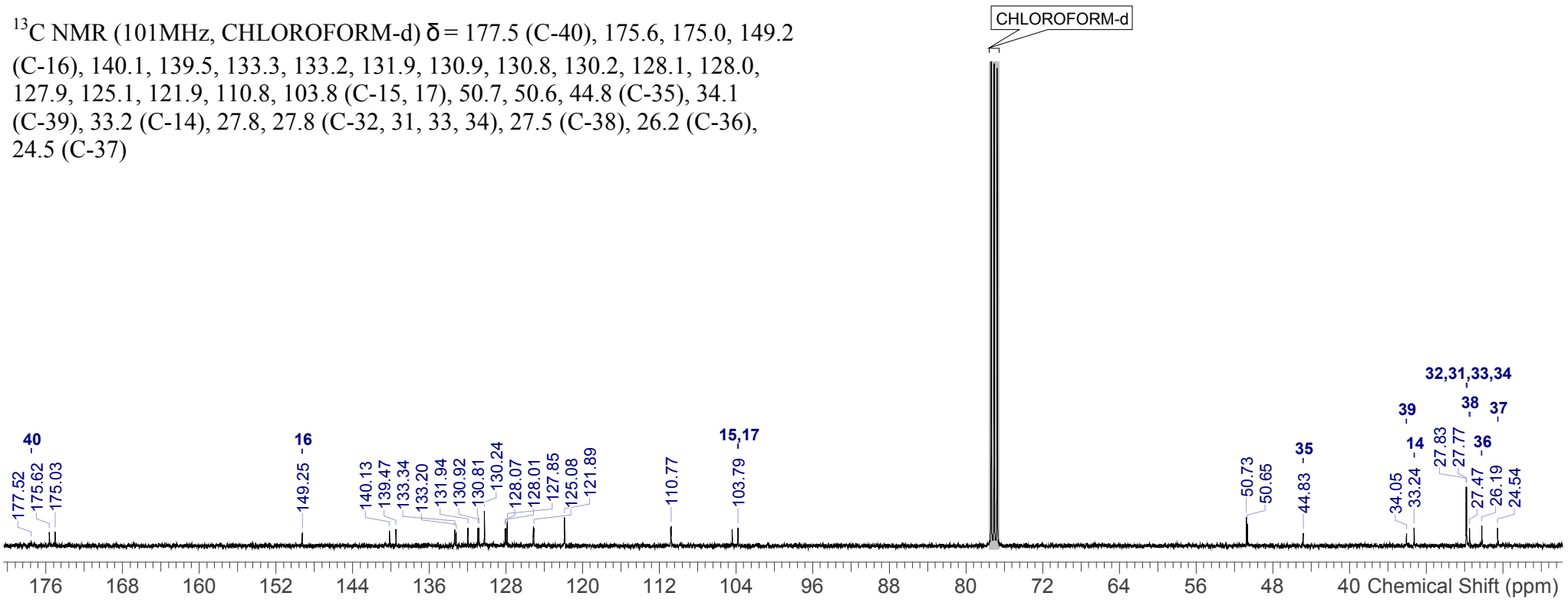
^{1}H NMR (400MHz, CHLOROFORM-d) δ = 8.58 - 8.74 (2H, m, H-16), 8.12 (3H, d, J = 8.5 Hz, M09), 7.95 (6H, br d, J = 8.6 Hz, M10), 7.59 - 7.67 (3H, m, M11), 7.38 - 7.56 (7H, m, M12), 7.17 - 7.33 (2H, m, H-15, 17), 4.34 (2H, br t, J = 7.6 Hz, H-35), 3.93 (4H, s, H-14), 2.46 (2H, br t, J = 6.7 Hz, H-39), 2.05 (15H, d, J = 1.2 Hz, H-38), 1.66 - 1.83 (5H, m, H-36, 37)



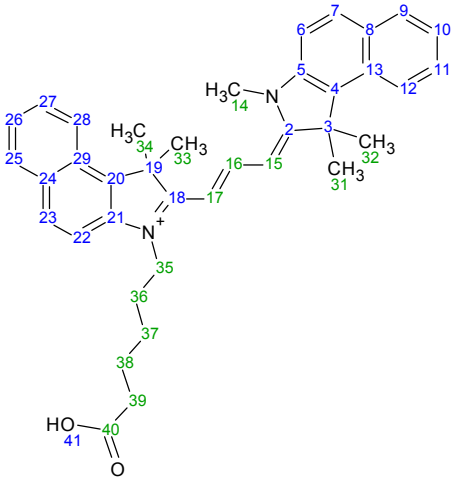
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/48 Cy3.5 Carboxylic acid
Date	27 Aug 2014 22:27:31		
Date Stamp	27 Aug 2014 22:27:31		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\au2714mm1 Cy3.5 Carboxylic acid\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	10060.3320	***Spectrum Type***	standard
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	24.998



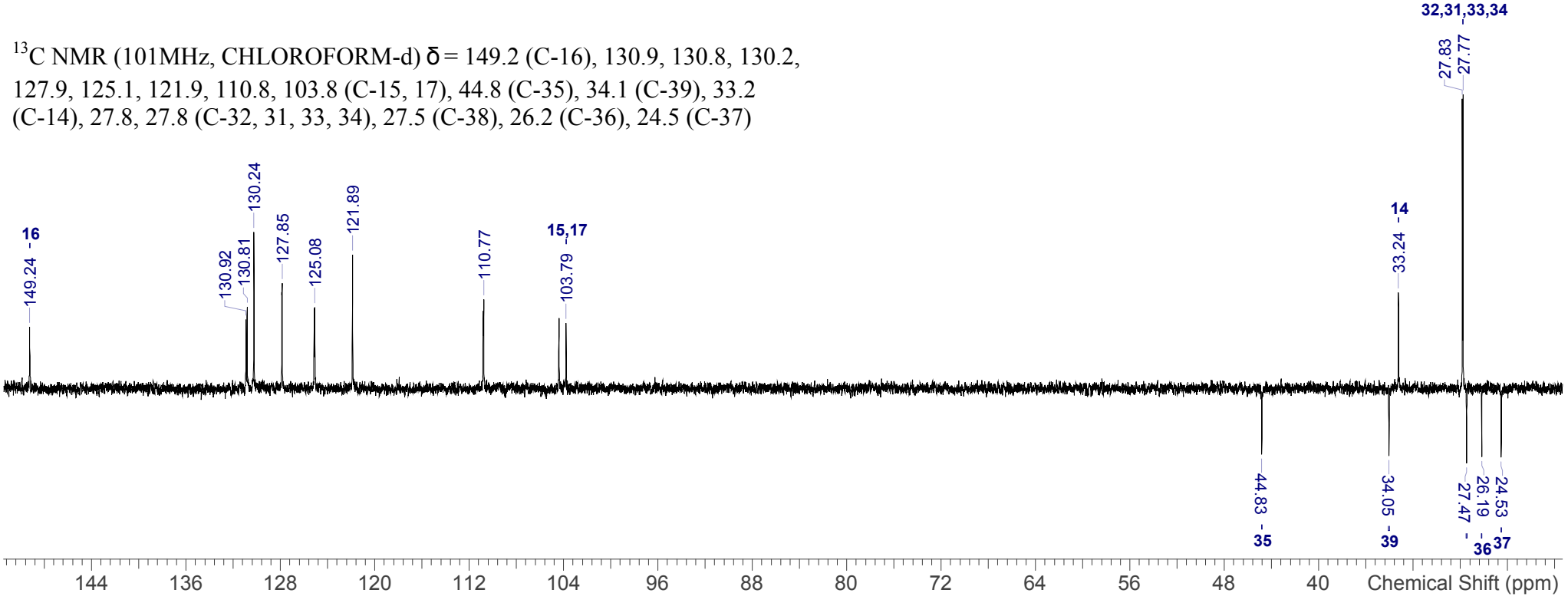
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 177.5 (C-40), 175.6, 175.0, 149.2 (C-16), 140.1, 139.5, 133.3, 133.2, 131.9, 130.9, 130.8, 130.2, 128.1, 128.0, 127.9, 125.1, 121.9, 110.8, 103.8 (C-15, 17), 50.7, 50.6, 44.8 (C-35), 34.1 (C-39), 33.2 (C-14), 27.8, 27.8 (C-32, 31, 33, 34), 27.5 (C-38), 26.2 (C-36), 24.5 (C-37)



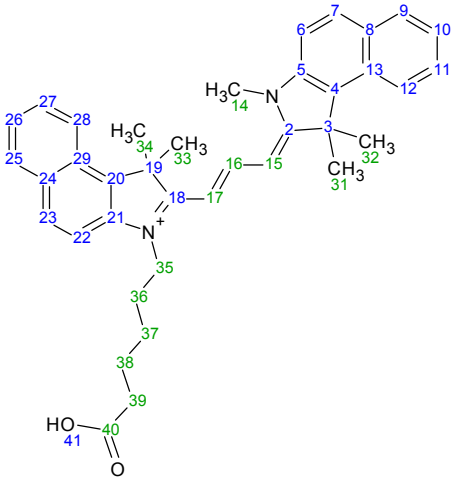
Acquisition Time (sec)	2.0316	***Comment***	mm/7316/48 Cy3.5 Carboxylic acid
Date	27 Aug 2014 22:46:09		
Date Stamp	27 Aug 2014 22:46:09		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\au2714mm1 Cy3.5 Carboxylic acid\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	CHLOROFORM-d	***Spectrum Offset (Hz)***	8048.0767
Spectrum Type	DEPT135	***Sweep Width (Hz)***	16128.54
Temperature (degree C)	24.996		



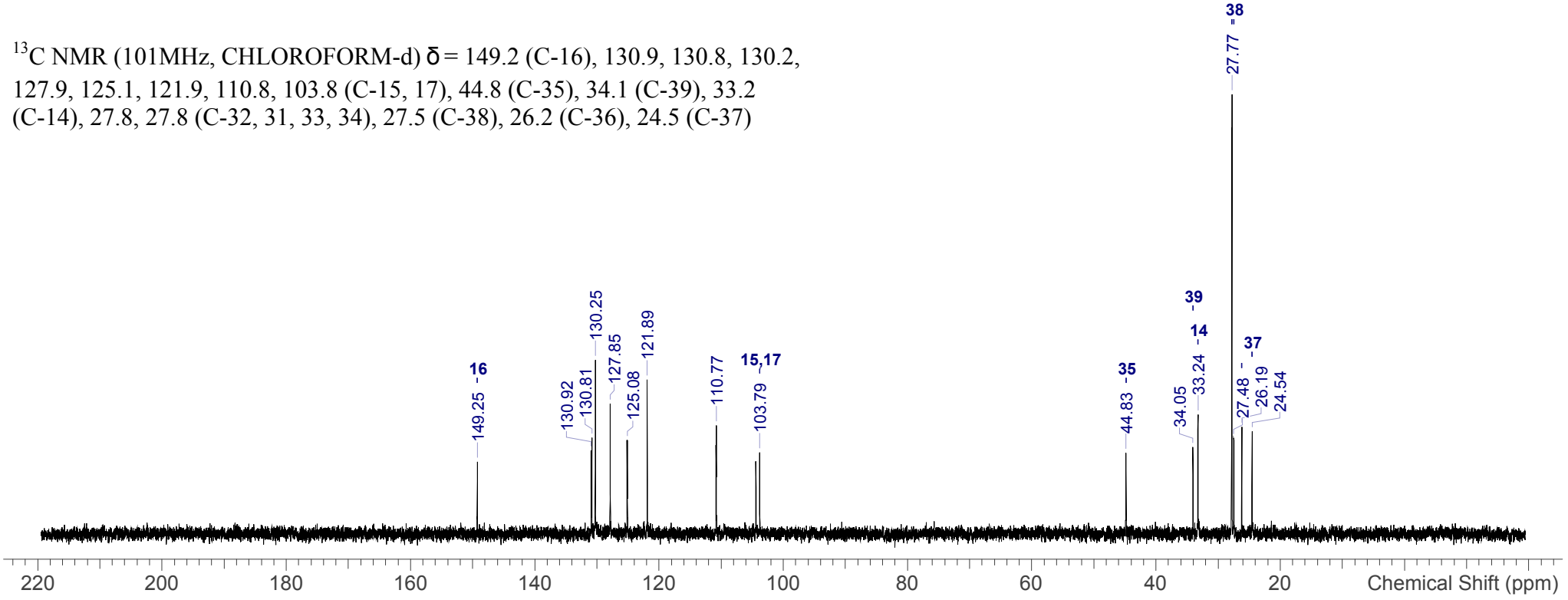
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 149.2 (C-16), 130.9, 130.8, 130.2, 127.9, 125.1, 121.9, 110.8, 103.8 (C-15, 17), 44.8 (C-35), 34.1 (C-39), 33.2 (C-14), 27.8, 27.8 (C-32, 31, 33, 34), 27.5 (C-38), 26.2 (C-36), 24.5 (C-37)



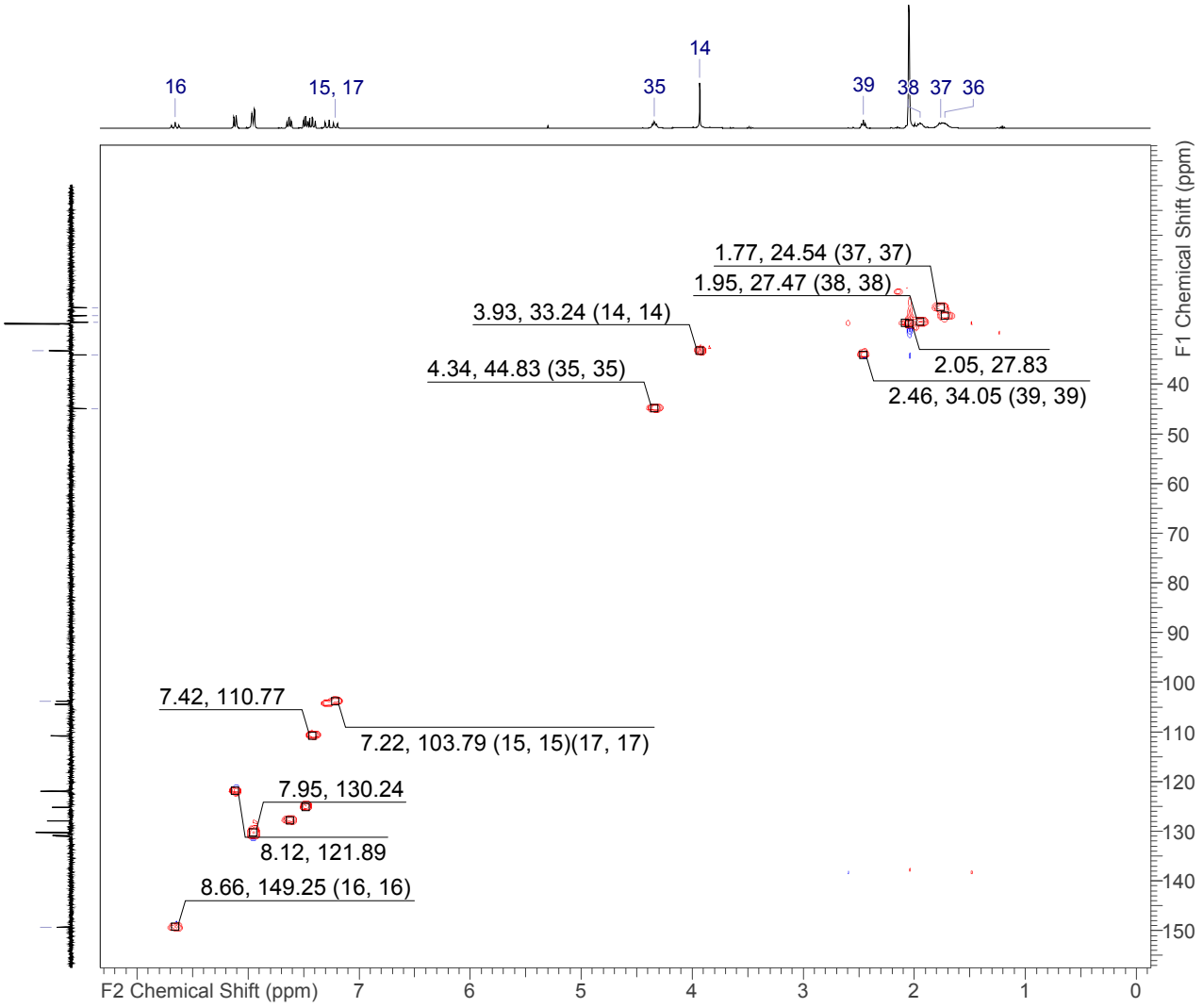
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/48 Cy3.5 Carboxylic acid
Date	27 Aug 2014 23:18:07		
Date Stamp	27 Aug 2014 23:18:07		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\au2714mm1 Cy3.5 Carboxylic acid\14\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp45
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d	***Spectrum Offset (Hz)***	10061.0684
Spectrum Type	DEPT45	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	25.000		

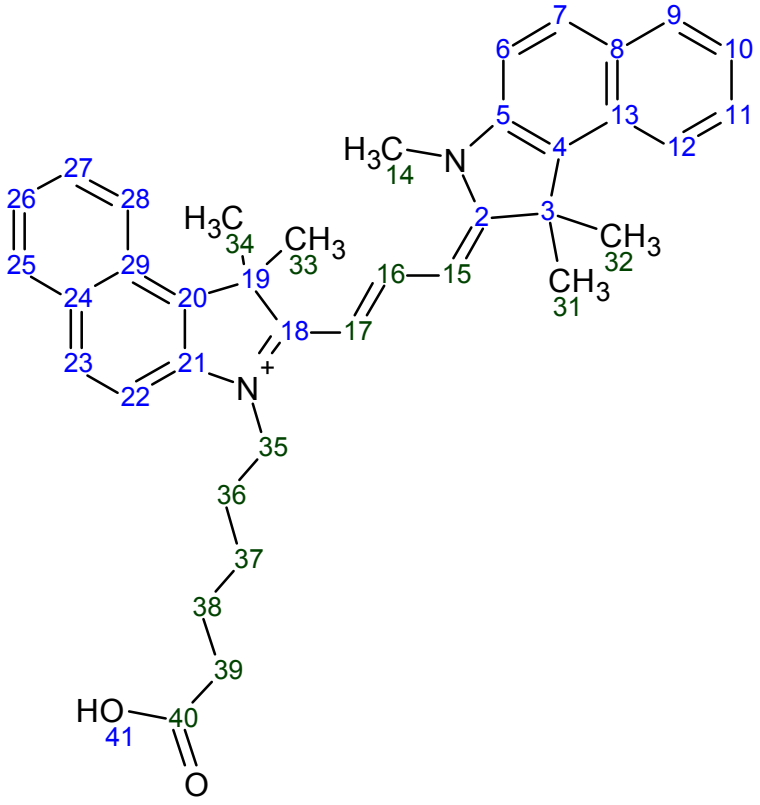


^{13}C NMR (101MHz, CHLOROFORM-d) δ = 149.2 (C-16), 130.9, 130.8, 130.2, 127.9, 125.1, 121.9, 110.8, 103.8 (C-15, 17), 44.8 (C-35), 34.1 (C-39), 33.2 (C-14), 27.8, 27.8 (C-32, 31, 33, 34), 27.5 (C-38), 26.2 (C-36), 24.5 (C-37)

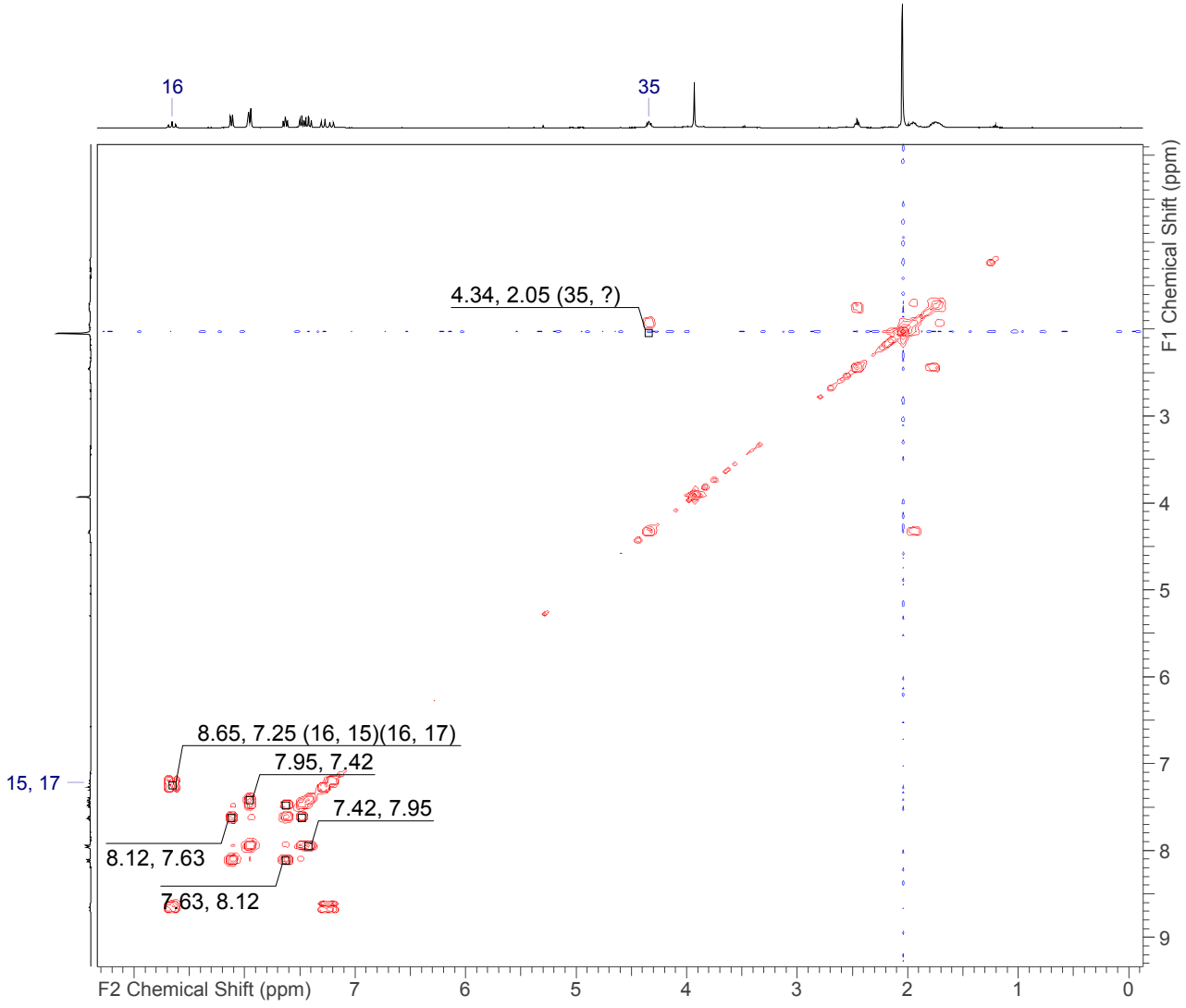


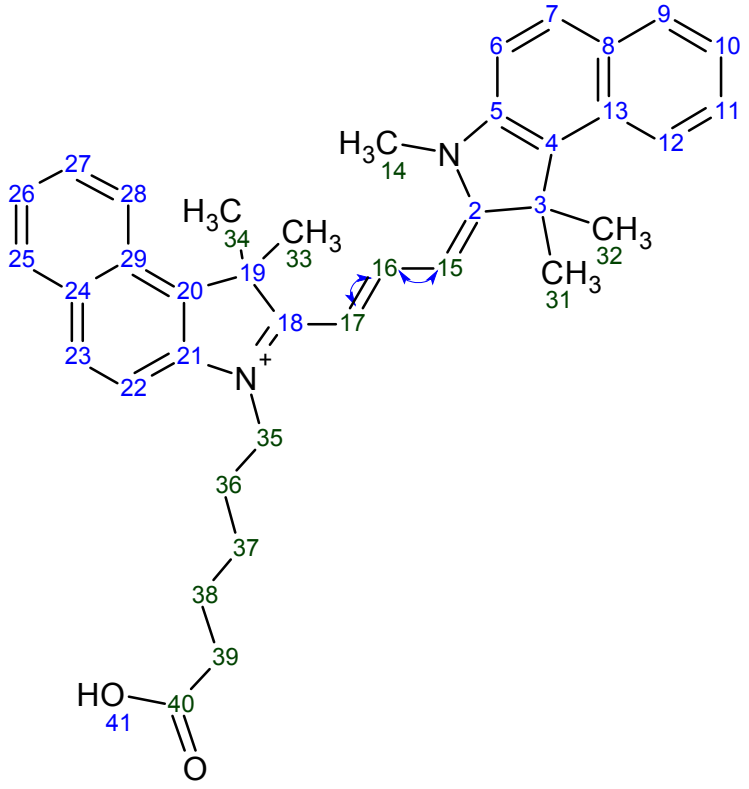
Acquisition Time (sec)	(0.1352, 0.0154)	***Comment***	mm/7316/48 Cy3.5 Carboxylic acid
Date	27 Aug 2014 23:33:00		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\au2714mm1 Cy3.5 Carboxylic acid\15\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)	***Nucleus***	(1H, 13C)
Number of Transients	2	***Origin***	spect
Original Points Count	(512, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	hsqcetgp
Solvent	CHLOROFORM-d	***Spectrum Type***	HSQC
Sweep Width (Hz)	(3784.18, 16650.39)	***Temperature (degree C)***	25.052
Title	mm/7316/48 Cy3.5 Carboxylic acid		





Acquisition Time (sec)	(0.2703, 0.0676)	***Comment***	mm/7316/48 Cy3.5 Carboxylic acid
Date	27 Aug 2014 23:02:00		
File Name	C:\Users\Mike Moazami\Desktop\PhD NMR Data RAW\au2714mm1 Cy3.5 Carboxylic acid\13\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)	***Nucleus***	(1H, 1H)
Number of Transients	2	***Origin***	spect
Original Points Count	(1024, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	cosygpqf
Solvent	CHLOROFORM-d	***Spectrum Type***	COSY
Sweep Width (Hz)	(3784.18, 3784.18)	***Temperature (degree C)***	24.998
Title	mm/7316/48 Cy3.5 Carboxylic acid		



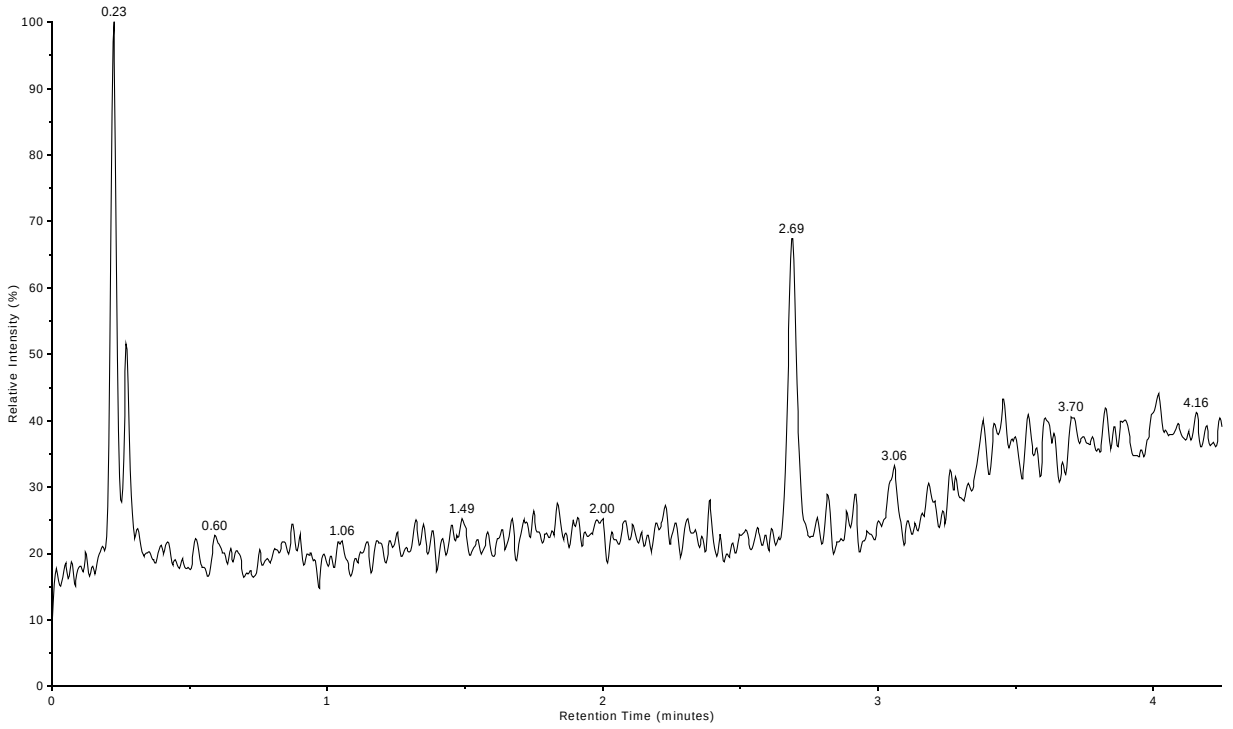


A.14 Dinitrophenylpyridinium chloride

Top 5 Peak Report

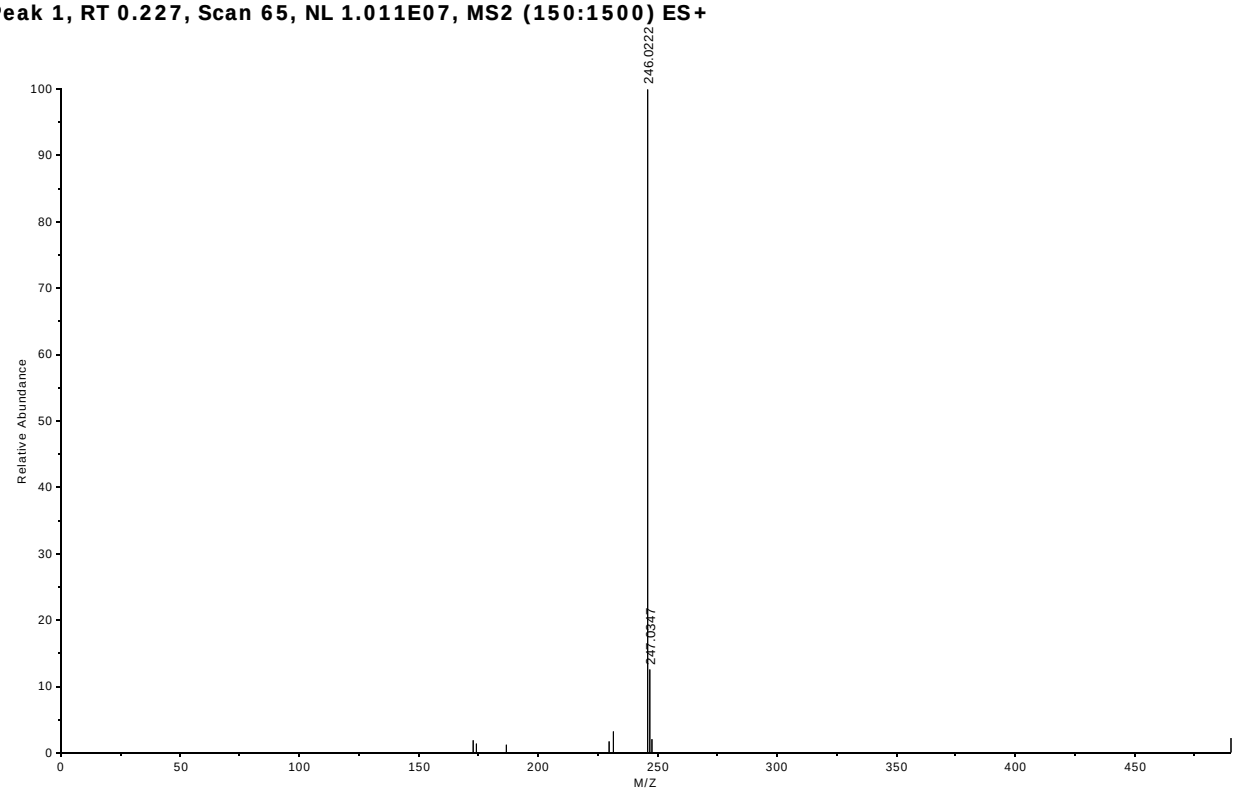
Group: Watts, J
Submitter: Michael Moazami
Project: RP LC C18 < 2 cmpds
Sample ID: mm/7500/90 pyridiniumdinitrobenzene
Experiment: BLUE ESIPOS C18 5 min
Filename: mm750090_pyridiniumdinitrobenzene_Michael_M
Instrument: Blue RP UHPLC-MS (B30:1023) ON-LINE
Acquisition Date: 28/07/2015 12:21:54

TIC, NL 1.039E07, MS2 (150:1500) ES+

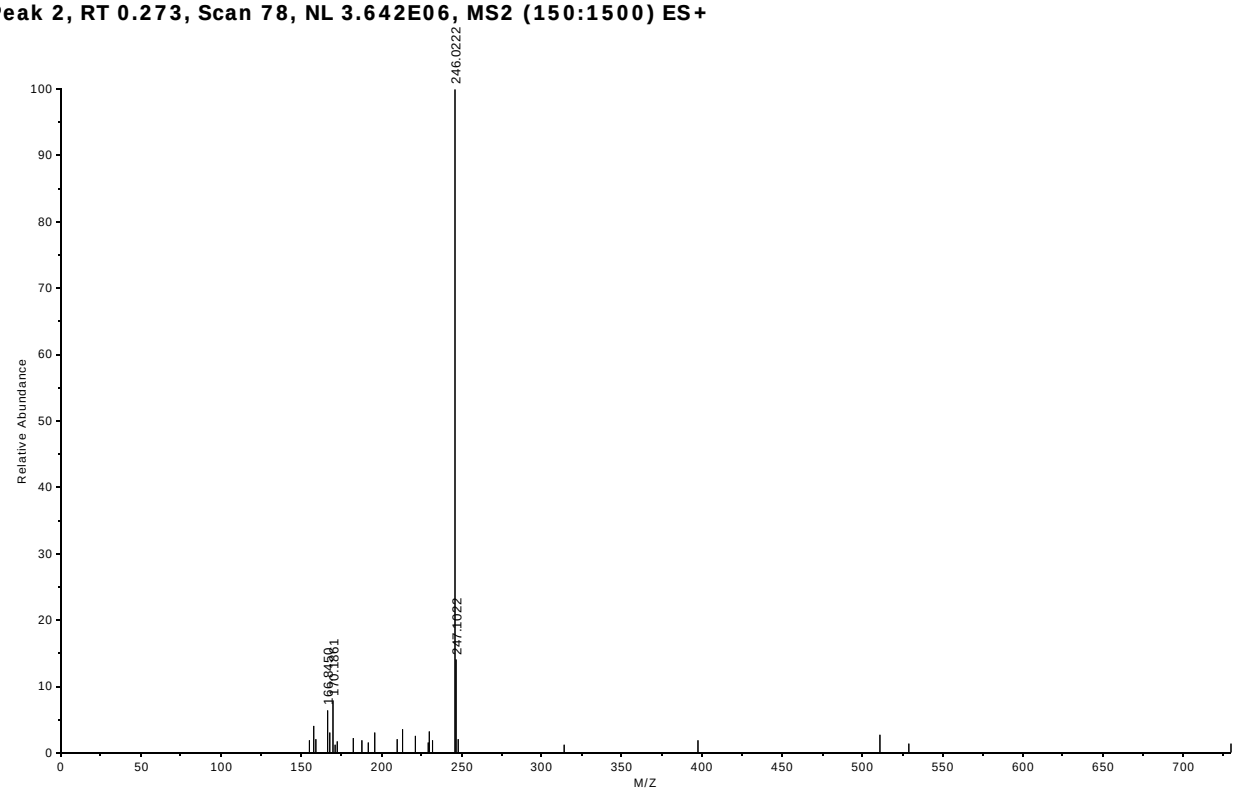


	RT Mins	Height	Height %	Area	Area %
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2	0.27	2758053	34.93	4077473	32.36
3	2.69	4693058	59.44	10899848	86.51
4	3.06	1088356	13.78	3293925	26.14
5	3.38	968319	12.26	2186950	17.36

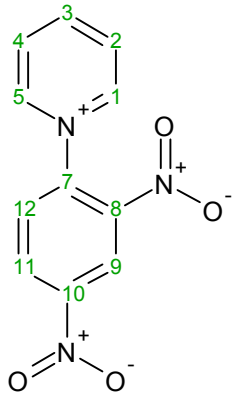
Peak 1, RT 0.227, Scan 65, NL 1.011E07, MS2 (150:1500) ES+



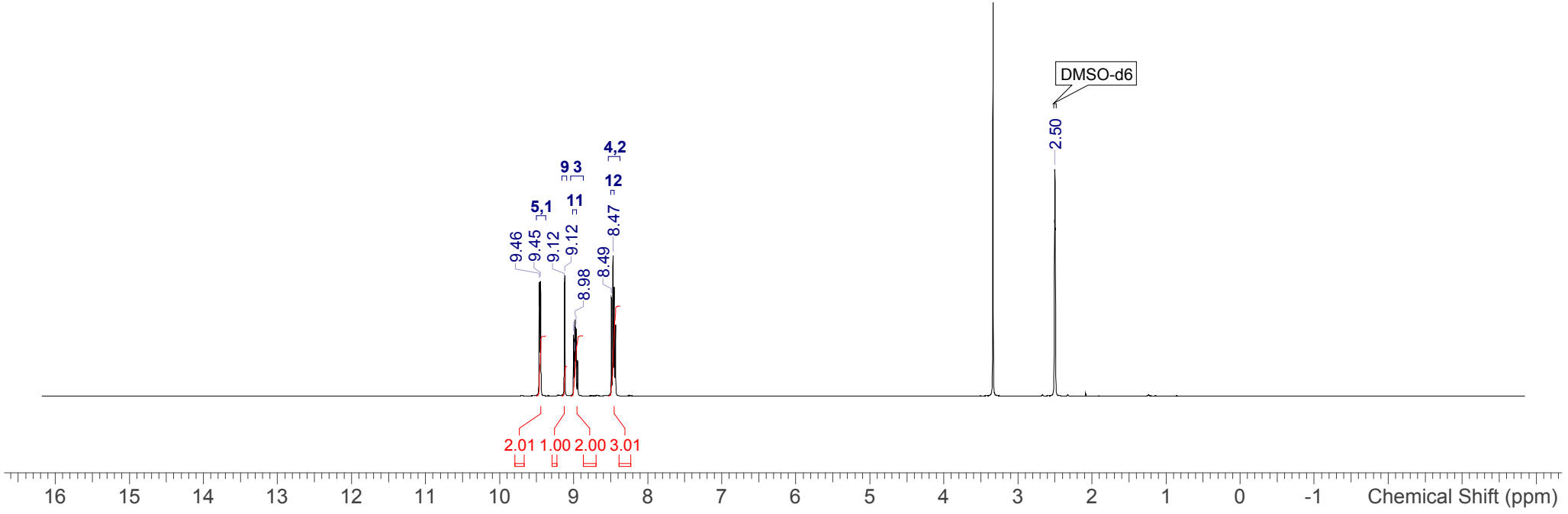
Peak 2, RT 0.273, Scan 78, NL 3.642E06, MS2 (150:1500) ES+



Acquisition Time (sec)	4.0894	***Comment***	mm/7500/90 pyridinium-2,4-dinitobenzene		
D	0.0002	***DE***	6.5	***DS***	2
Date	29 Jul 2015 10:25:50			***Date Stamp***	29 Jul 2015 10:25:50
File Name	\\filestore.soton.ac.uk\users\mm29g10\mydesktop\jy2915mm3 pyridinyldinitrobenzene\10\PDATA\1\1r				
Frequency (MHz)	400.1300	***GB***	0	***INSTRUM***	<spect>
LB	0.3	***NS***	16	***Nucleus***	1H
Number of Transients	16	***Origin***	spect	***Original Points Count***	32768
Owner	nmr	***PC***	1		
PROBHD	<5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218 >			***PULPROG***	<zg30>
Points Count	65536	***Pulse Sequence***	zg30	***Receiver Gain***	100.45
SF	400.13	***SFO1***	400.132470966543		
SI	65536	***SSB***	0	***SW(cyclical) (Hz)***	8012.82
SWH	8012.82051282051			***Solvent***	DMSO-d6
Spectrum Offset (Hz)	2467.8057	***Spectrum Type***	standard	***Sweep Width (Hz)***	8012.70
TD	65536	***TD0***	1	***TE***	297.9971
Temperature (degree C)	24.997	***UNC1***	<1H>	***WDW***	1

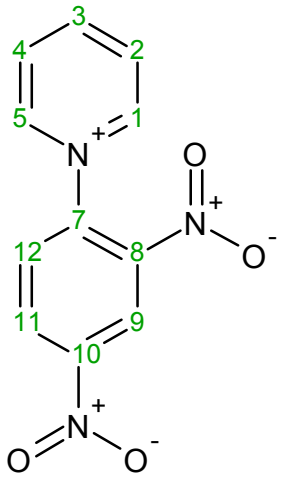


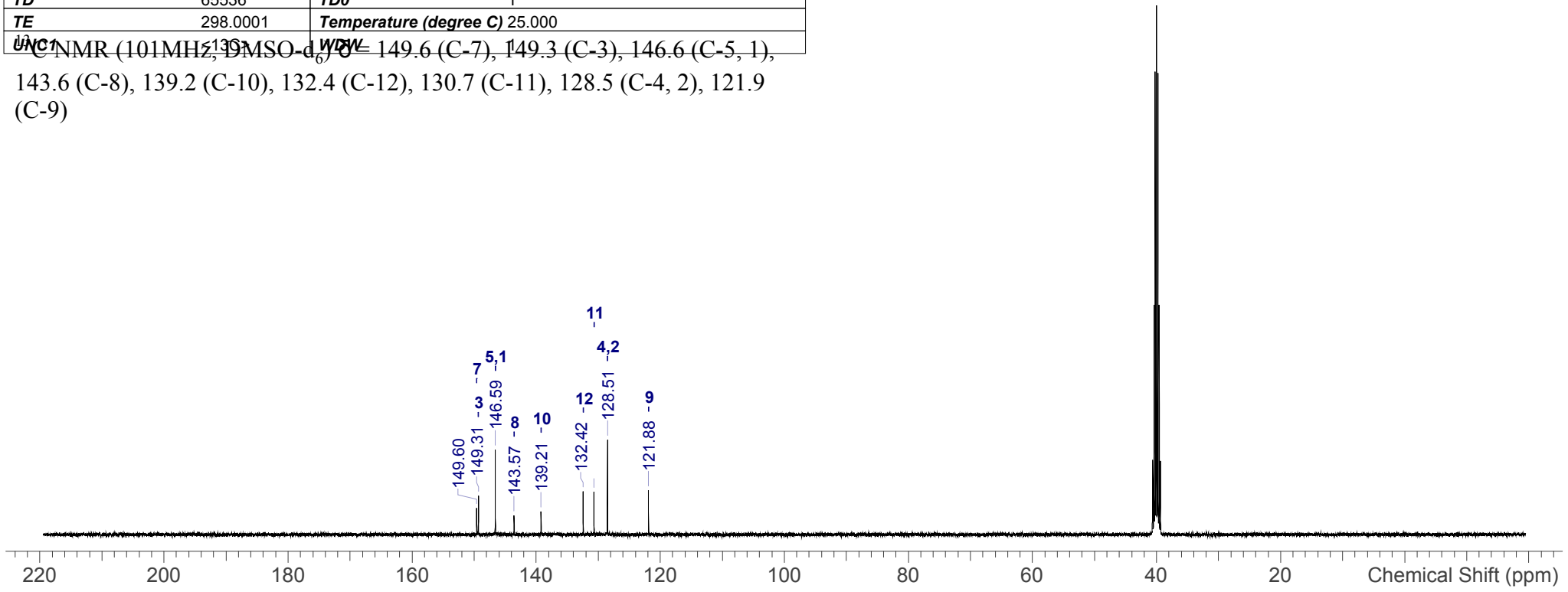
^{1}H NMR (400MHz, DMSO-d_6) δ = 9.45 (2H, d, J = 6.0 Hz, H-5, 1), 9.12 (1H, d, J = 2.4 Hz, H-9), 8.96 - 9.01 (1H, m, H-11), 8.87 - 9.04 (1H, m, H-3), 8.48 (1H, d, J = 8.4 Hz, H-12), 8.37 - 8.53 (2H, m, H-4, 2)



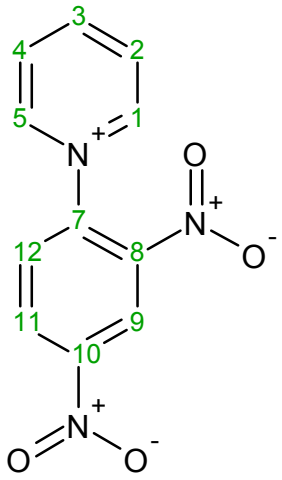
Acquisition Time (sec)	1.3631	***Comment***	mm/7500/90 pyridinium-2,4-dinitobenzene
D	0.03	***DE***	6.5
DS	4	***Date***	29 Jul 2015 21:39:56
Date Stamp	29 Jul 2015 21:39:56		
File Name	\\filestore.soton.ac.uk\users\mm29g10\mydesktop\jy2915mm3 pyridinyldinitrobenzene\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***GB***	0
INSTRUM	<spect>	***LB***	1
NS	512	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
PC	1.4		
PROBHD	<5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218 >		
PULPROG	<zgpg30>	***Points Count***	32768
Pulse Sequence	zgpg30	***Receiver Gain***	212.69
SF	100.612769	***SFO1***	100.622829328806
SI	32768	***SSB***	0
SW(cyclical) (Hz)	24038.46	***SWH***	24038.4615384615
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	10060.3320
Spectrum Type	standard	***Sweep Width (Hz)***	24037.73
TD	65536	***TD0***	1
TE	298.0001	***Temperature (degree C)***	25.000
UNC1	<13C>	***WDW***	1

^{13}C-NMR (101MHz, DMSO-d_6) δ = 149.6 (C-7), 149.3 (C-3), 146.6 (C-5, 1), 143.6 (C-8), 139.2 (C-10), 132.4 (C-12), 130.7 (C-11), 128.5 (C-4, 2), 121.9 (C-9)

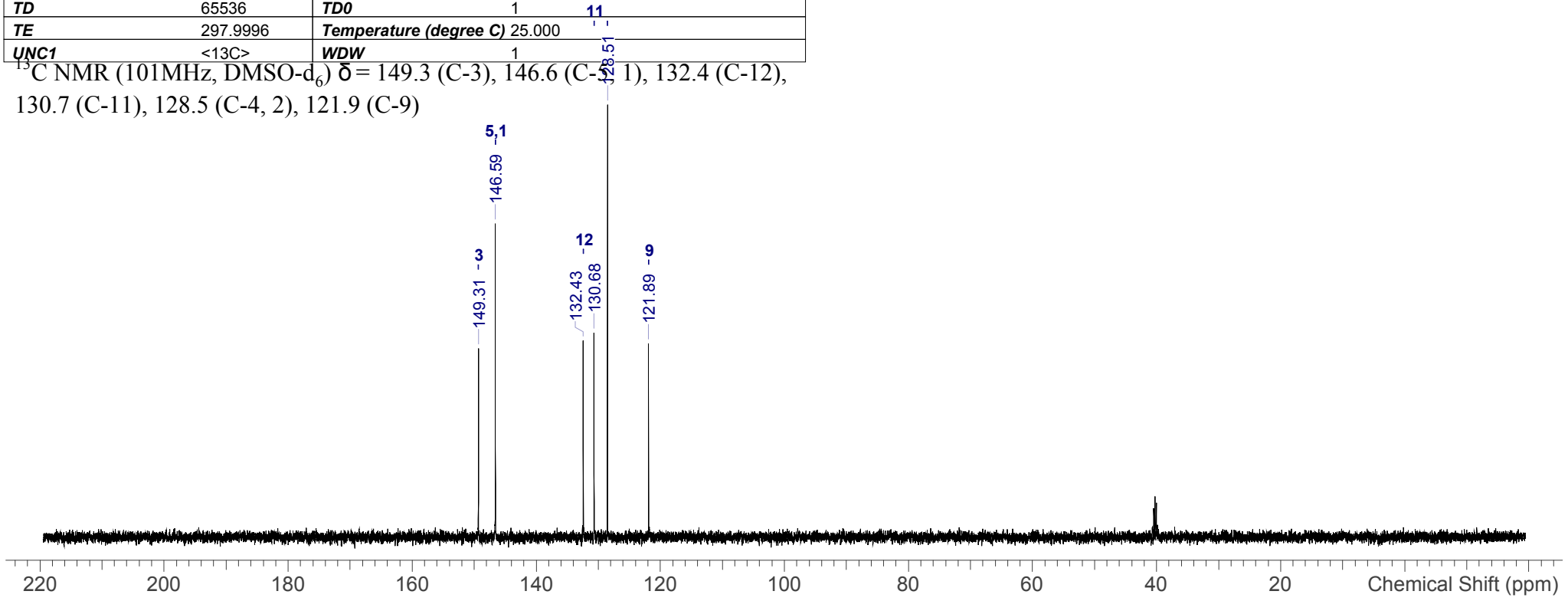




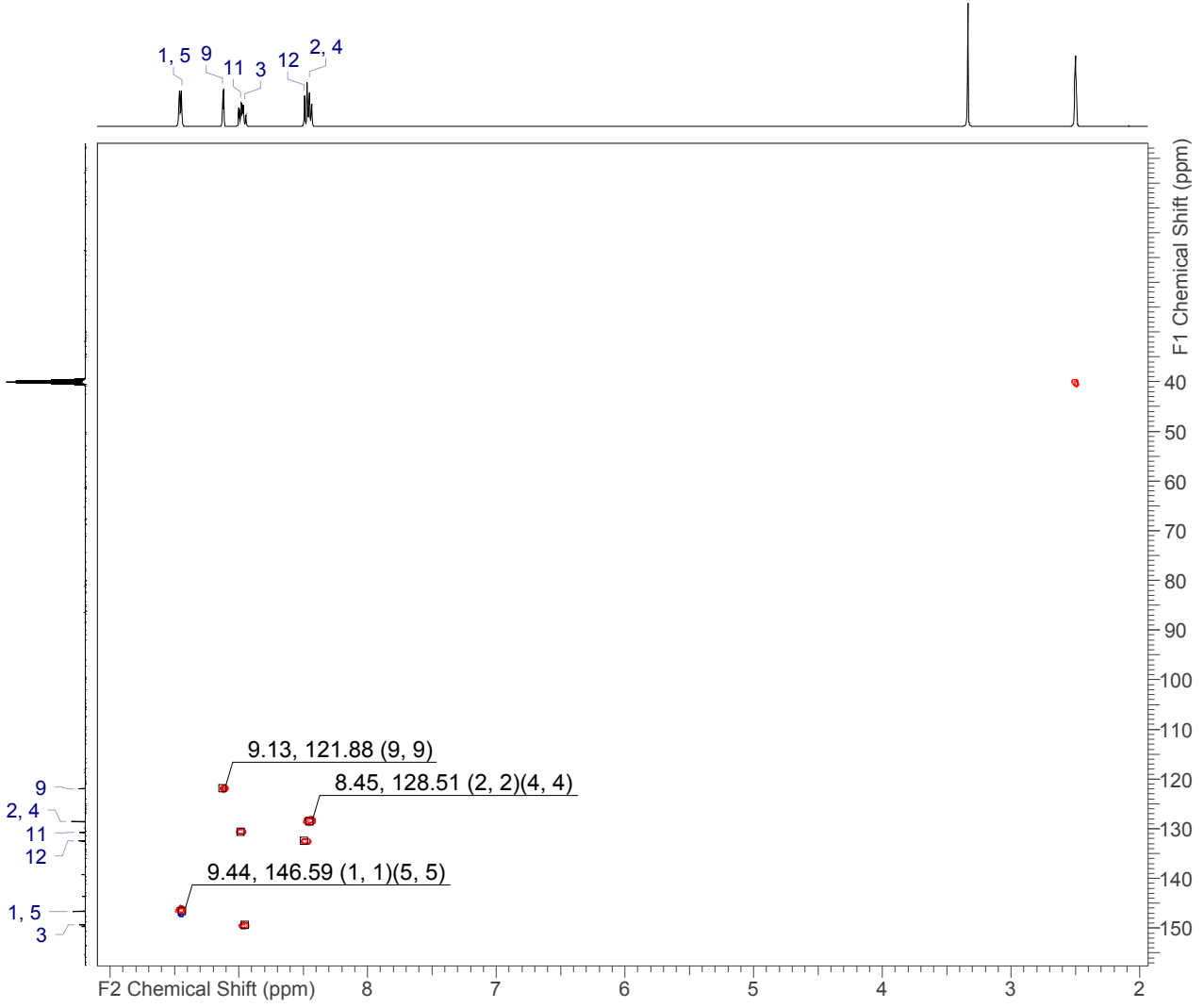
Acquisition Time (sec)	1.3631	***Comment***	mm/7500/90 pyridinium-2,4-dinitobenzene
D	0.003448276	***DE***	6.5
DS	4	***Date***	29 Jul 2015 22:11:59
Date Stamp	29 Jul 2015 22:11:59		
File Name	\\filestore.soton.ac.uk\users\mm29g10\mydesktop\jy2915mm3 pyridinyldinitrobenzene\13\PDATA\1\1r		
Frequency (MHz)	100.6128	***GB***	0
INSTRUM	<spect>	***LB***	1
NS	256	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
PC	1.4		
PROBHD	<5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218 >		
PULPROG	<deptsp45>	***Points Count***	32768
Pulse Sequence	deptsp45	***Receiver Gain***	212.69
SF	100.612769	***SFO1***	100.622829802853
SI	32768	***SSB***	0
SW(cyclical) (Hz)	24038.46	***SWH***	24038.4615384615
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	10061.0684
Spectrum Type	DEPT45	***Sweep Width (Hz)***	24037.73
TD	65536	***TD0***	1
TE	297.9996	***Temperature (degree C)***	25.000
UNC1	<13C>	***WDW***	1

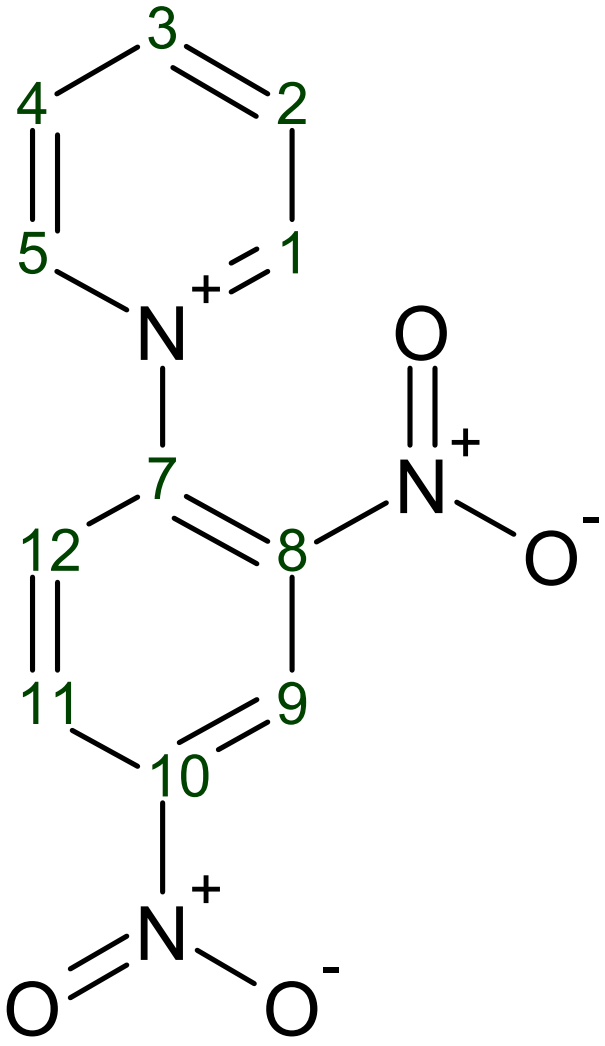


^{13}C NMR (101MHz, DMSO-d_6) δ = 149.3 (C-3), 146.6 (C-5, 1), 132.4 (C-12), 130.7 (C-11), 128.5 (C-4, 2), 121.9 (C-9)

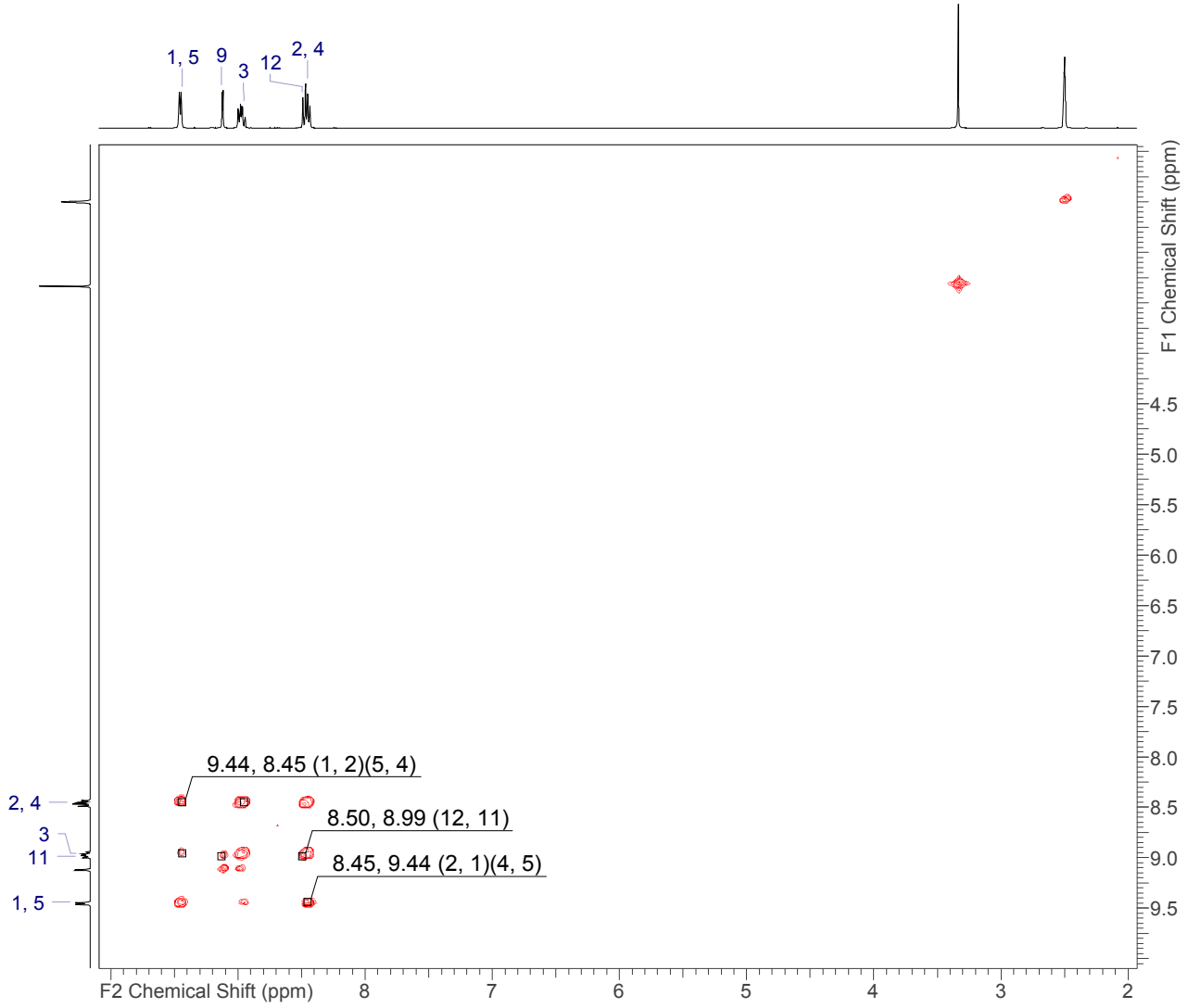


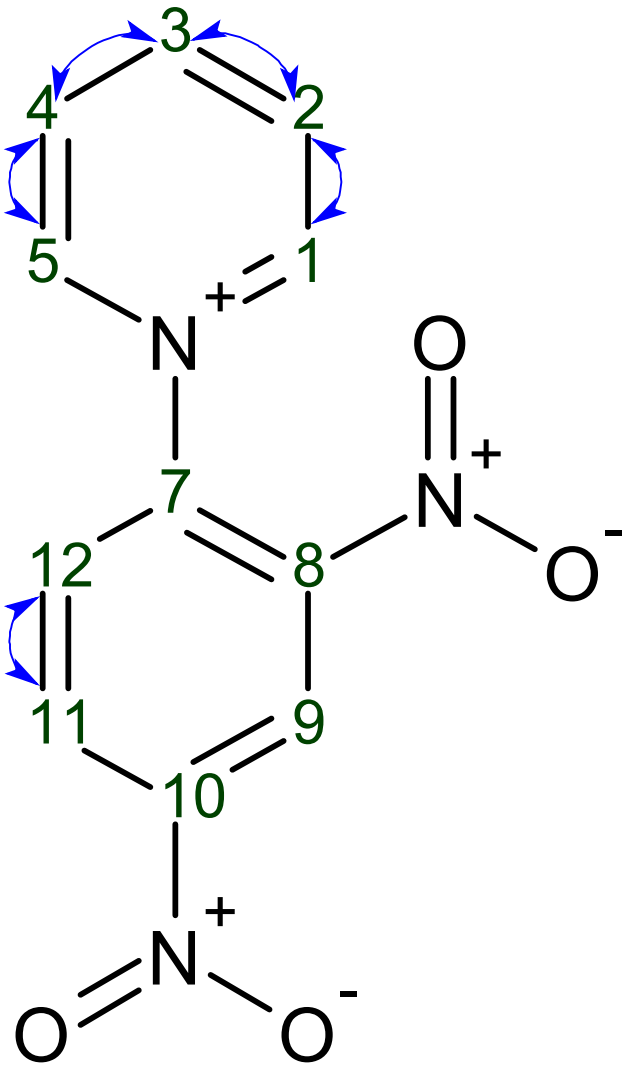
Acquisition Time (sec)	(0.1567, 0.0154)	***Comment***	mm/7500/90 pyridinium-2,4-dinitobenzene
Date	29 Jul 2015 22:27:00		
File Name	\\filestore.soton.ac.uk\users\mm29g10\mydesktop\jy2915mm3 pyridinyldinitrobenzene\14\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)	***Nucleus***	(1H, 13C)
Number of Transients	2	***Origin***	spect
Original Points Count	(512, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	hsqcetgp
Solvent	DMSO-d6	***Spectrum Type***	HSQC
Sweep Width (Hz)	(3264.78, 16650.39)	***Temperature (degree C)***	25.056
Title	mm/7500/90 pyridinium-2,4-dinitobenzene		



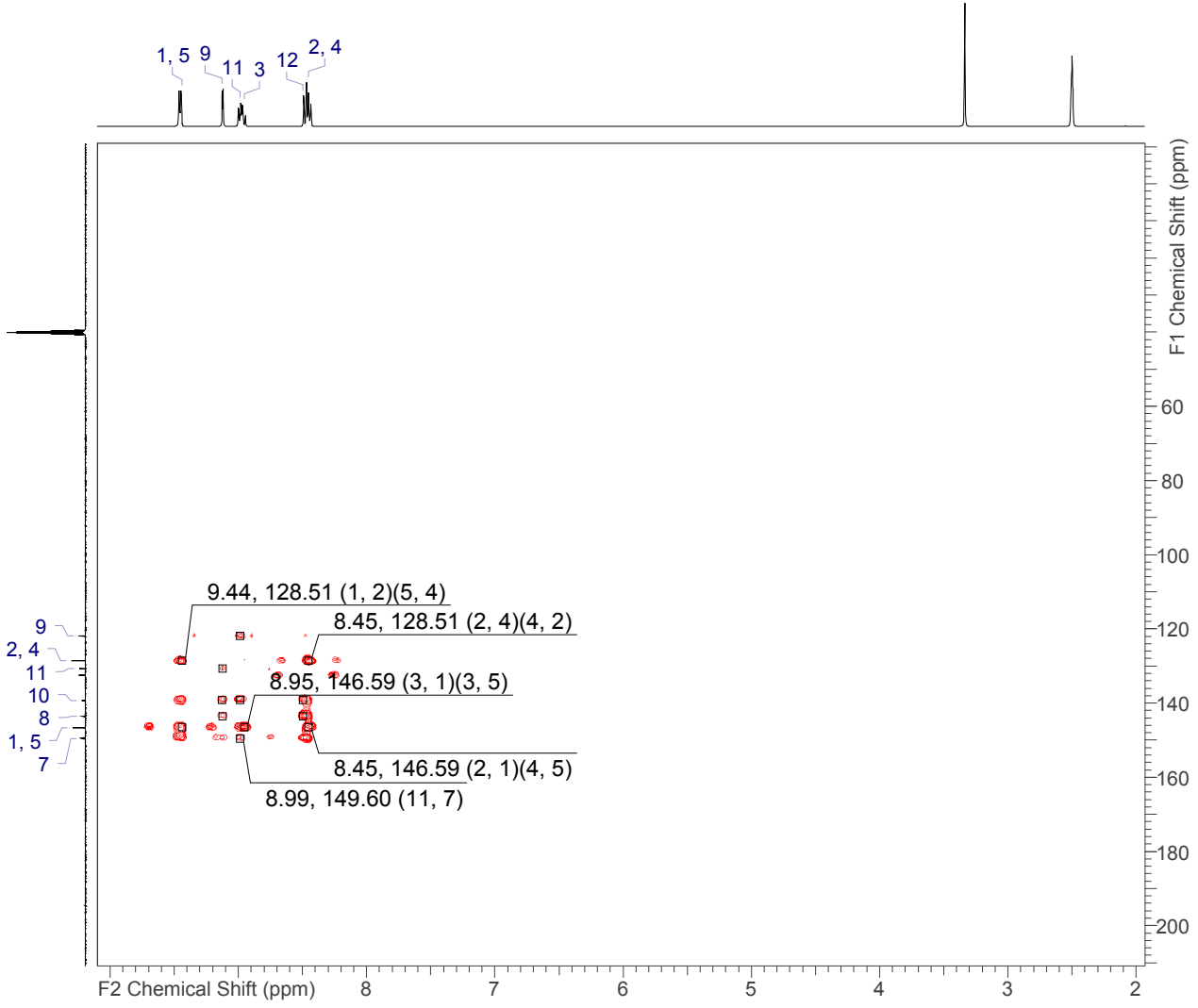


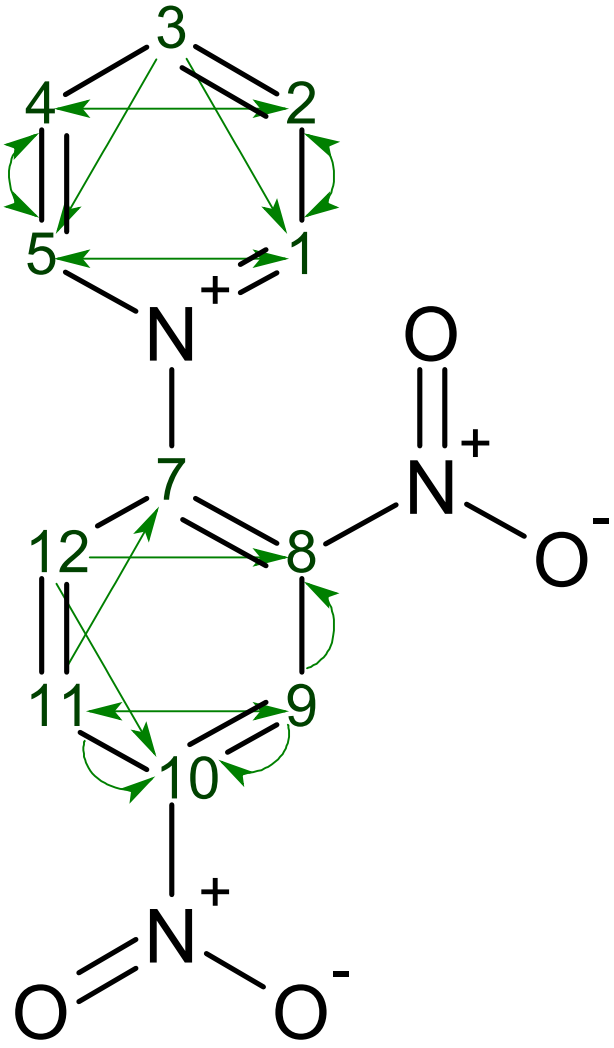
Acquisition Time (sec)	(0.3133, 0.0783)	***Comment***	mm/7500/90 pyridinium-2,4-dinitobenzene
Date	29 Jul 2015 21:56:00		
File Name	\\filestore.soton.ac.uk\users\mm29g10\mydesktop\jy2915mm3 pyridinyldinitrobenzene\12\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)	***Nucleus***	(1H, 1H)
Number of Transients	2	***Origin***	spect
Original Points Count	(1024, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	cosygpqf
Solvent	DMSO-d6	***Spectrum Type***	COSY
Sweep Width (Hz)	(3264.78, 3264.78)	***Temperature (degree C)***	24.986
Title	mm/7500/90 pyridinium-2,4-dinitobenzene		





Acquisition Time (sec)	(0.3133, 0.0115)	***Comment***	mm/7500/90 pyridinium-2,4-dinitobenzene
Date	29 Jul 2015 22:58:00		
File Name	\\filestore.soton.ac.uk\users\mm29g10\mydesktop\jy2915mm3 pyridinyldinitrobenzene\15\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)	***Nucleus***	(1H, 13C)
Number of Transients	4	***Origin***	spect
Original Points Count	(1024, 256)	***Owner***	nmr
Points Count	(2048, 1024)	***Pulse Sequence***	hmbcgplpndqf
Solvent	DMSO-d6	***Spectrum Type***	HMBC
Sweep Width (Hz)	(3266.38, 22299.63)	***Temperature (degree C)***	25.001
Title	mm/7500/90 pyridinium-2,4-dinitobenzene		



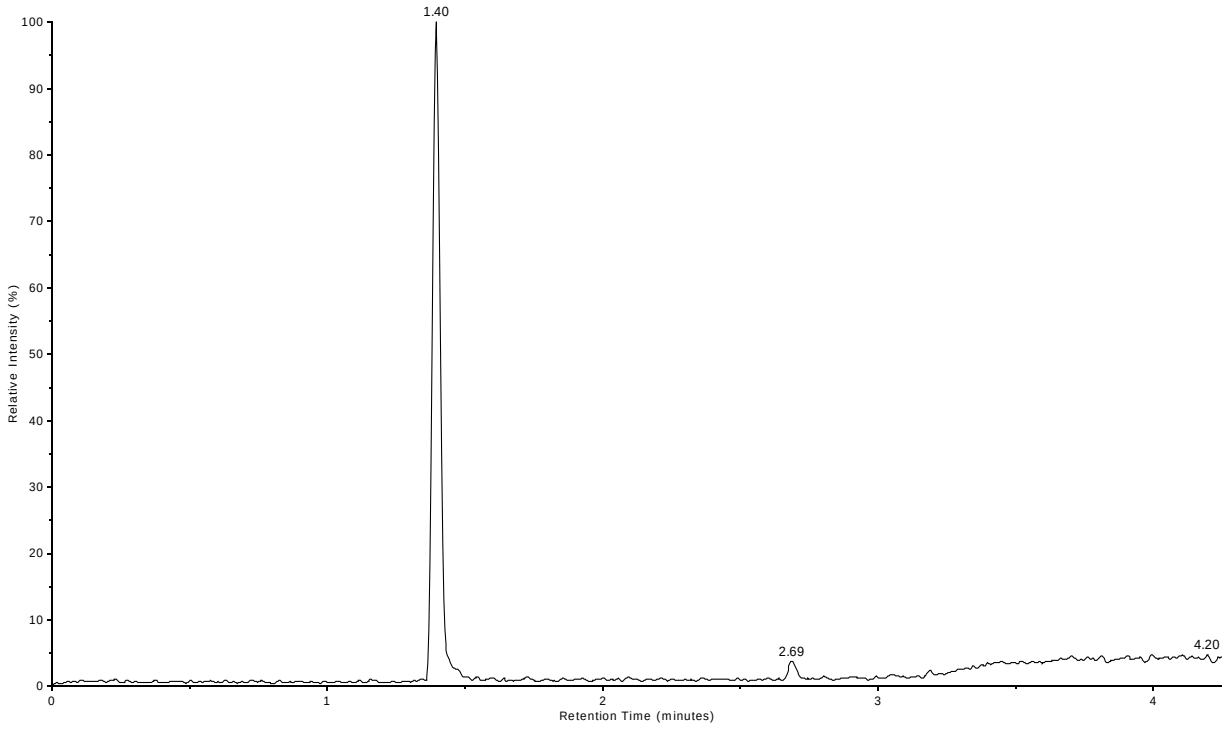


A.15 Glutacodianil

Top 5 Peak Report

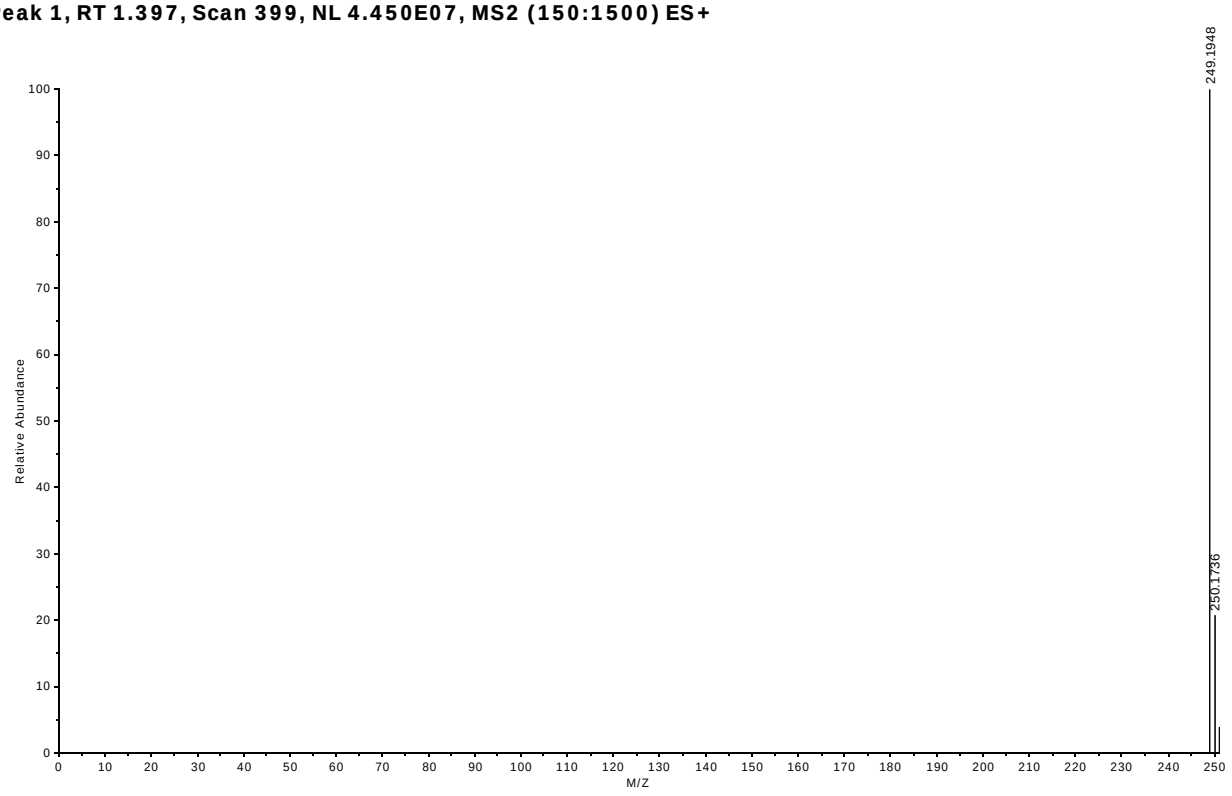
Group: Watts, J
Submitter: Michael Moazami
Project: RP LC C18 < 2 cmpds
Sample ID: mm/7500/105 Cy7 linker
Experiment: BLUE ESIPOS C18 5 min
Filename: mm7500105_Cy7_linker_Michael_Moazami__Wa
Instrument: Blue RP UHPLC-MS (B30:1023) ON-ACN
Acquisition Date: 05/08/2015 12:30:03

TIC, NL 5.512E07, MS2 (150:1500) ES+

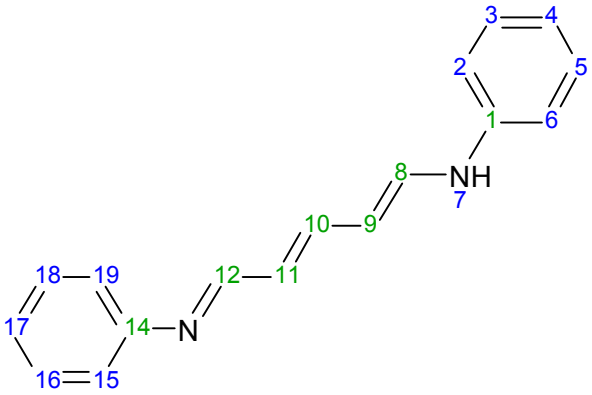


	RT Mins	Height	Height %	Area	Area %
1	1.40	54648086	100.00	111291961	100.00

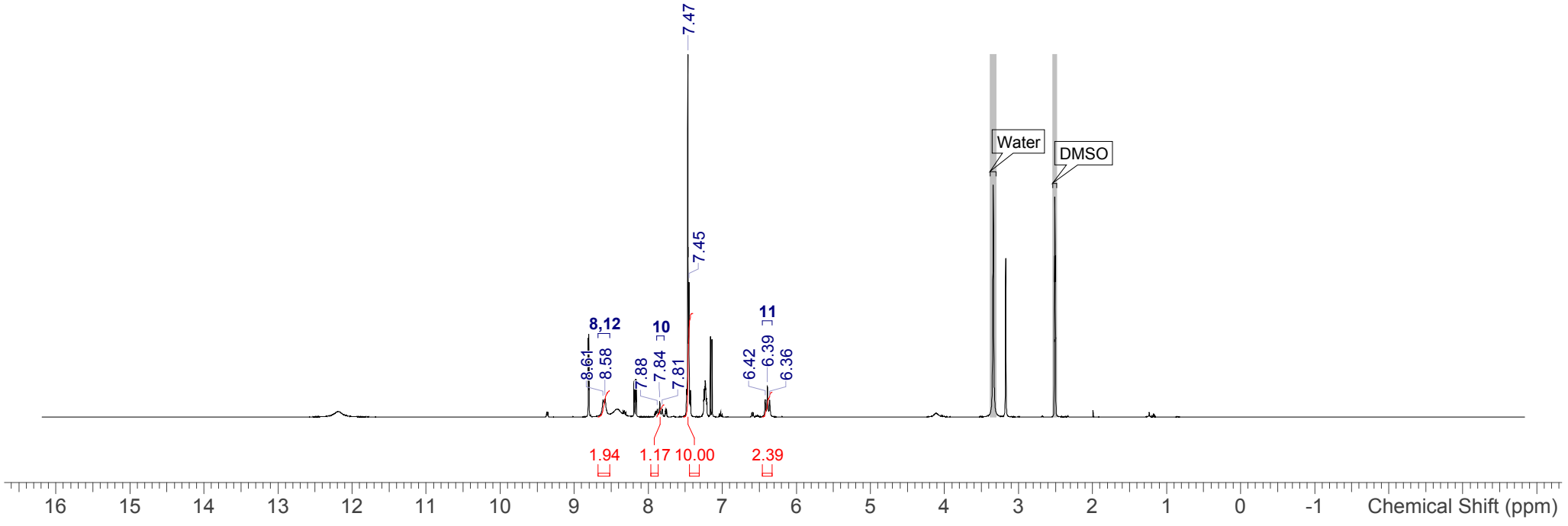
Peak 1, RT 1.397, Scan 399, NL 4.450E07, MS2 (150:1500) ES+



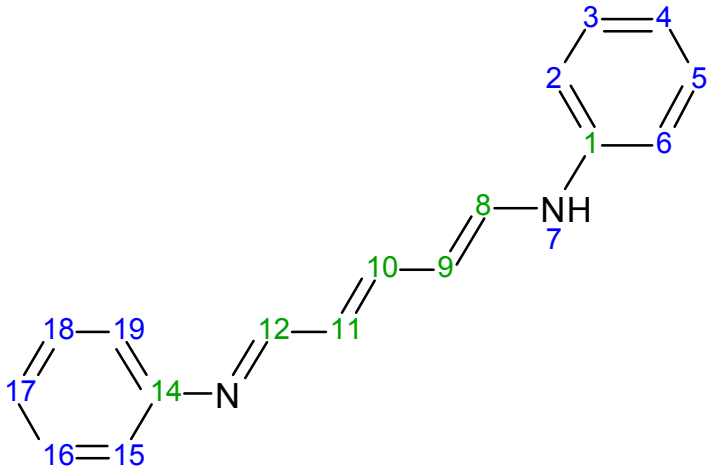
Acquisition Time (sec)	4.0894	***Comment***	mm/7500/105 Cy7 linker		
Date	05 Aug 2015 05:34:07				
Date Stamp	05 Aug 2015 05:34:07				
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au0415mm2 Cy7 linker\10\PDATA\1\1r				
Frequency (MHz)	400.1300	***Nucleus***	1H	***Number of Transients***	16
Origin	spect	***Original Points Count***	32768	***Owner***	nmr
Points Count	65536	***Pulse Sequence***	zg30	***Receiver Gain***	128.28
SW(cyclical) (Hz)	8012.82	***Solvent***	DMSO-d6	***Spectrum Offset (Hz)***	2470.9668
Spectrum Type	standard	***Sweep Width (Hz)***	8012.70	***Temperature (degree C)***	25.001



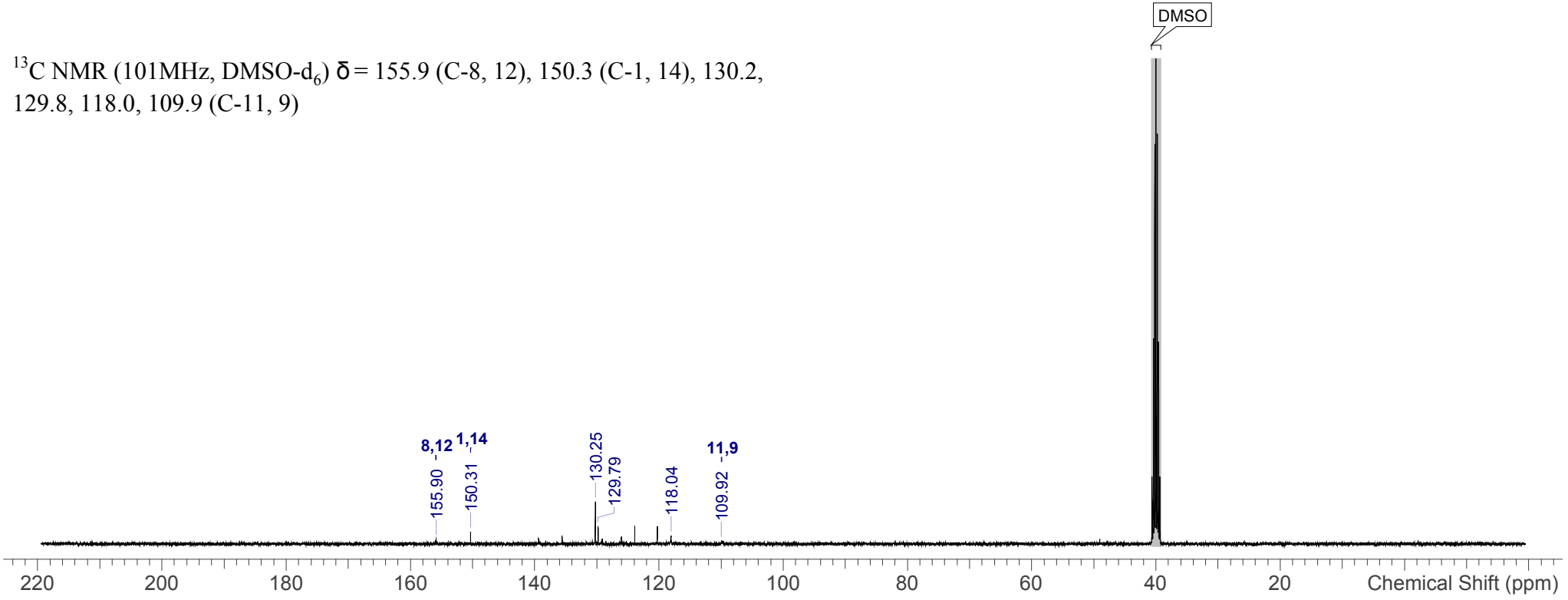
^{1}H NMR (400MHz, DMSO-d_6) δ = 8.60 (2H, br d, J = 10.8 Hz, H-8, 12), 7.84 (1H, br t, J = 13.0 Hz, H-10), 7.40 - 7.53 (10H, m, M01), 6.39 (2H, br t, J = 12.3 Hz, H-11)



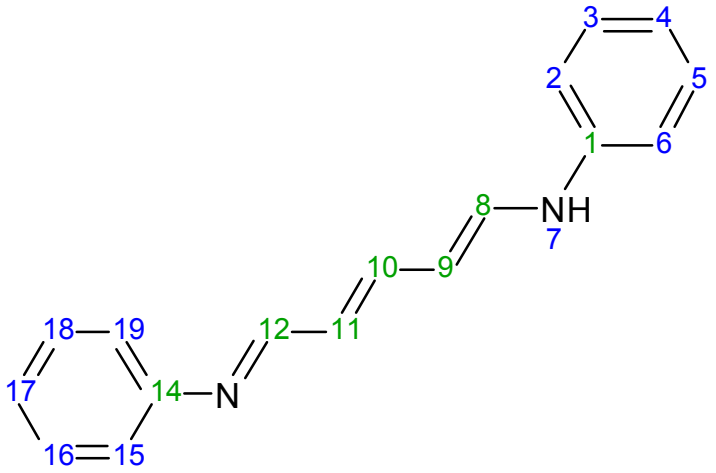
Acquisition Time (sec)	1.3631	***Comment***	mm/7500/105 Cy7 linker
Date	05 Aug 2015 06:04:34		
Date Stamp	05 Aug 2015 06:04:34		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au0415mm2 Cy7 linker\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	10060.3320
Spectrum Type	standard	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	24.999		



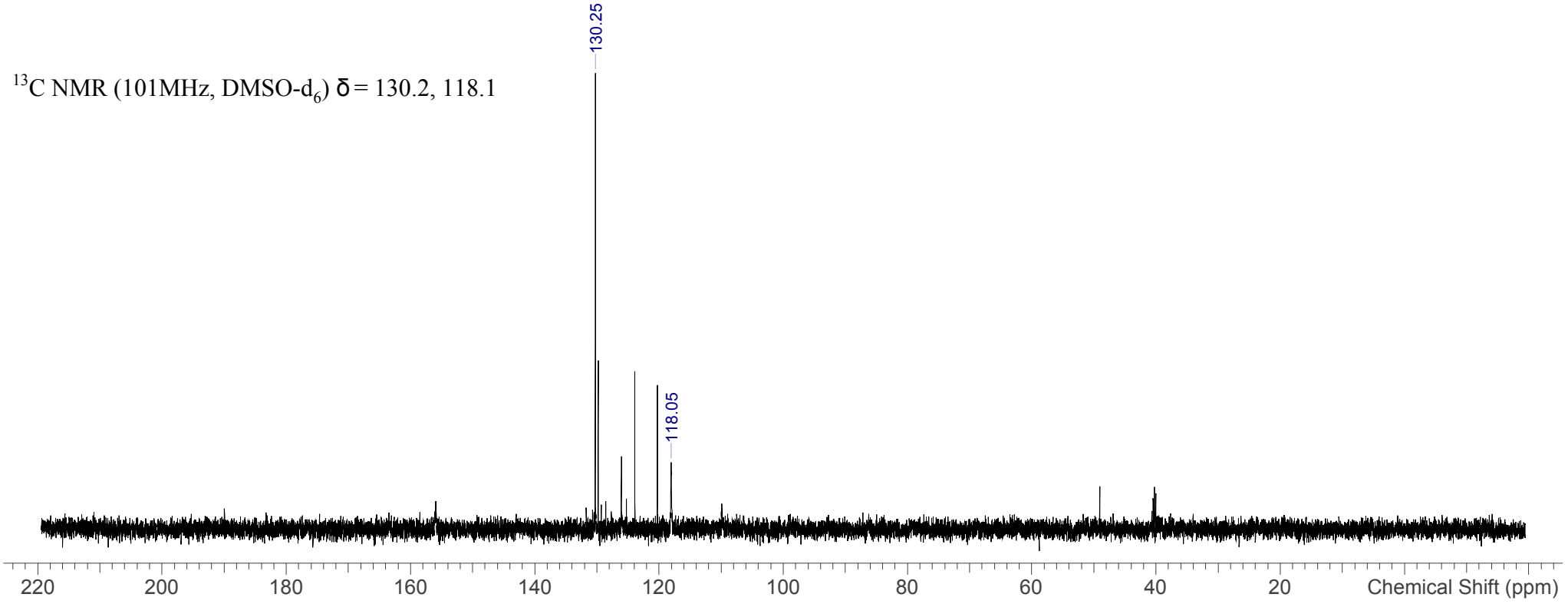
^{13}C NMR (101MHz, DMSO-d_6) δ = 155.9 (C-8, 12), 150.3 (C-1, 14), 130.2, 129.8, 118.0, 109.9 (C-11, 9)



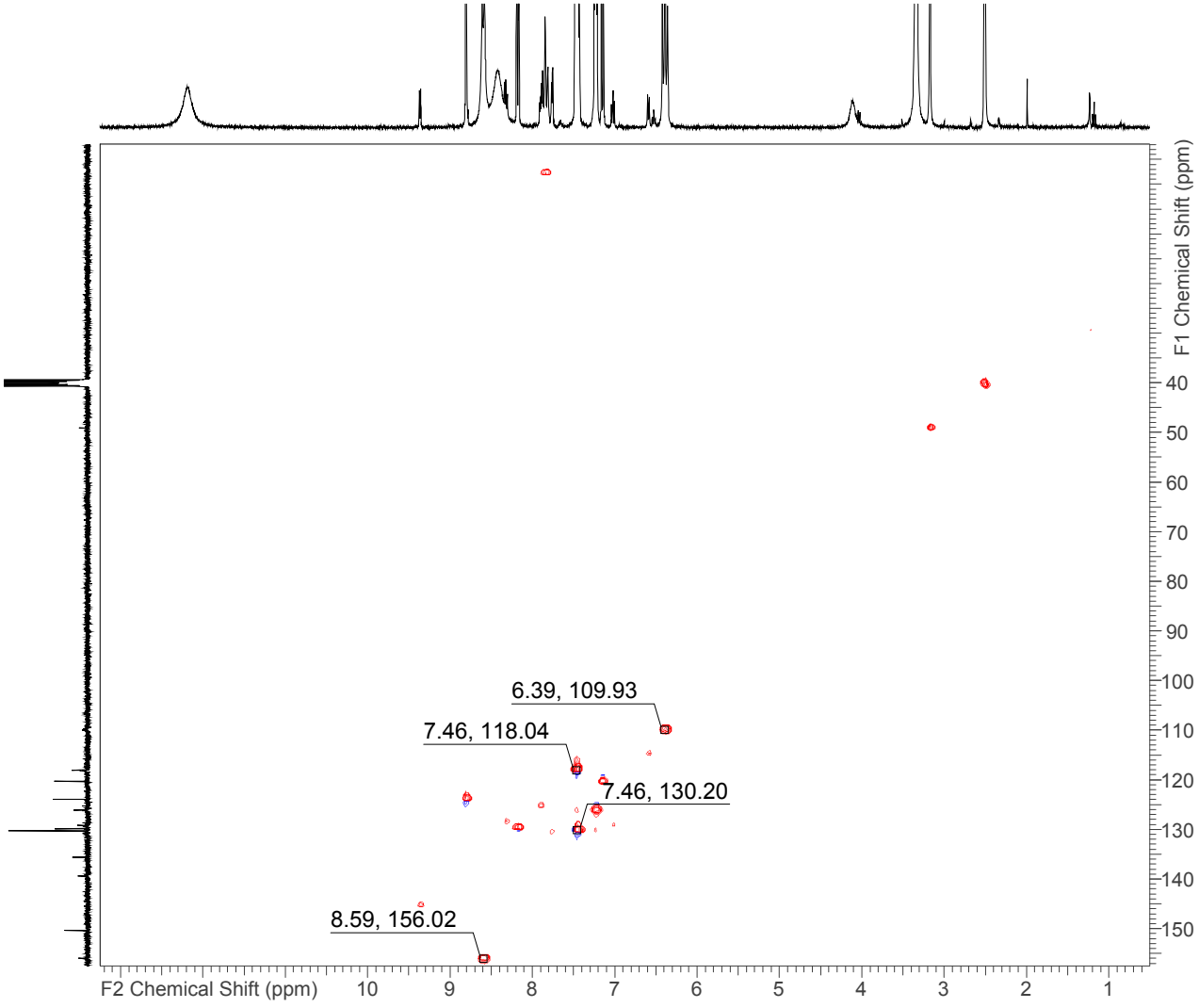
Acquisition Time (sec)	1.3631	***Comment***	mm/7500/105 Cy7 linker
Date	05 Aug 2015 06:36:29		
Date Stamp	05 Aug 2015 06:36:29		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au0415mm2 Cy7 linker\13\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp45
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	10061.0684
Spectrum Type	DEPT45	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	25.004		

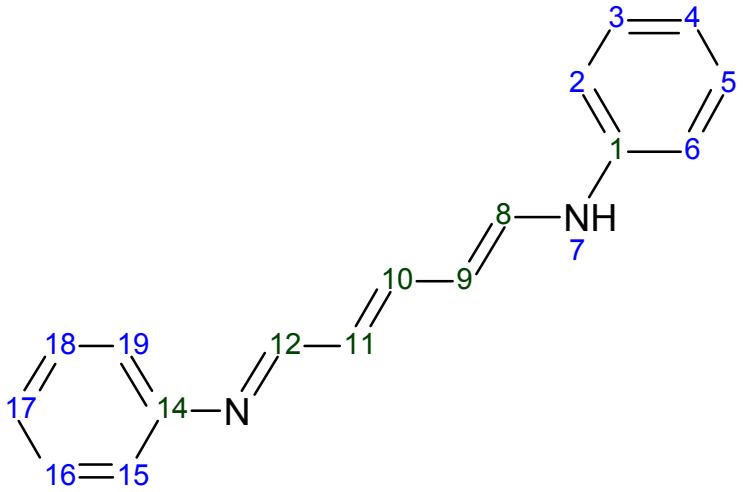


^{13}C NMR (101MHz, DMSO-d_6) δ = 130.2, 118.1



Acquisition Time (sec)	(0.1004, 0.0154)	***Comment***	mm/7500/105 Cy7 linker
Date	05 Aug 2015 06:51:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au0415mm2 Cy7 linker\14\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)		
Nucleus	(1H, 13C)	***Number of Transients***	2
Origin	spect	***Original Points Count***	(512, 256)
Owner	nmr	***Points Count***	(1024, 1024)
Pulse Sequence	hsqcetgp	***Solvent***	DMSO-d6
Spectrum Type	HSQC	***Sweep Width (Hz)***	(5097.06, 16650.39)
Temperature (degree C)	25.039	***Title***	mm/7500/105 Cy7 linker



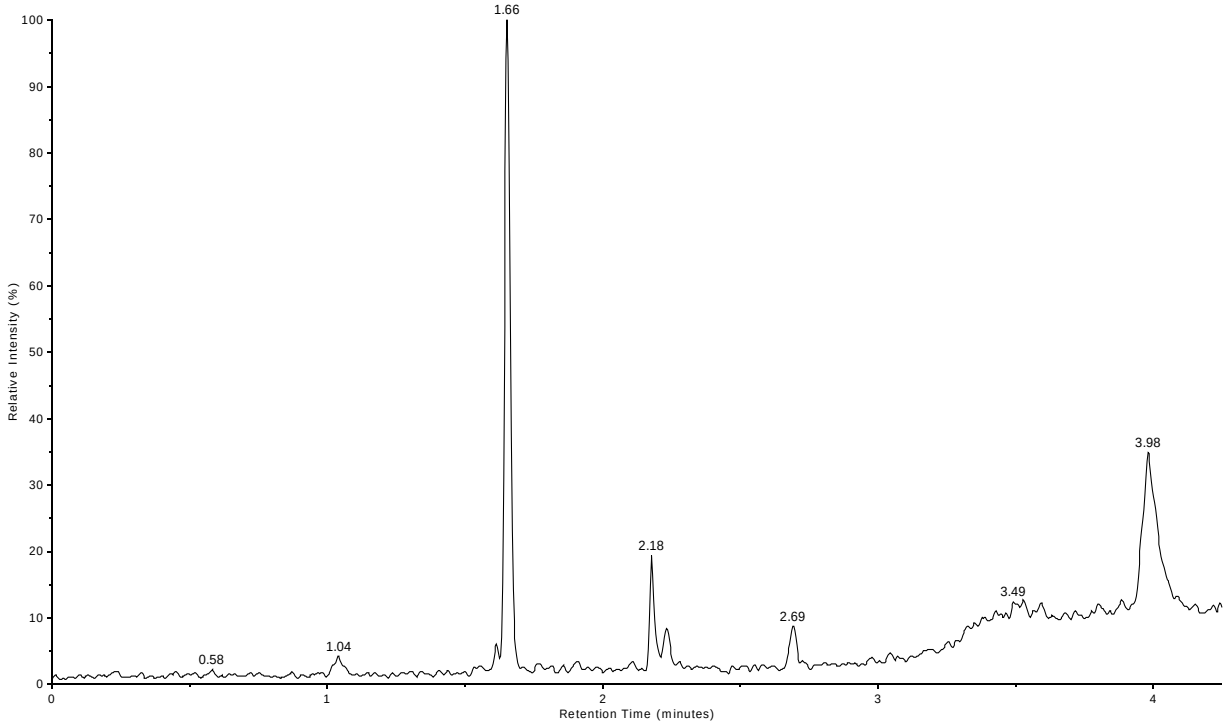


A.16 Cy7-Hemi

Top 5 Peak Report

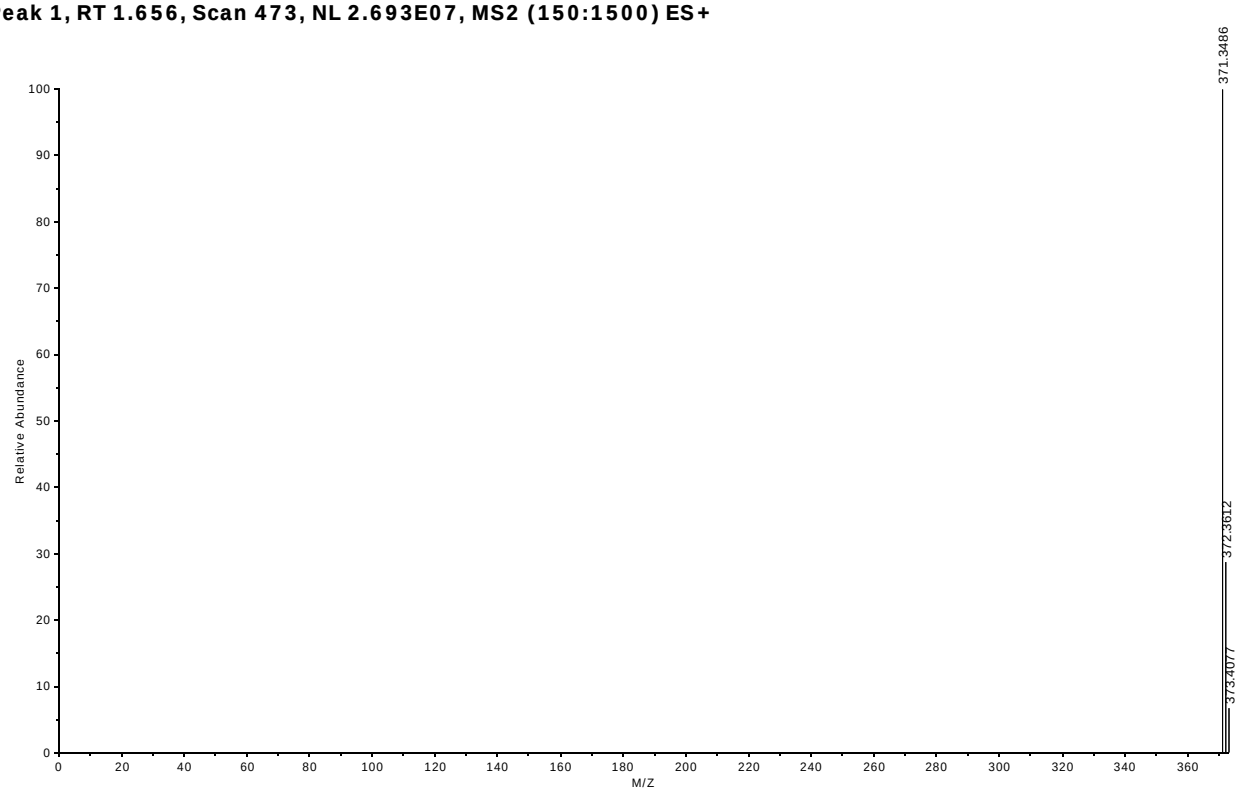
Group: Watts, J
Submitter: Michael Moazami
Project: RP LC C18 < 2 cmpds
Sample ID: mm/7500/106 Cy7 Hemicyanine N-Me
Experiment: BLUE ESIPOS C18 5 min
Filename: mm7500106_Cy7_Hemicyanine_NMe_Michael_M
Instrument: Blue RP UHPLC-MS (B30:1023) ON-AcNE
Acquisition Date: 06/08/2015 11:40:50

TIC, NL 3.000E07, MS2 (150:1500) ES+

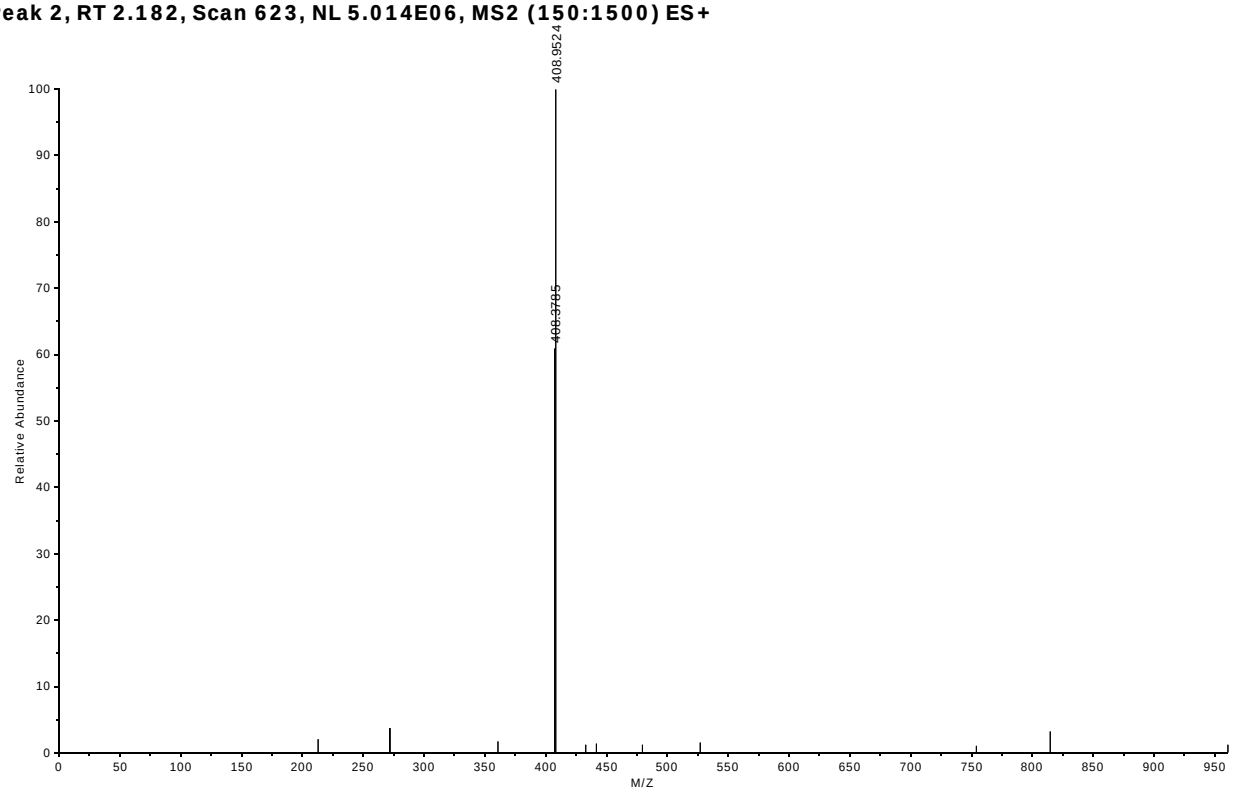


	RT Mins	Height	Height %	Area	Area %
1	1.66	29063050	100.00	42407768	100.00
2	2.18	4884081	16.81	7018188	16.55
3	2.69	1823852	6.28	3759008	8.86
4	3.98	6837668	23.53	27669889	65.25

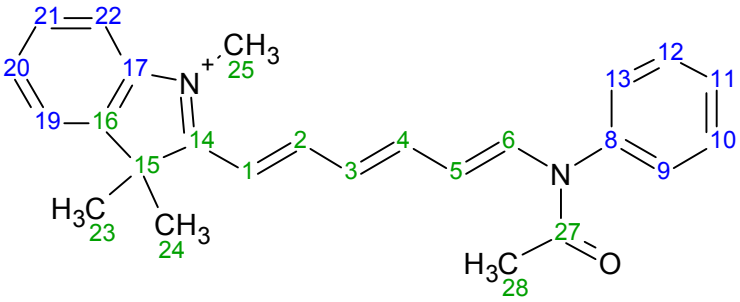
Peak 1, RT 1.656, Scan 473, NL 2.693E07, MS2 (150:1500) ES+



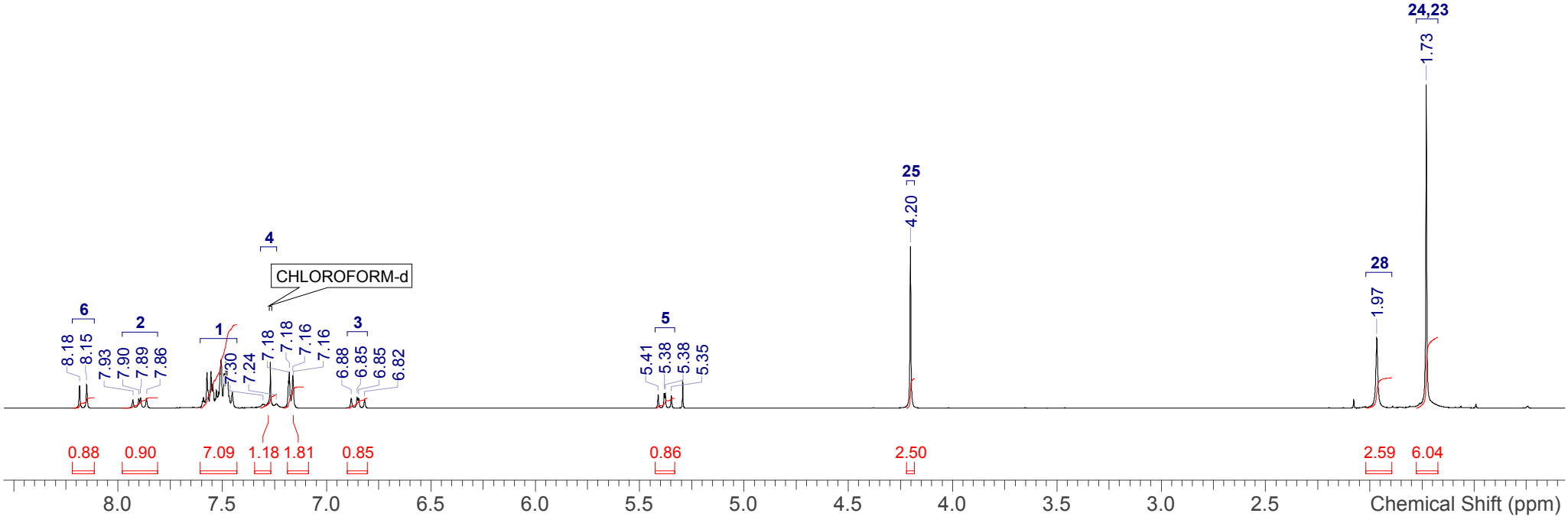
Peak 2, RT 2.182, Scan 623, NL 5.014E06, MS2 (150:1500) ES+



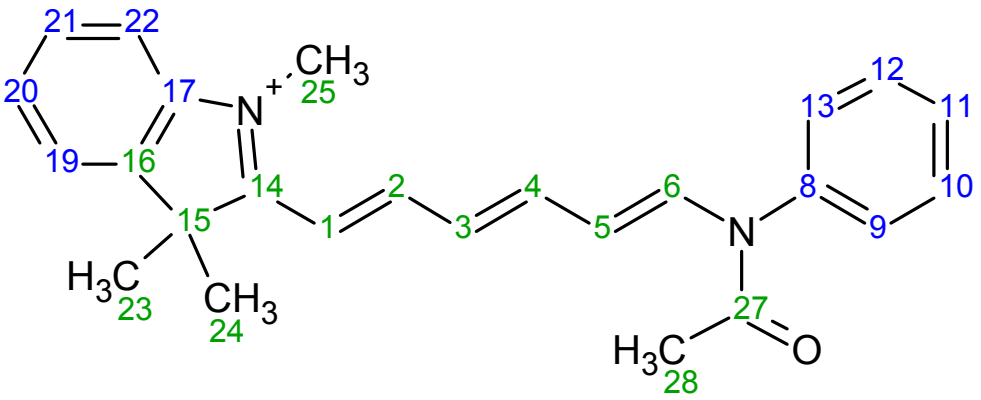
Acquisition Time (sec)	4.0894	***Comment***	mm/7500/117 F14-22
Date	20 Aug 2015 19:44:55		
Date Stamp	20 Aug 2015 19:44:55		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au2015mm1 Cy7 Hemicyanine later fractions\10\PDATA\1\1r		
Frequency (MHz)	400.1300	***Nucleus***	1H
Number of Transients	16	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	65536	***Pulse Sequence***	zg30
Receiver Gain	100.45	***SW(cyclical) (Hz)***	8012.82
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	2464.9165	***Spectrum Type***	standard
Sweep Width (Hz)	8012.70	***Temperature (degree C)***	24.999



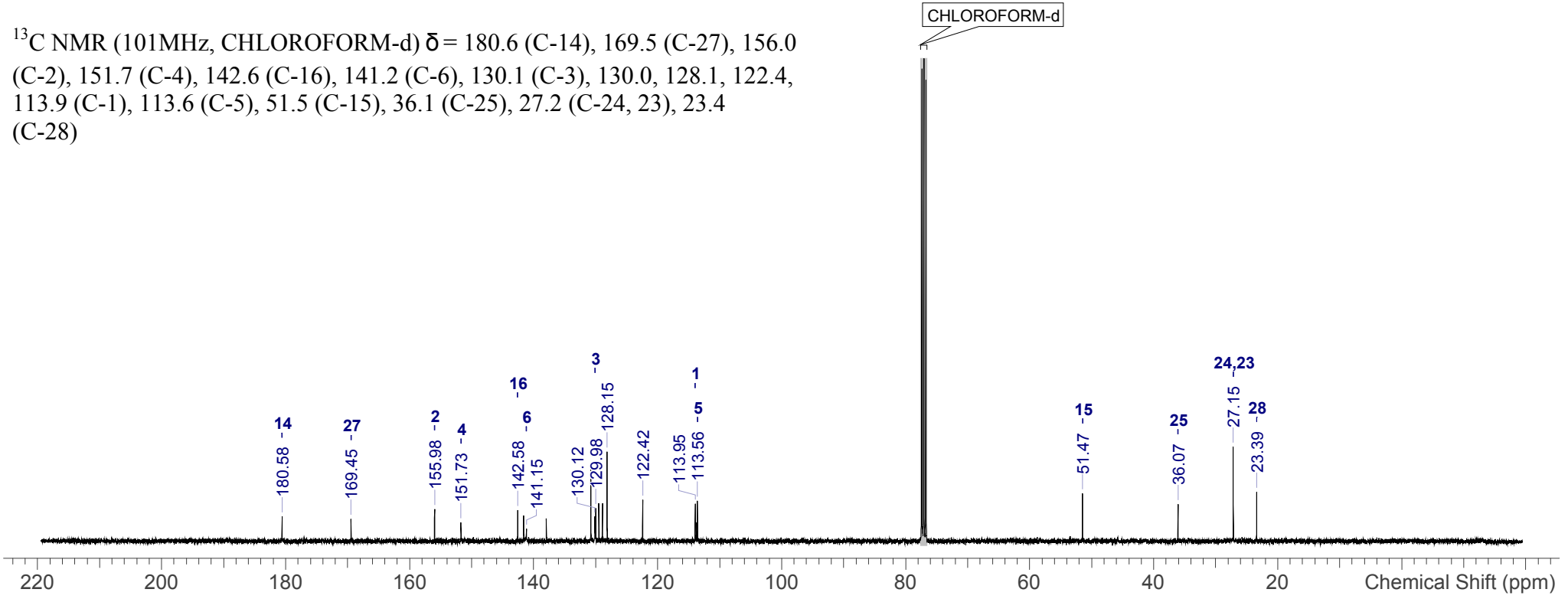
^{1}H NMR (400MHz, CHLOROFORM-d) δ = 8.17 (1H, d, J = 13.7 Hz, H-6), 7.90 (1H, dd, J = 14.9 Hz, J = 11.4 Hz, H-2), 7.43 - 7.61 (7H, m, H-1), 7.24 - 7.32 (1H, m, H-4), 7.17 (2H, dd, J = 8.0 Hz, J = 1.3 Hz, M06), 6.85 (1H, dd, J = 14.2 Hz, J = 11.4 Hz, H-3), 5.38 (1H, dd, J = 13.7 Hz, J = 11.5 Hz, H-5), 4.20 (3H, s, H-25), 1.97 (3H, s, H-28), 1.73 (6H, s, H-24, 23)



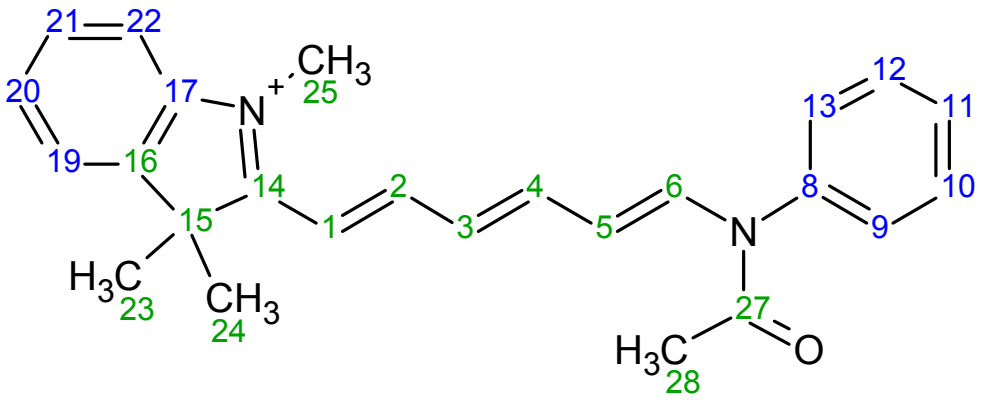
Acquisition Time (sec)	1.3631	***Comment***	mm/7500/117 F14-22
Date	20 Aug 2015 20:15:22		
Date Stamp	20 Aug 2015 20:15:22		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au2015mm1 Cy7 Hemicyanine later fractions\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	10060.3320	***Spectrum Type***	standard
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	24.999



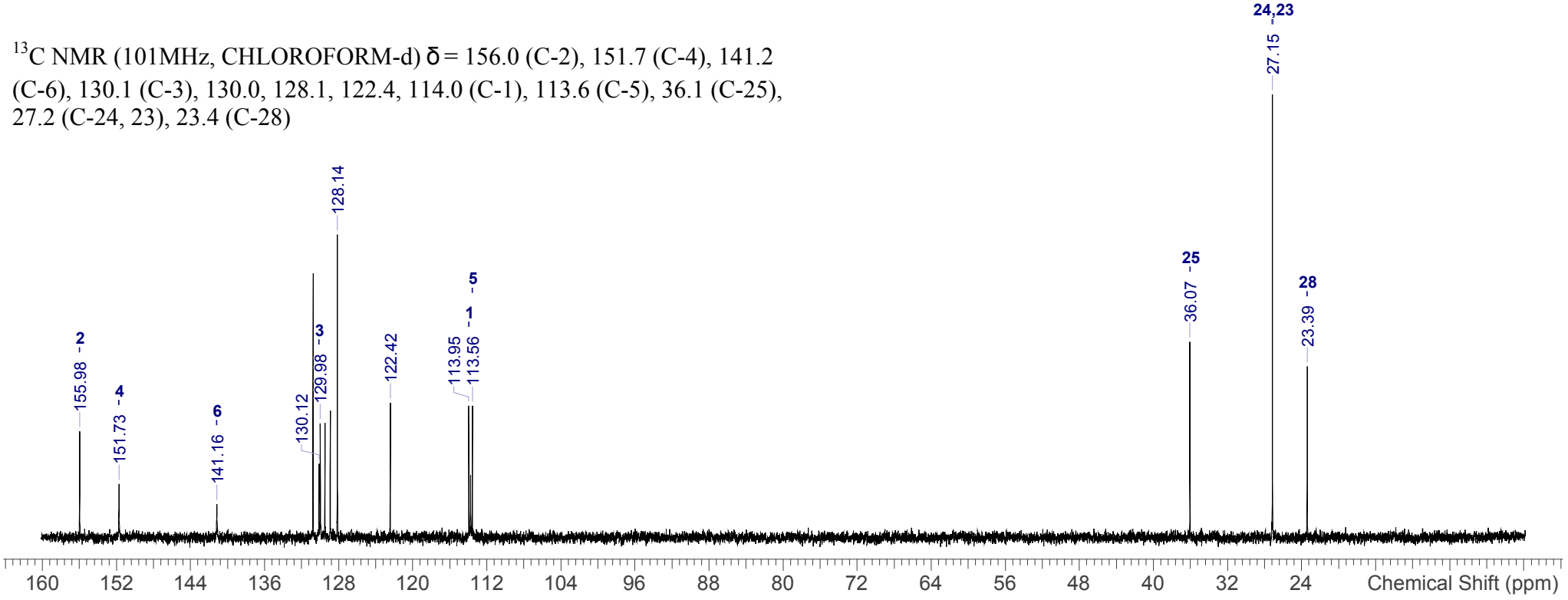
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 180.6 (C-14), 169.5 (C-27), 156.0 (C-2), 151.7 (C-4), 142.6 (C-16), 141.2 (C-6), 130.1 (C-3), 130.0, 128.1, 122.4, 113.9 (C-1), 113.6 (C-5), 51.5 (C-15), 36.1 (C-25), 27.2 (C-24, 23), 23.4 (C-28)



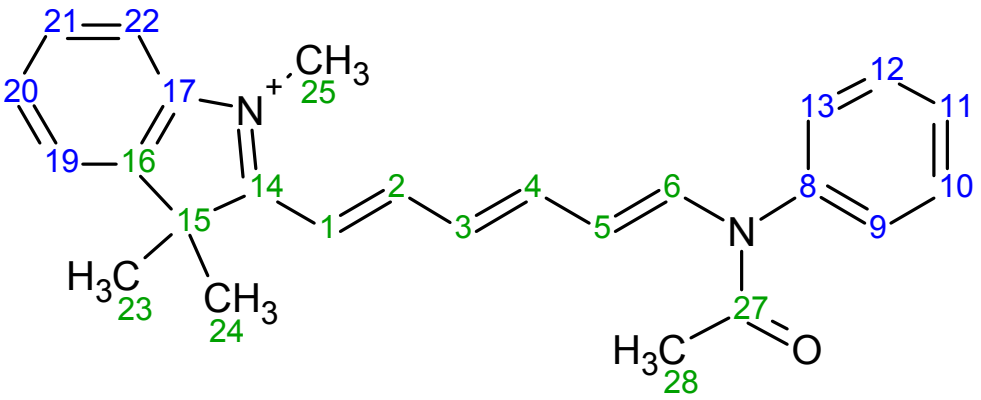
Acquisition Time (sec)	2.0316	***Comment***	mm/7500/117 F14-22
Date	20 Aug 2015 20:34:00		
Date Stamp	20 Aug 2015 20:34:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au2015mm1 Cy7 Hemicyanine later fractions\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	CHLOROFORM-d	***Spectrum Offset (Hz)***	8047.7617
Spectrum Type	DEPT135	***Sweep Width (Hz)***	16128.54
Temperature (degree C)	25.000		



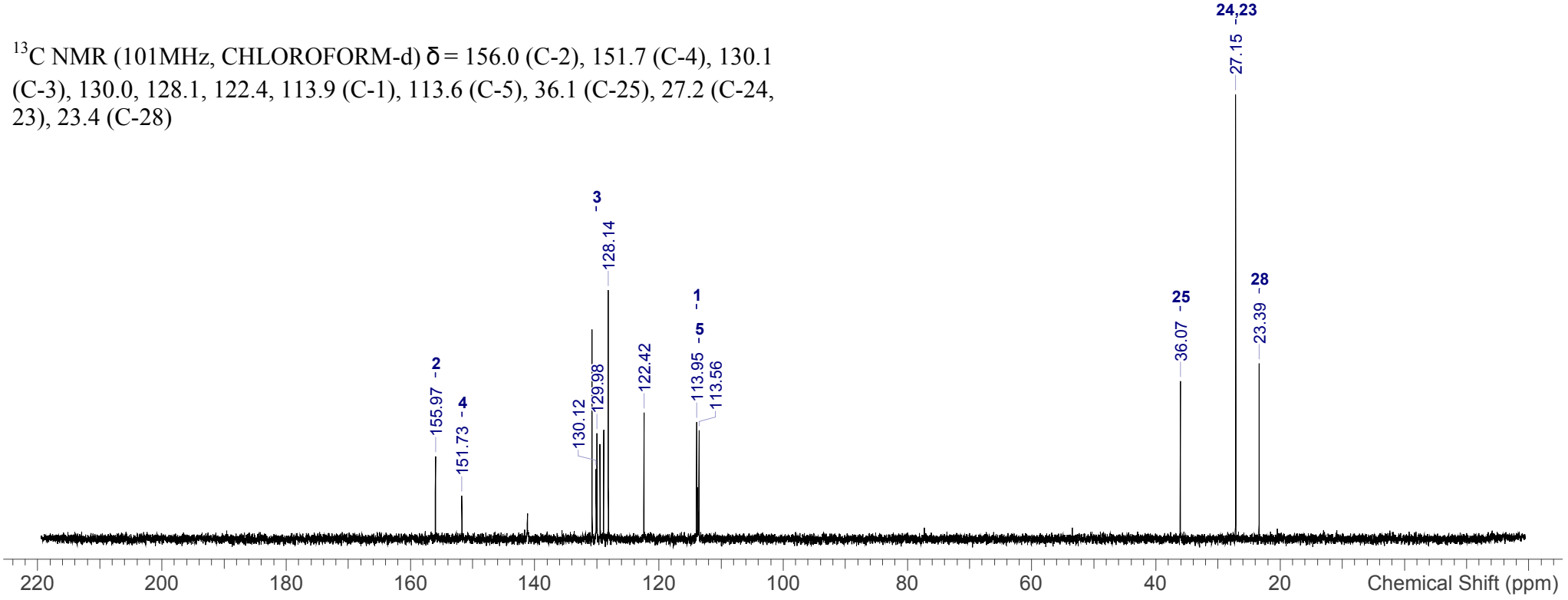
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 156.0 (C-2), 151.7 (C-4), 141.2 (C-6), 130.1 (C-3), 130.0, 128.1, 122.4, 114.0 (C-1), 113.6 (C-5), 36.1 (C-25), 27.2 (C-24, 23), 23.4 (C-28)



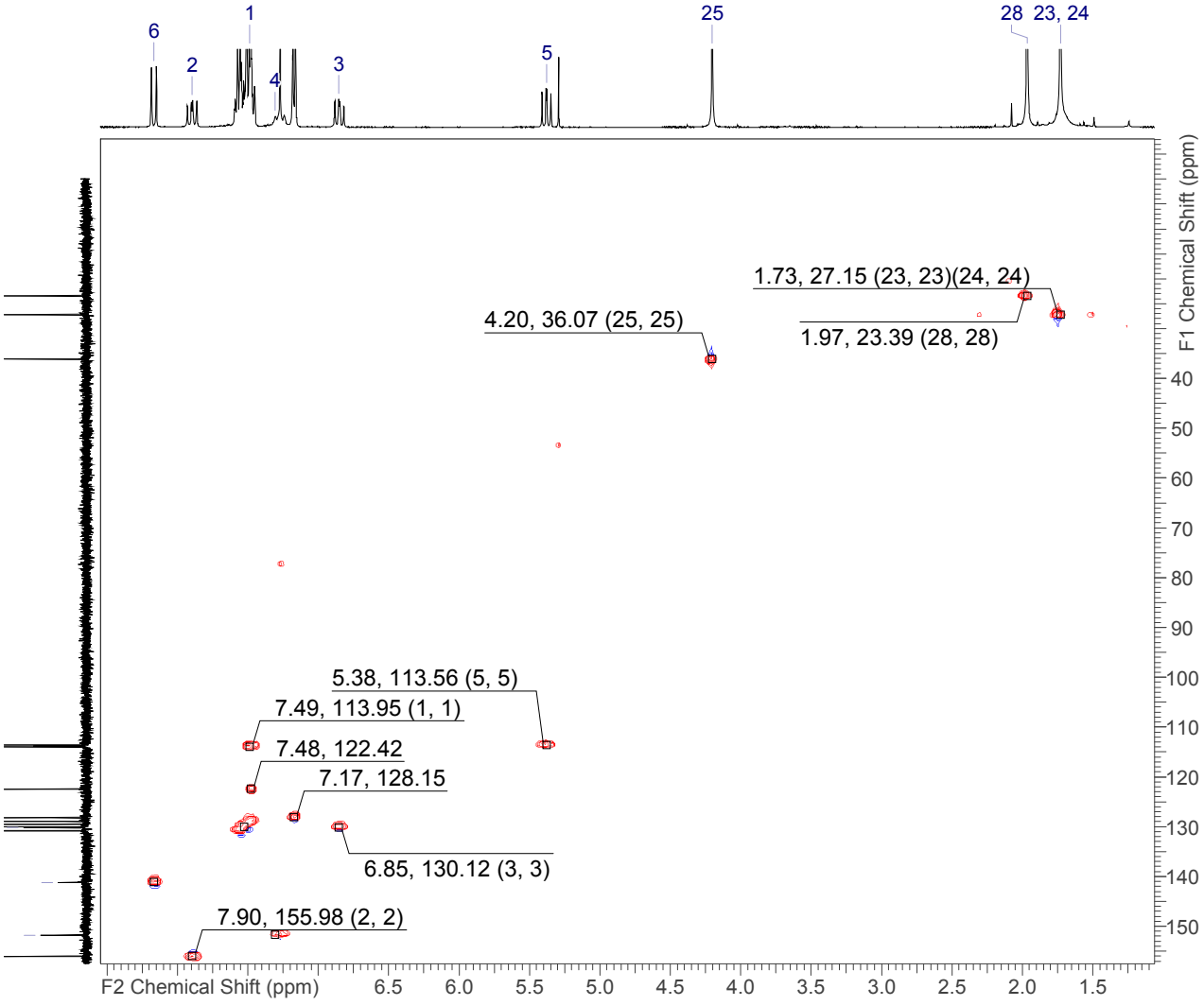
Acquisition Time (sec)	1.3631	***Comment***	mm/7500/117 F14-22
Date	20 Aug 2015 21:27:37		
Date Stamp	20 Aug 2015 21:27:37		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au2015mm1 Cy7 Hemicyanine later fractions\15\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp45
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d	***Spectrum Offset (Hz)***	10060.3564
Spectrum Type	DEPT45	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	24.999		

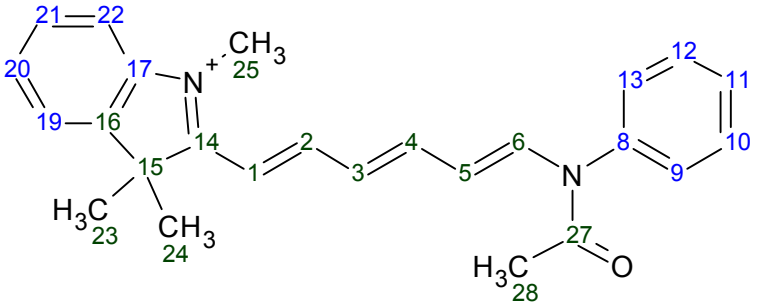


^{13}C NMR (101MHz, CHLOROFORM-d) δ = 156.0 (C-2), 151.7 (C-4), 130.1 (C-3), 130.0, 128.1, 122.4, 113.9 (C-1), 113.6 (C-5), 36.1 (C-25), 27.2 (C-24, 23), 23.4 (C-28)

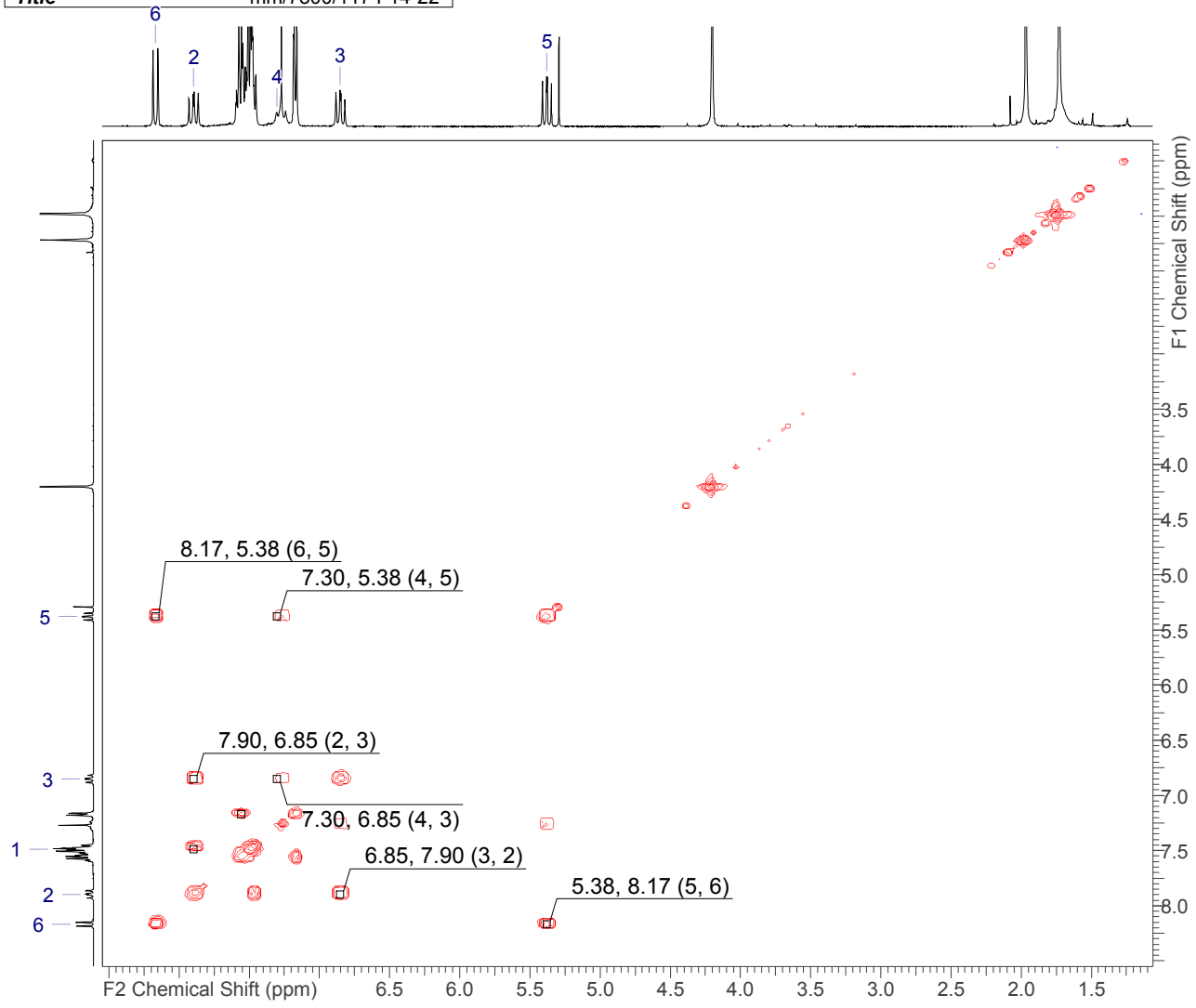


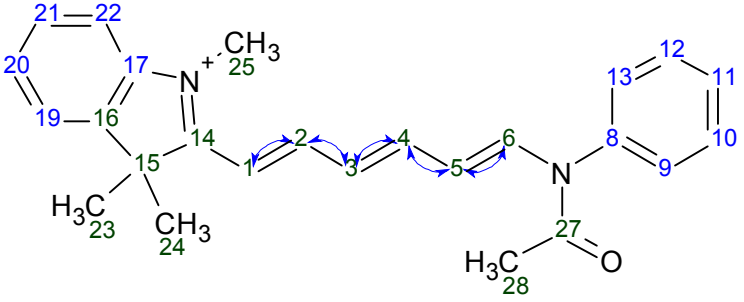
Acquisition Time (sec)	(0.1546, 0.0154)	***Comment***	mm/7500/117 F14-22
Date	20 Aug 2015 21:42:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au2015mm1 Cy7 Hemicyanine later fractions\16\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)	***Nucleus***	(1H, 13C)
Number of Transients	2	***Origin***	spect
Original Points Count	(512, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	hsqcetgp
Solvent	CHLOROFORM-d	***Spectrum Type***	HSQC
Sweep Width (Hz)	(3308.02, 16650.39)	***Temperature (degree C)***	25.059
Title	mm/7500/117 F14-22		





Acquisition Time (sec)	(0.3092, 0.0773)	***Comment***	mm/7500/117 F14-22
Date	20 Aug 2015 20:50:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au2015mm1 Cy7 Hemicyanine later fractions\13\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)	***Nucleus***	(1H, 1H)
Number of Transients	2	***Origin***	spect
Original Points Count	(1024, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	cosygpqf
Solvent	CHLOROFORM-d	***Spectrum Type***	COSY
Sweep Width (Hz)	(3308.02, 3308.02)	***Temperature (degree C)***	24.998
Title	mm/7500/117 F14-22		



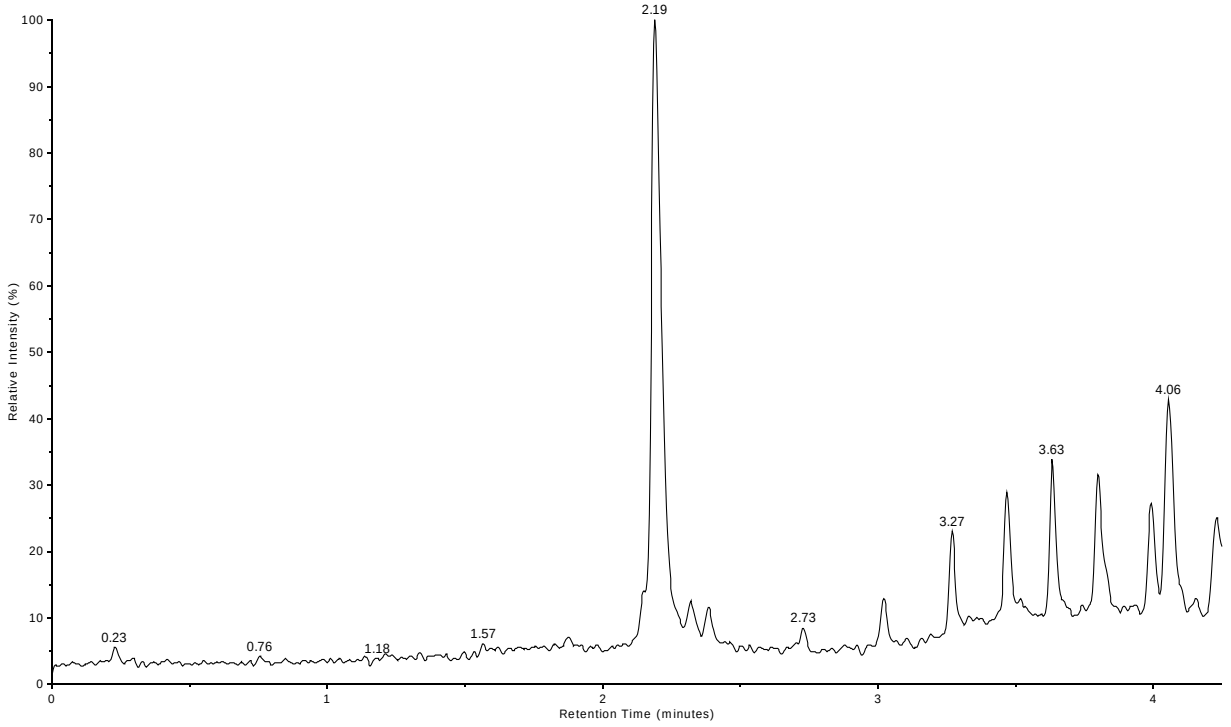


A.17 Cy7-COOH

Top 5 Peak Report

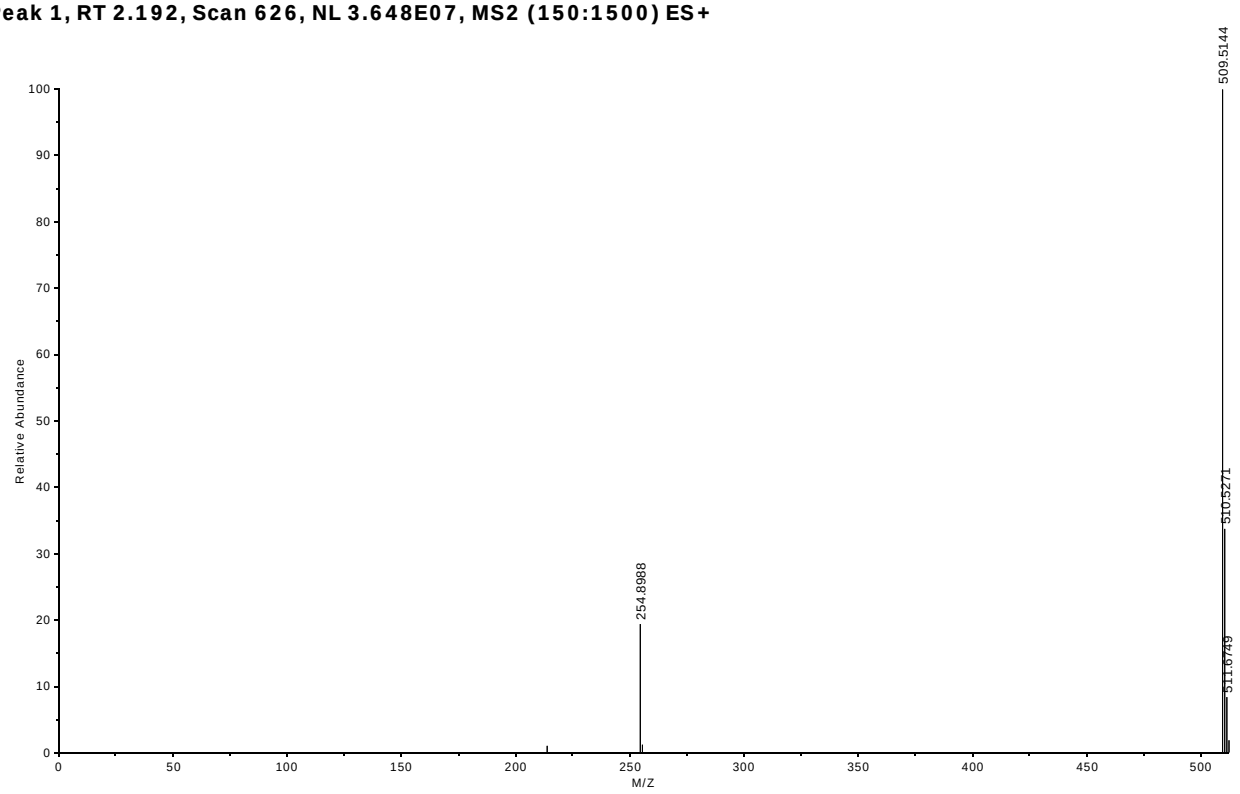
Group: Watts, J
Submitter: Michael Moazami
Project: RP LC C18 < 2 cmpds
Sample ID: mm/7500/119 Cy7 COOH
Experiment: BLUE ESIPOS C18 5 min
Filename: mm7500119_Cy7_COOH_Michael_Moazami__Wa
Instrument: Blue RP UHPLC-MS (B30:1023) ON-ACN
Acquisition Date: 26/08/2015 12:48:32

TIC, NL 5.926E07, MS2 (150:1500) ES+

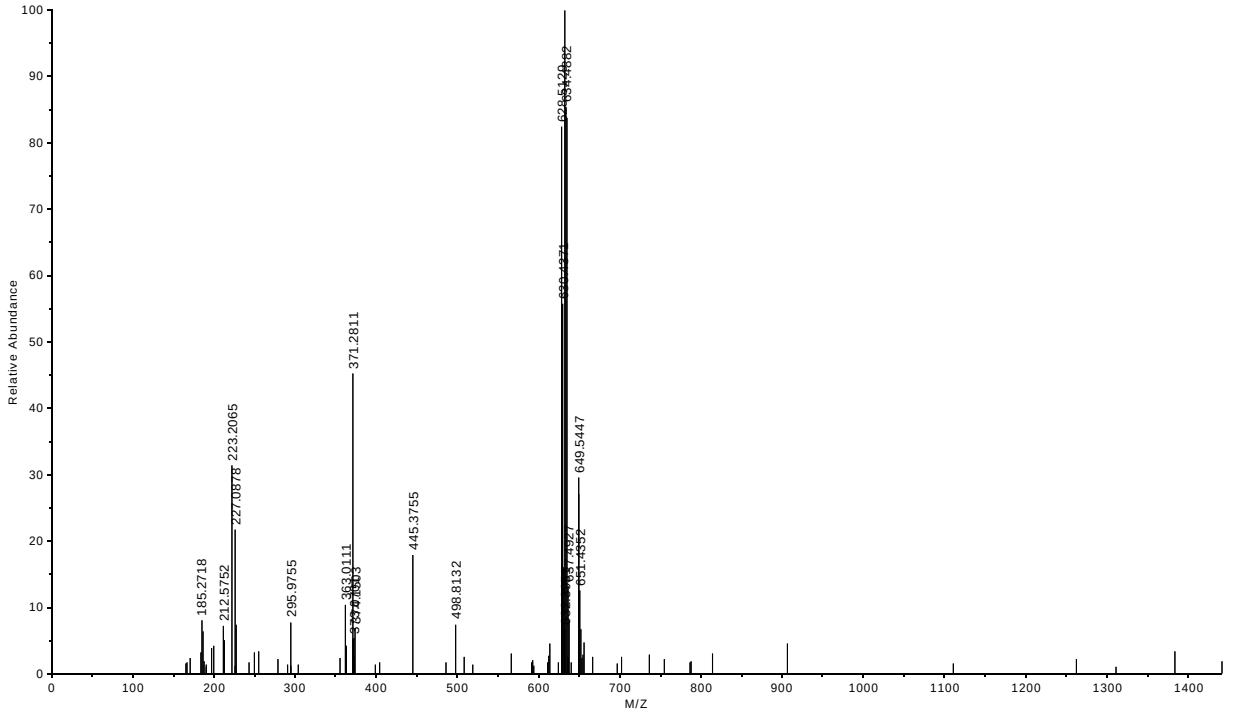


	RT Mins	Height	Height %	Area	Area %
1	2.19	52650642	100.00	141514369	100.00
2	3.47	10662237	20.25	19872569	14.04
3	3.63	13991629	26.57	24699989	17.45
4	3.80	11976867	22.75	25116205	17.75
5	4.06	18175085	34.52	44481528	31.43

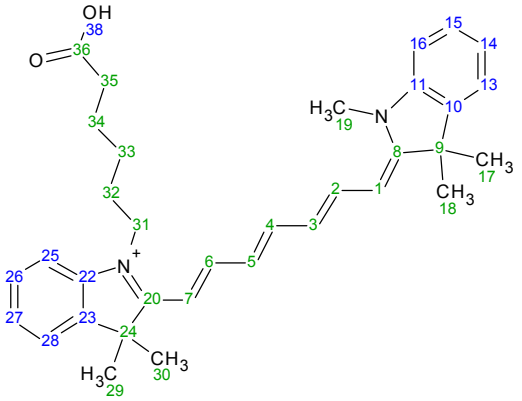
Peak 1, RT 2.192, Scan 626, NL 3.648E07, MS2 (150:1500) ES+



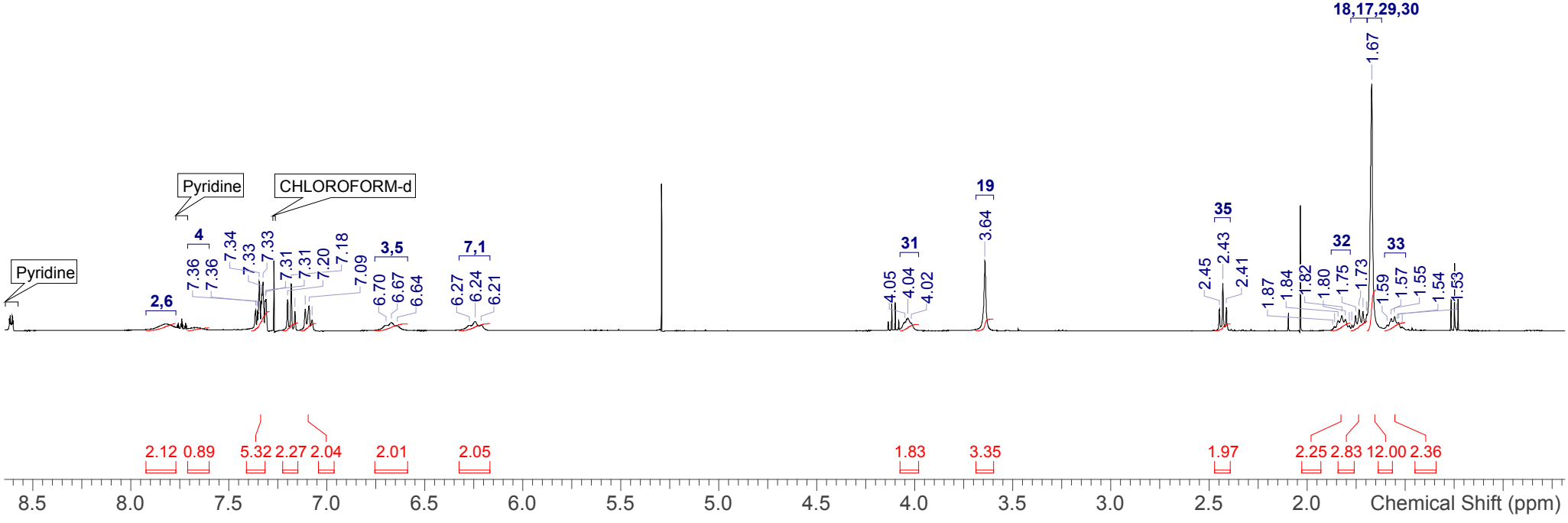
Peak 2, RT 3.470, Scan 991, NL 2.219E06, MS2 (150:1500) ES+



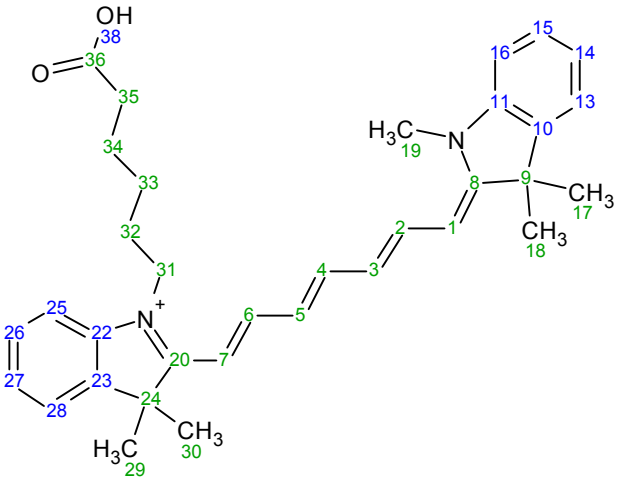
Acquisition Time (sec)	4.0894	***Comment***	mm/7500/119 Cy7COOH		
Date	25 Aug 2015 08:12:54				
Date Stamp	25 Aug 2015 08:12:54				
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au2415mm2 Cy7 COOH\10\PDATA\1\1r				
Frequency (MHz)	400.1300	***Nucleus***	1H	***Number of Transients***	16
Origin	spect	***Original Points Count***	32768	***Owner***	nmr
Points Count	262144	***Pulse Sequence***	zg30	***Receiver Gain***	72.67
SW(cyclical) (Hz)	8012.82	***Solvent***	CHLOROFORM-d		
Spectrum Offset (Hz)	2464.8303	***Spectrum Type***	standard	***Sweep Width (Hz)***	8012.79
Temperature (degree C)	25.002				



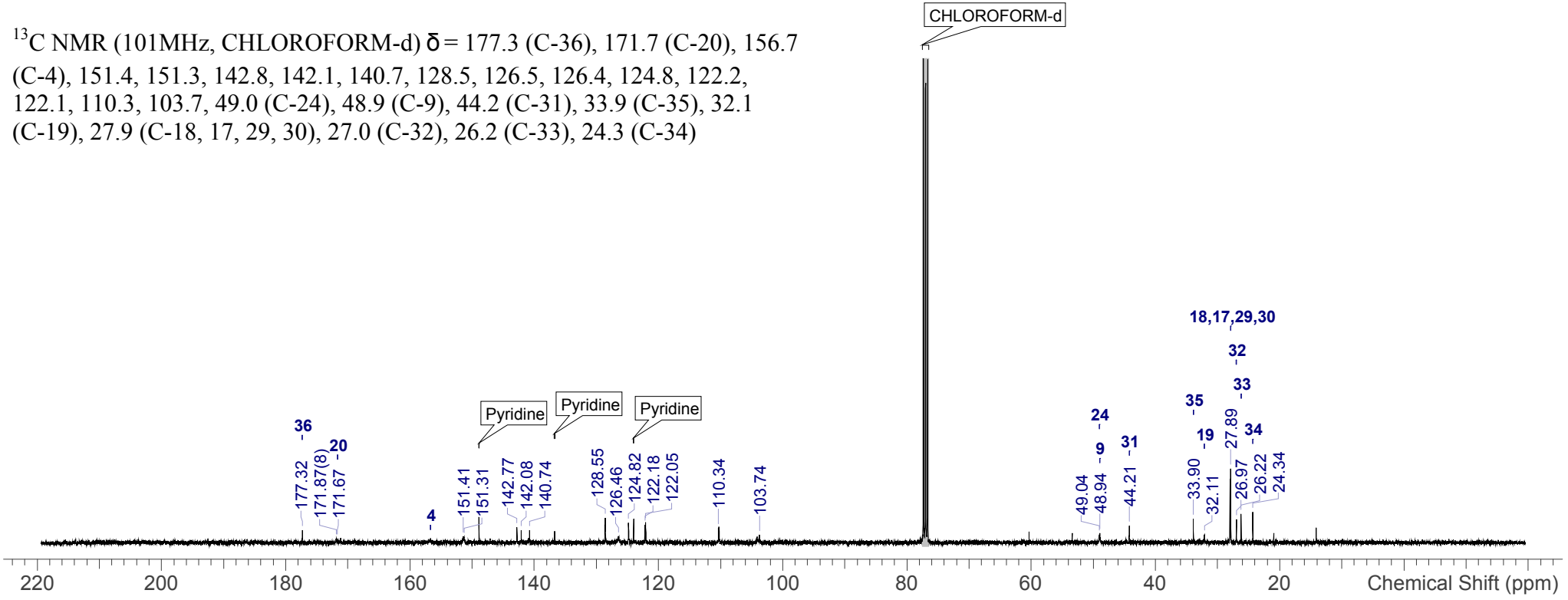
^{1}H NMR (400MHz, CHLOROFORM-d) δ = 7.77 - 7.92 (2H, m, H-2, 6), 7.60 - 7.71 (1H, m, H-4), 7.29 - 7.35 (2H, m, M15), 7.29 - 7.39 (3H, m, M11), 7.15 - 7.22 (2H, m, M10), 7.06 - 7.13 (2H, m, M09), 6.67 (2H, br t, J = 11.5 Hz, H-3, 5), 4.04 (2H, br t, J = 6.6 Hz, H-7, 1), 4.04 (2H, br t, J = 6.6 Hz, H-31), 3.64 (3H, s, H-19), 2.43 (2H, t, J = 7.2 Hz, H-35), 1.78 - 1.88 (2H, m, H-32), 1.69 - 1.78 (3H, m, H-34), 1.67 (12H, s, H-18, 17, 29, 30), 1.50 - 1.61 (2H, m, H-33)



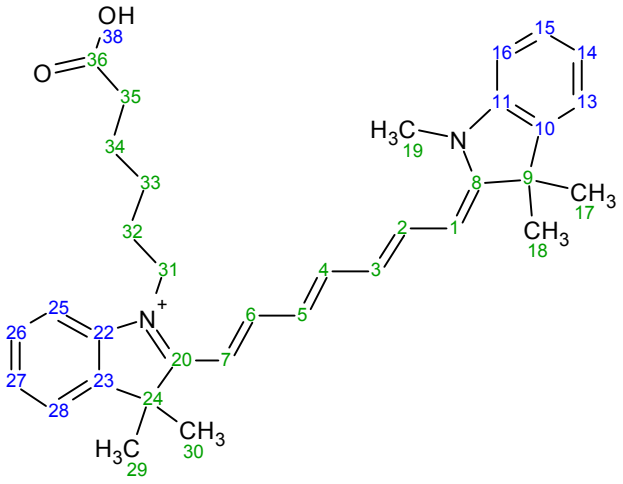
Acquisition Time (sec)	1.3631	***Comment***	mm/7500/119 Cy7COOH
Date	25 Aug 2015 08:43:42		
Date Stamp	25 Aug 2015 08:43:42		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au2415mm2 Cy7 COOH\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	10052.5049	***Spectrum Type***	standard
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	25.005



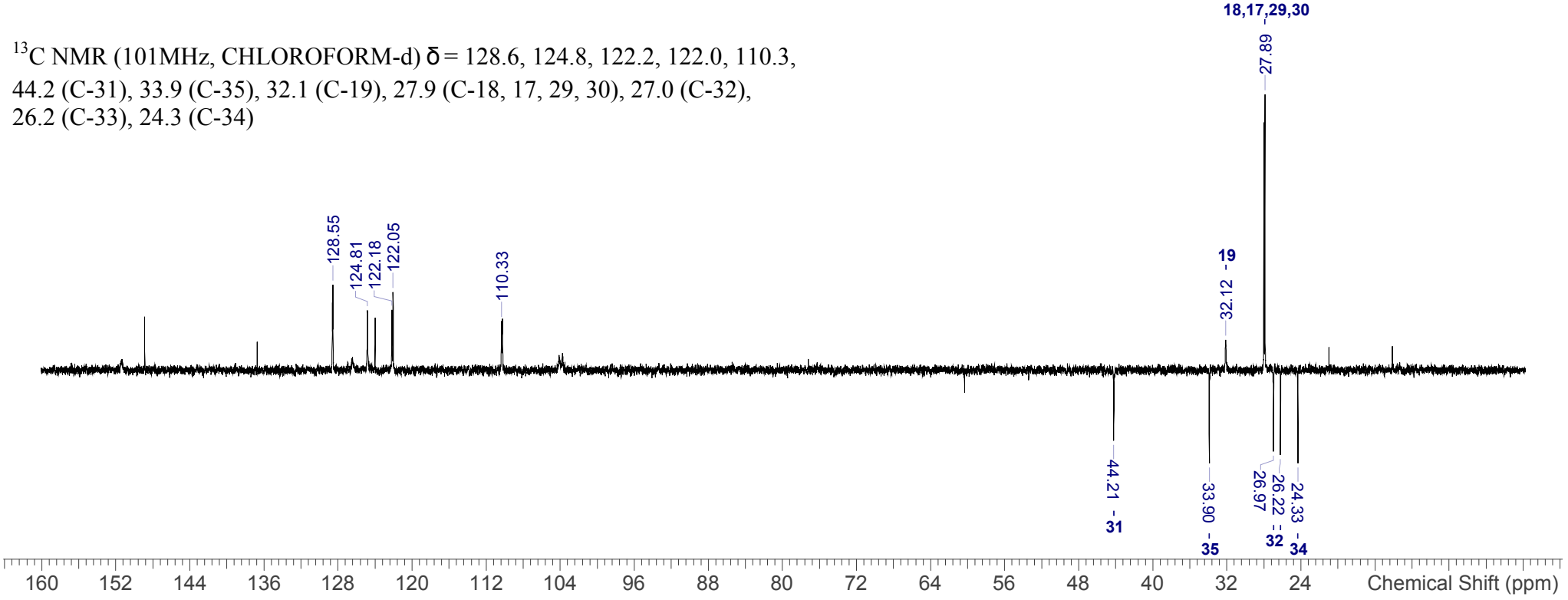
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 177.3 (C-36), 171.7 (C-20), 156.7 (C-4), 151.4, 151.3, 142.8, 142.1, 140.7, 128.5, 126.5, 126.4, 124.8, 122.2, 122.1, 110.3, 103.7, 49.0 (C-24), 48.9 (C-9), 44.2 (C-31), 33.9 (C-35), 32.1 (C-19), 27.9 (C-18, 17, 29, 30), 27.0 (C-32), 26.2 (C-33), 24.3 (C-34)



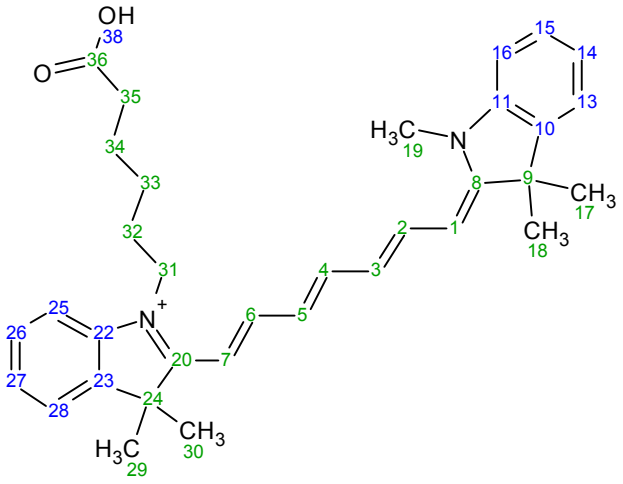
Acquisition Time (sec)	2.0316	***Comment***	mm/7500/119 Cy7COOH
Date	25 Aug 2015 10:41:28		
Date Stamp	25 Aug 2015 10:41:28		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au2415mm2 Cy7 COOH\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	8040.0107	***Spectrum Type***	DEPT135
Sweep Width (Hz)	16128.54	***Temperature (degree C)***	24.999



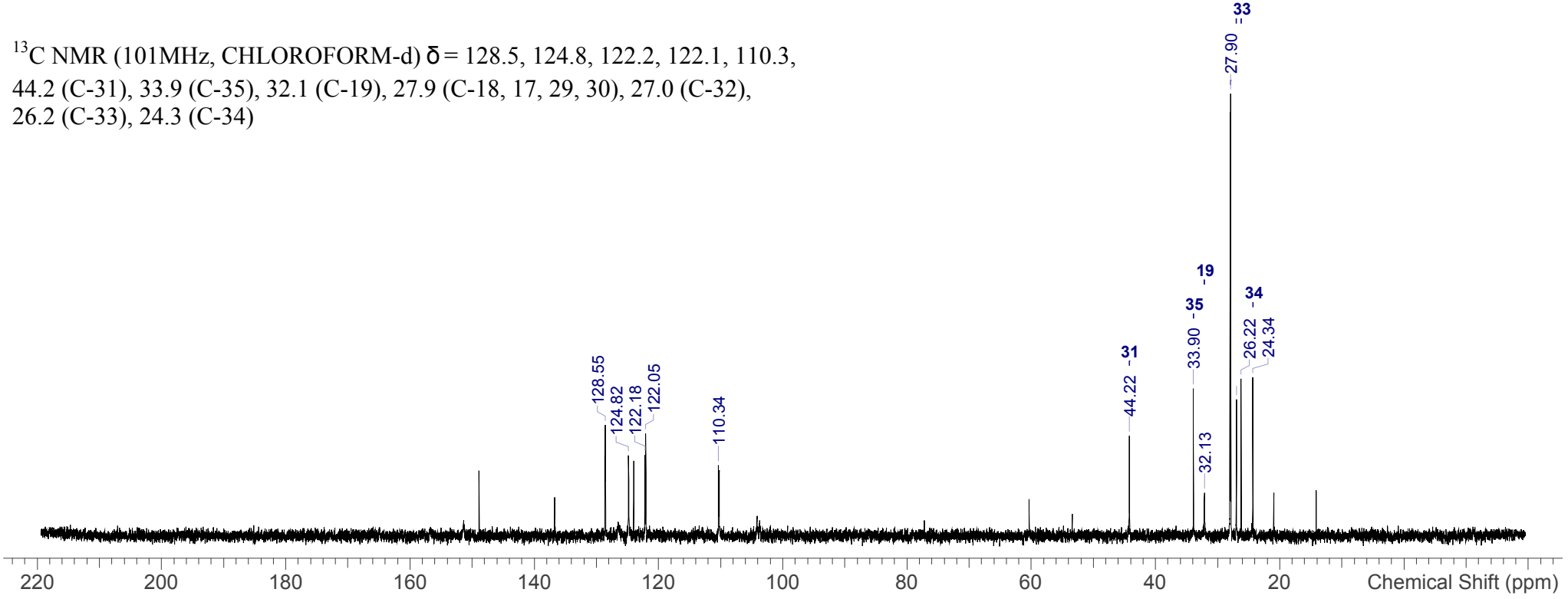
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 128.6, 124.8, 122.2, 122.0, 110.3, 44.2 (C-31), 33.9 (C-35), 32.1 (C-19), 27.9 (C-18, 17, 29, 30), 27.0 (C-32), 26.2 (C-33), 24.3 (C-34)



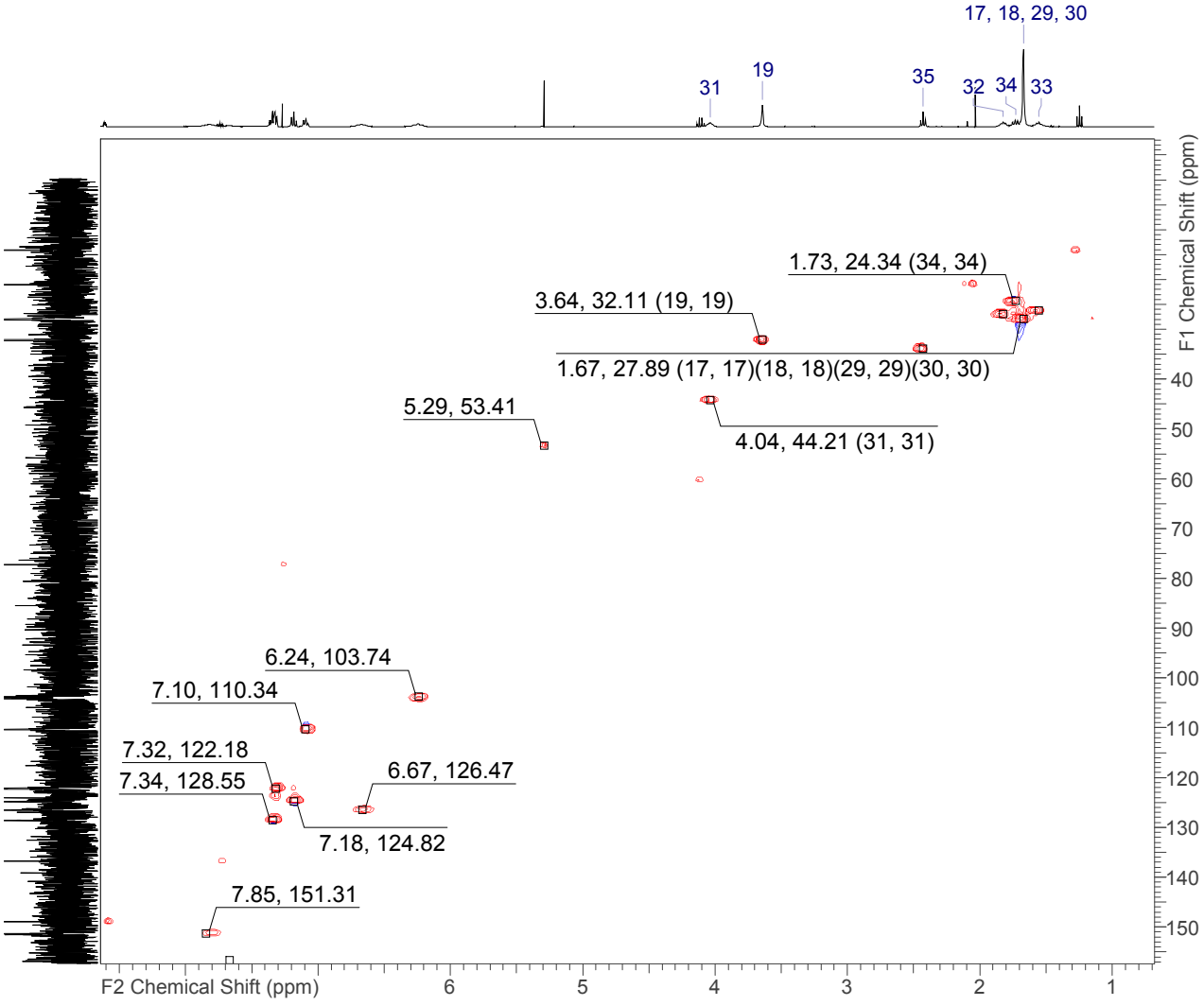
Acquisition Time (sec)	1.3631	***Comment***	mm/7500/119 Cy7COOH
Date	25 Aug 2015 11:52:54		
Date Stamp	25 Aug 2015 11:52:54		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au2415mm2 Cy7 COOH\15\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp45
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	10053.2412	***Spectrum Type***	DEPT45
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	24.998

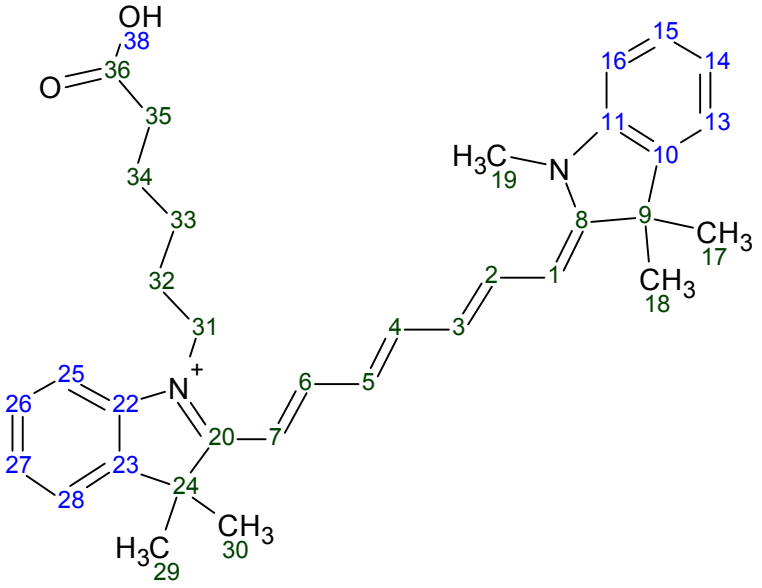


^{13}C NMR (101MHz, CHLOROFORM-d) δ = 128.5, 124.8, 122.2, 122.1, 110.3, 44.2 (C-31), 33.9 (C-35), 32.1 (C-19), 27.9 (C-18, 17, 29, 30), 27.0 (C-32), 26.2 (C-33), 24.3 (C-34)

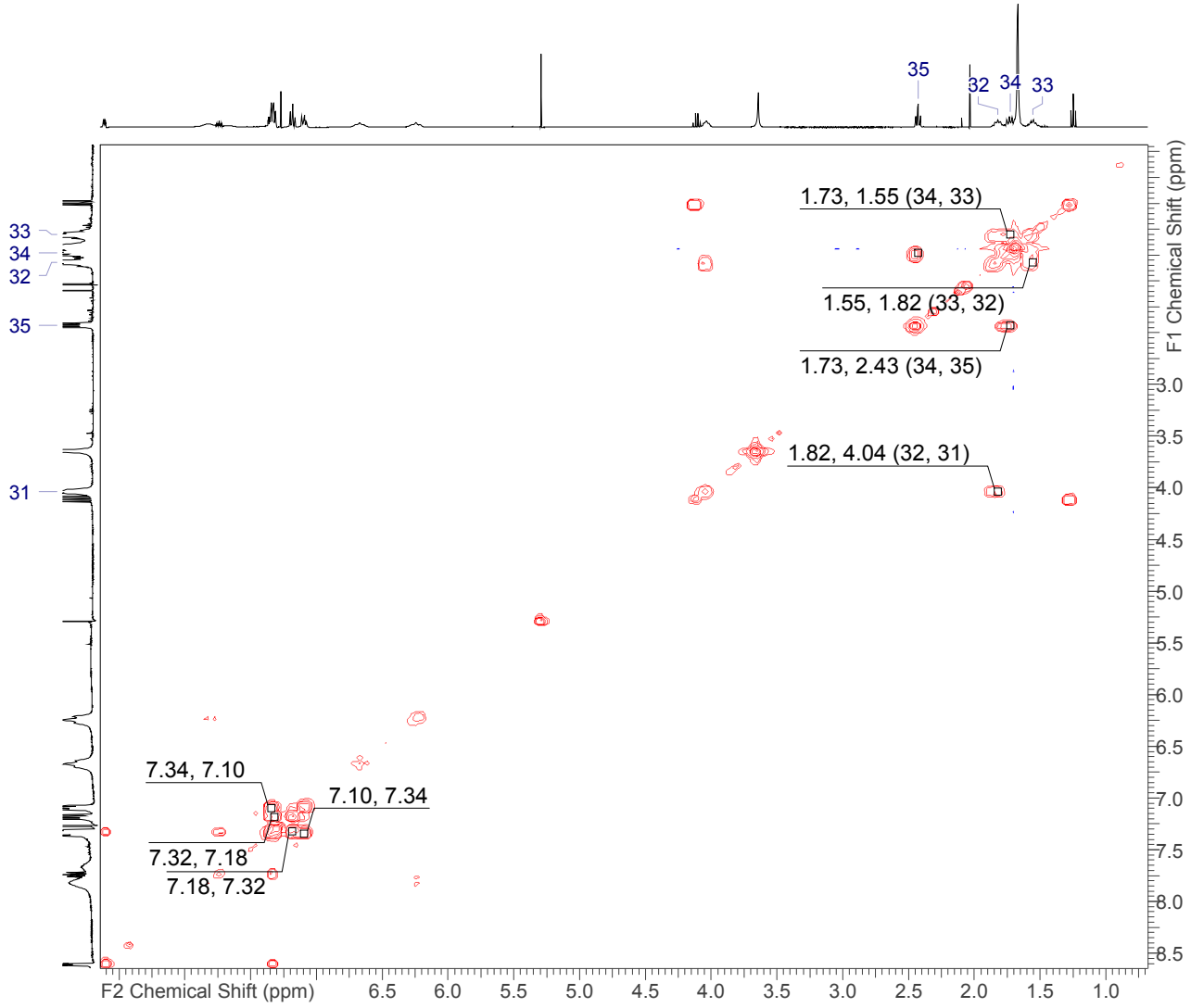


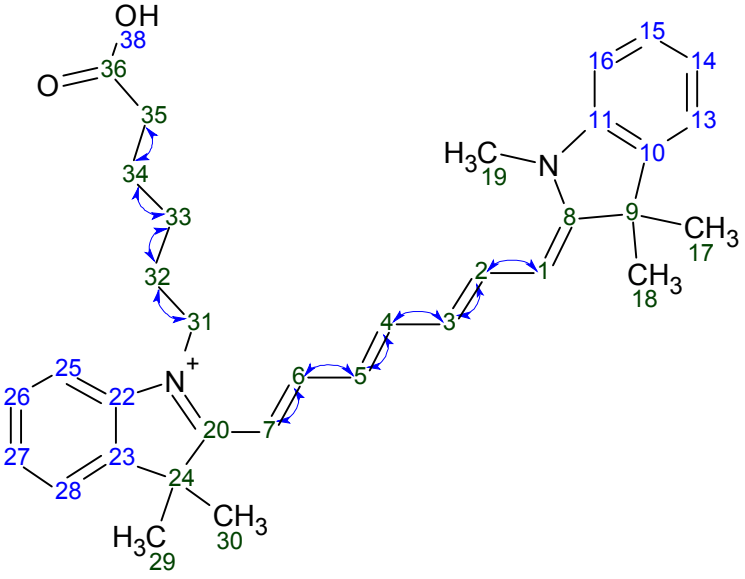
Acquisition Time (sec)	(0.1352, 0.0154)	***Comment***	mm/7500/119 Cy7COOH
Date	25 Aug 2015 12:08:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au2415mm2 Cy7 COOH\16\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)	***Nucleus***	(1H, 13C)
Number of Transients	2	***Origin***	spect
Original Points Count	(512, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	hsqcetgp
Solvent	CHLOROFORM-d	***Spectrum Type***	HSQC
Sweep Width (Hz)	(3784.18, 16650.39)	***Temperature (degree C)***	25.049
Title	mm/7500/119 Cy7COOH		





Acquisition Time (sec)	(0.2642, 0.0660)	***Comment***	mm/7500/119 Cy7COOH
Date	25 Aug 2015 10:57:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au2415mm2 Cy7 COOH\13\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)	***Nucleus***	(1H, 1H)
Number of Transients	2	***Origin***	spect
Original Points Count	(1024, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	cosygpqf
Solvent	CHLOROFORM-d	***Spectrum Type***	COSY
Sweep Width (Hz)	(3872.18, 3872.18)	***Temperature (degree C)***	24.995
Title	mm/7500/119 Cy7COOH		



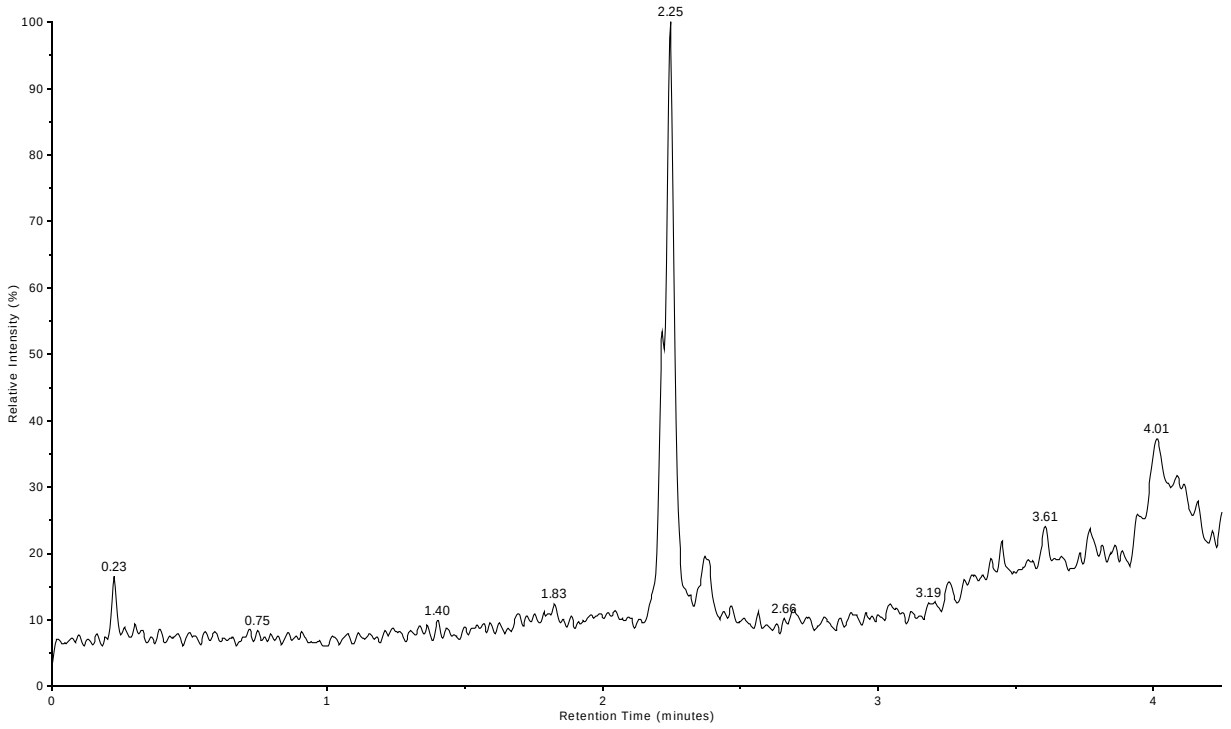


A.18 Cy7-N3

Top 5 Peak Report

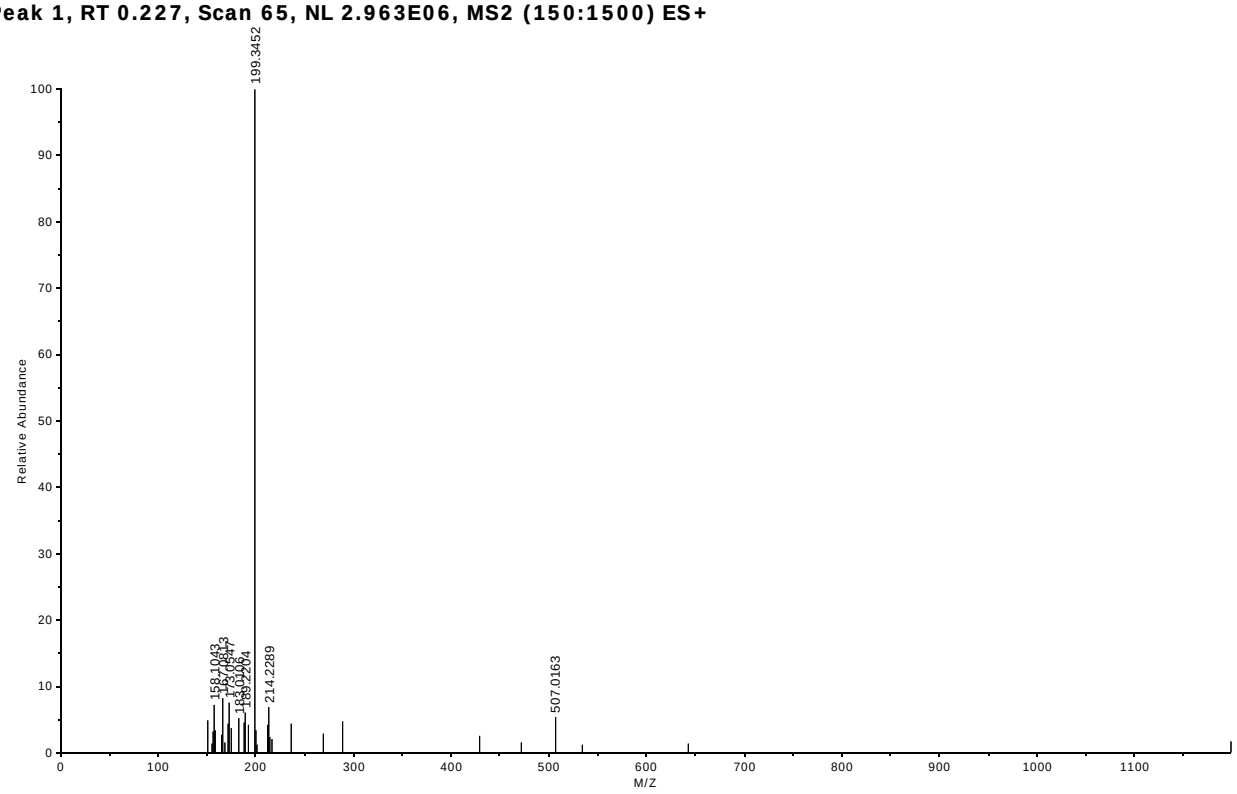
Group: Watts, J
Submitter: Michael Moazami
Project: RP LC C18 < 2 cmpds
Sample ID: mm/7500/120 Cy7-N3
Experiment: BLUE ESIPOS C18 5 min
Filename: mm7500120_Cy7N3_Michael_Moazami__Watts_
Instrument: Blue RP UHPLC-MS (B30:1023) ON-LINE
Acquisition Date: 26/08/2015 13:28:57

TIC, NL 2.973E07, MS2 (150:1500) ES+

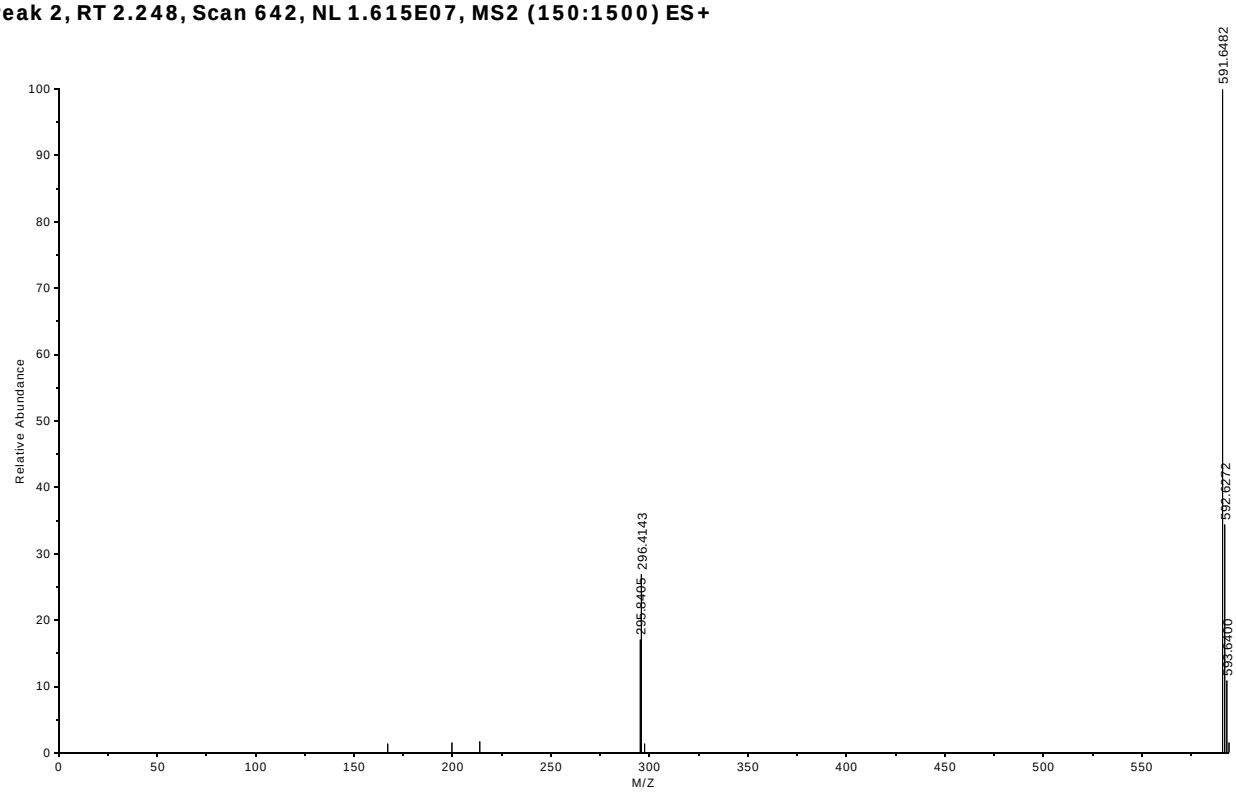


	RT Mins	Height	Height %	Area	Area %
1	0.23	2720905	13.48	3653806	7.25
2	2.25	20185991	100.00	50379042	100.00
3	2.37	2500144	12.39	8308751	16.49
4	3.94	2330011	11.54	4942177	9.81
5	4.01	2781386	13.78	10700693	21.24

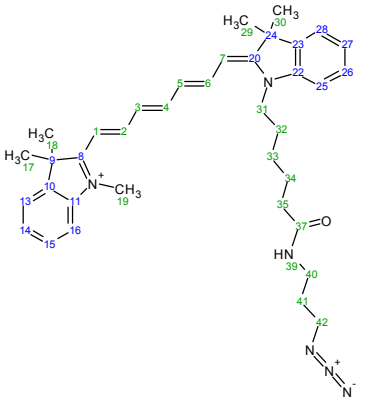
Peak 1, RT 0.227, Scan 65, NL 2.963E06, MS2 (150:1500) ES+



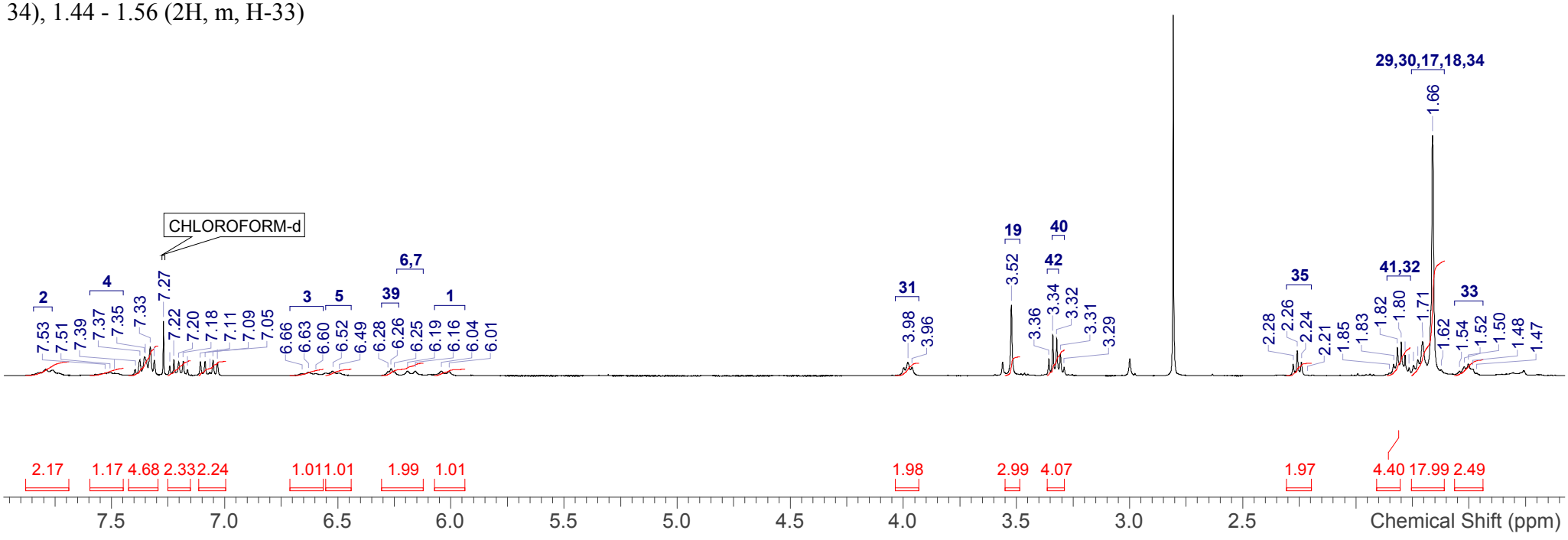
Peak 2, RT 2.248, Scan 642, NL 1.615E07, MS2 (150:1500) ES+



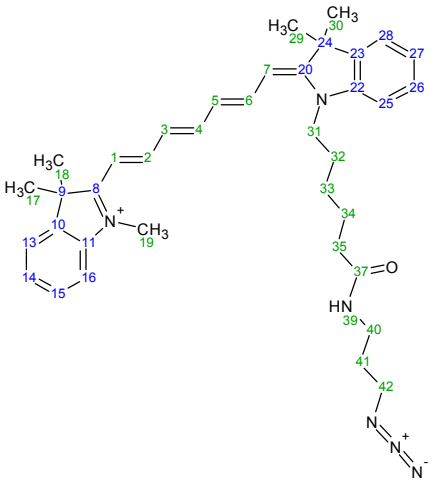
Acquisition Time (sec)	4.0894	***Comment***	mm/7500/120 Cy7-N3		
Date	27 Aug 2015 01:20:28				
Date Stamp	27 Aug 2015 01:20:28				
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au2615mm1 Cy7-N3\10\PDATA\1\1r				
Frequency (MHz)	400.1300	***Nucleus***	1H	***Number of Transients***	16
Origin	spect	***Original Points Count***	32768	***Owner***	nmr
Points Count	65536	***Pulse Sequence***	zg30	***Receiver Gain***	89.37
SW(cyclical) (Hz)	8012.82	***Solvent***	CHLOROFORM-d		
Spectrum Offset (Hz)	2465.2971	***Spectrum Type***	standard	***Sweep Width (Hz)***	8012.70
Temperature (degree C)	25.002				



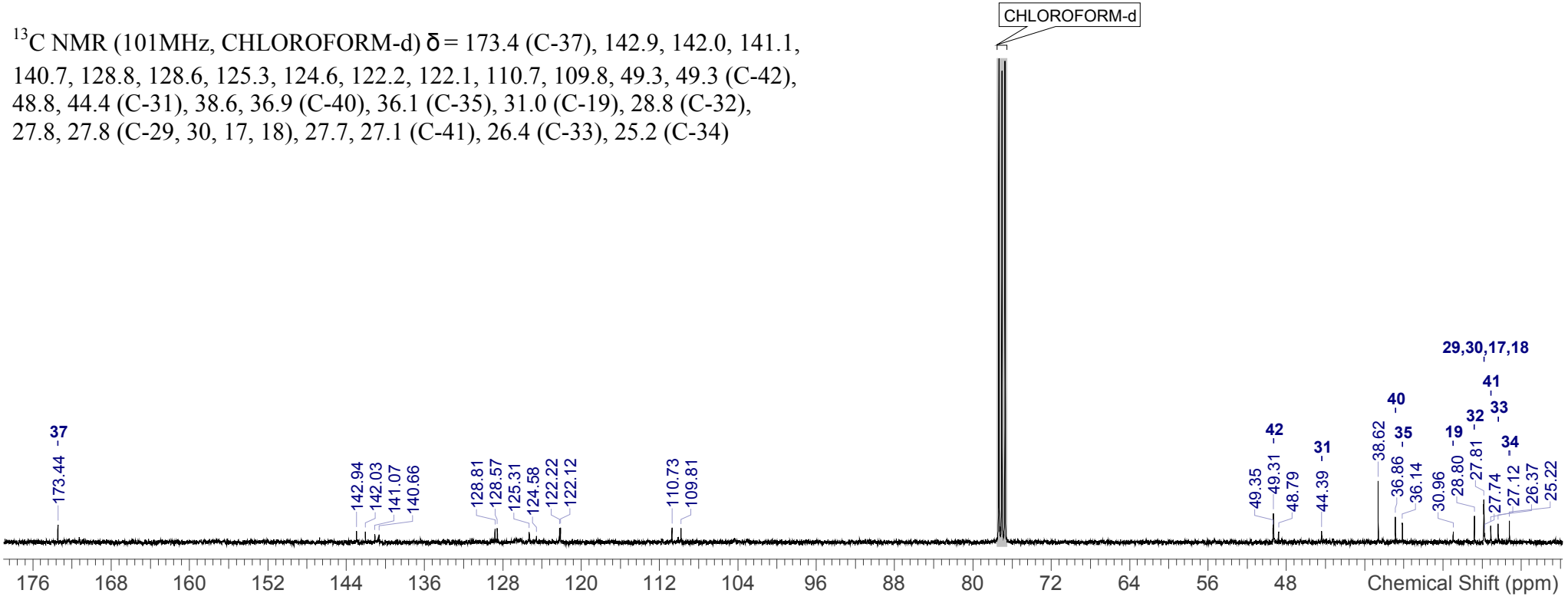
^{1}H NMR (400MHz, CHLOROFORM-d) δ = 7.76 - 7.84 (1H, m, H-2), 7.69 - 7.88 (1H, m, M12), 7.45 - 7.60 (1H, m, H-4), 7.29 - 7.42 (5H, m, M14), 7.15 - 7.25 (1H, m, M21), 7.15 - 7.25 (1H, m, M20), 7.15 - 7.25 (1H, m, M16), 7.00 - 7.11 (2H, m, M15), 6.63 (1H, br t, J = 12.2 Hz, H-3), 6.44 - 6.55 (1H, m, H-5), 6.26 (1H, br t, J = 5.5 Hz, H-39), 6.17 (1H, br d, J = 13.7 Hz, H-6, 7), 6.02 (1H, br d, J = 13.8 Hz, H-1), 3.98 (2H, br t, J = 7.5 Hz, H-31), 3.52 (3H, s, H-19), 3.36 (2H, s, H-42), 3.29 - 3.34 (2H, m, H-40), 2.26 (2H, t, J = 7.3 Hz, H-35), 1.76 - 1.86 (4H, m, H-41, 32), 1.61 - 1.75 (18H, m, H-29, 30, 17, 18, 34), 1.44 - 1.56 (2H, m, H-33)



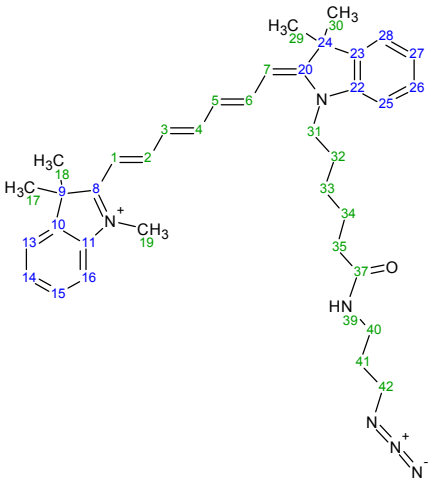
Acquisition Time (sec)	1.3631	***Comment***	mm/7500/120 Cy7-N3
Date	27 Aug 2015 01:50:53		
Date Stamp	27 Aug 2015 01:50:53		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au2615mm1 Cy7-N3\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	10060.3320	***Spectrum Type***	standard
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	24.999



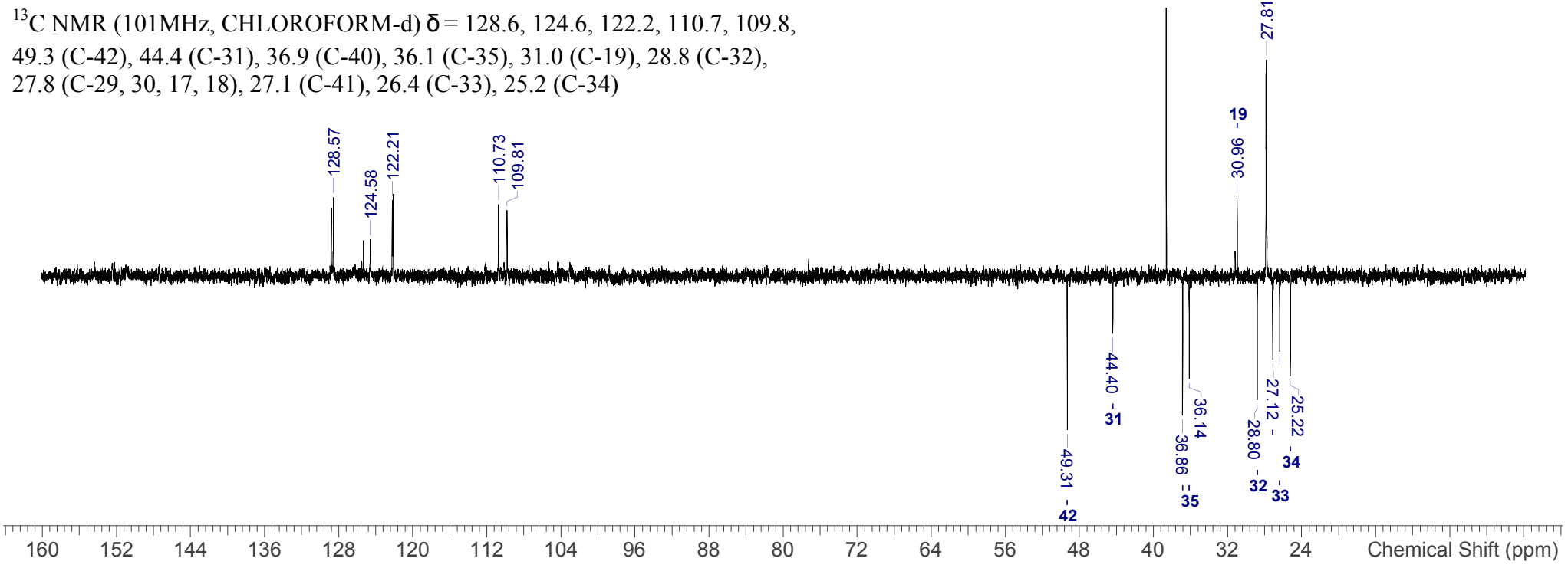
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 173.4 (C-37), 142.9, 142.0, 141.1, 140.7, 128.8, 128.6, 125.3, 124.6, 122.2, 122.1, 110.7, 109.8, 49.3, 49.3 (C-42), 48.8, 44.4 (C-31), 38.6, 36.9 (C-40), 36.1 (C-35), 31.0 (C-19), 28.8 (C-32), 27.8, 27.8 (C-29, 30, 17, 18), 27.7, 27.1 (C-41), 26.4 (C-33), 25.2 (C-34)



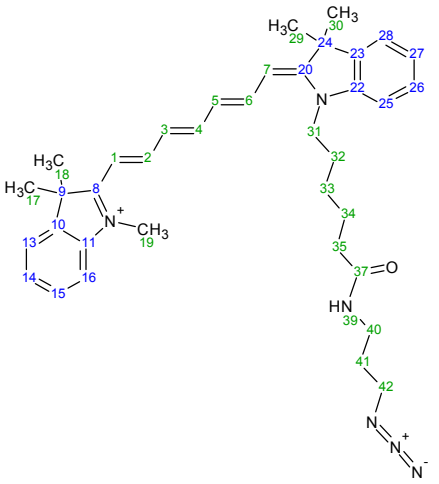
Acquisition Time (sec)	2.0316	***Comment***	mm/7500/120 Cy7-N3
Date	27 Aug 2015 02:09:32		
Date Stamp	27 Aug 2015 02:09:32		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au2615mm1 Cy7-N3\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	8047.8672	***Spectrum Type***	DEPT135
Sweep Width (Hz)	16128.54	***Temperature (degree C)***	24.996



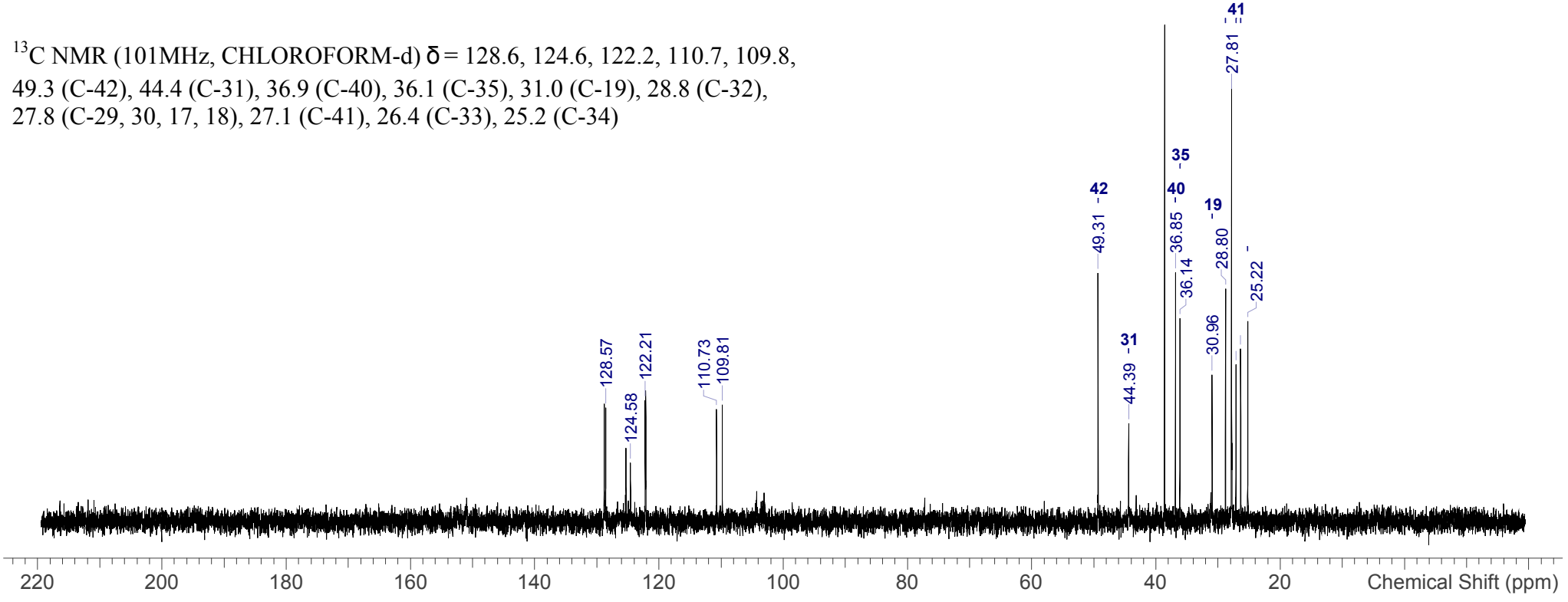
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 128.6, 124.6, 122.2, 110.7, 109.8, 49.3 (C-42), 44.4 (C-31), 36.9 (C-40), 36.1 (C-35), 31.0 (C-19), 28.8 (C-32), 27.8 (C-29, 30, 17, 18), 27.1 (C-41), 26.4 (C-33), 25.2 (C-34)



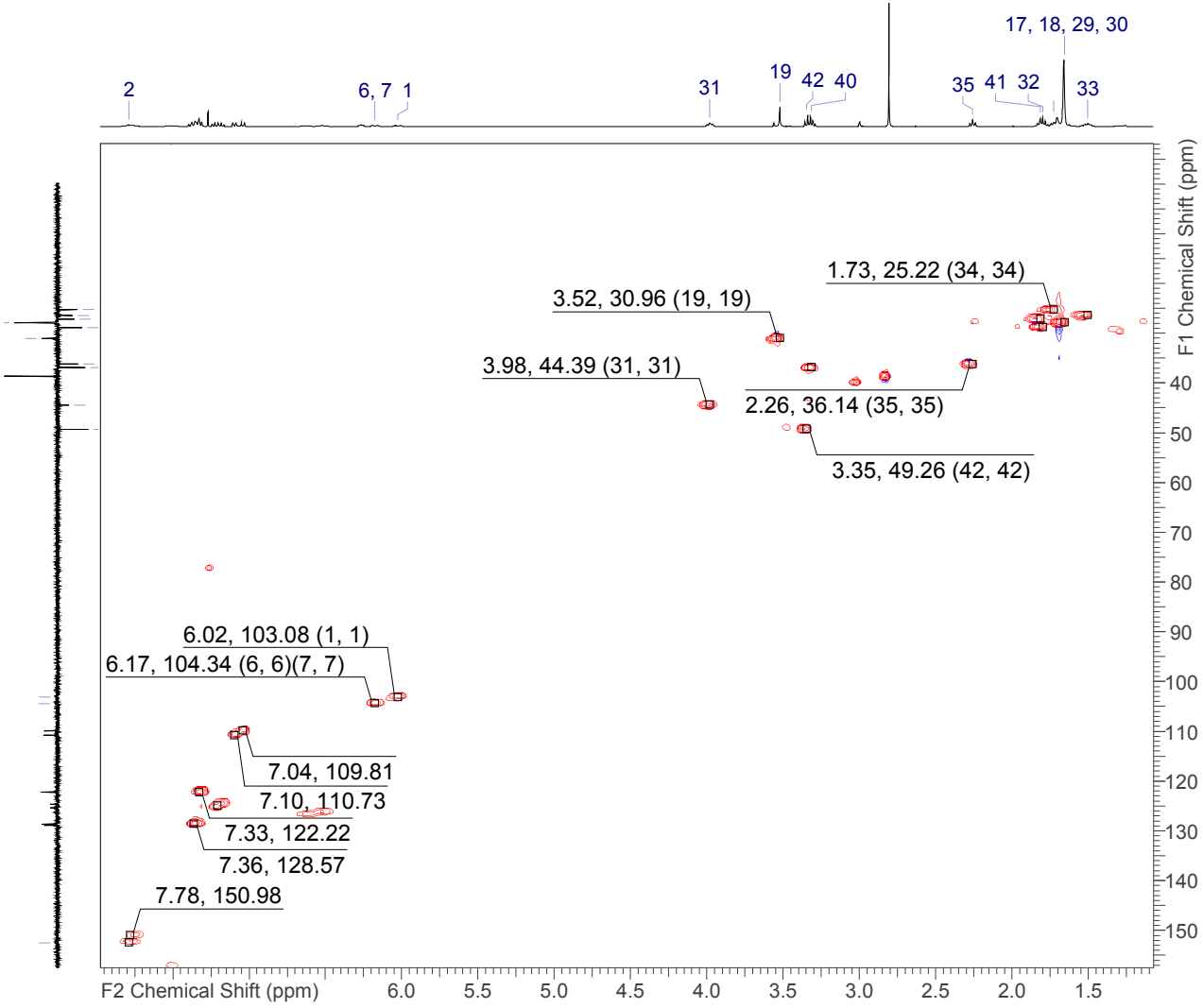
Acquisition Time (sec)	1.3631	***Comment***	mm/7500/120 Cy7-N3
Date	27 Aug 2015 03:03:12		
Date Stamp	27 Aug 2015 03:03:12		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au2615mm1 Cy7-N3\15\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp45
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	10060.3564	***Spectrum Type***	DEPT45
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	25.003

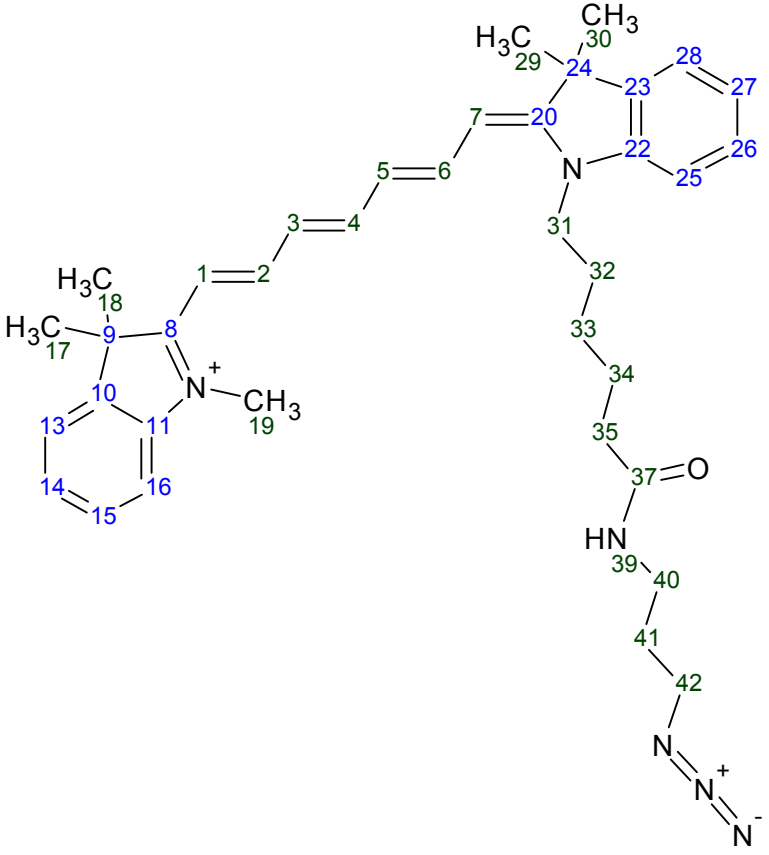


^{13}C NMR (101MHz, CHLOROFORM-d) δ = 128.6, 124.6, 122.2, 110.7, 109.8, 49.3 (C-42), 44.4 (C-31), 36.9 (C-40), 36.1 (C-35), 31.0 (C-19), 28.8 (C-32), 27.8 (C-29, 30, 17, 18), 27.1 (C-41), 26.4 (C-33), 25.2 (C-34)

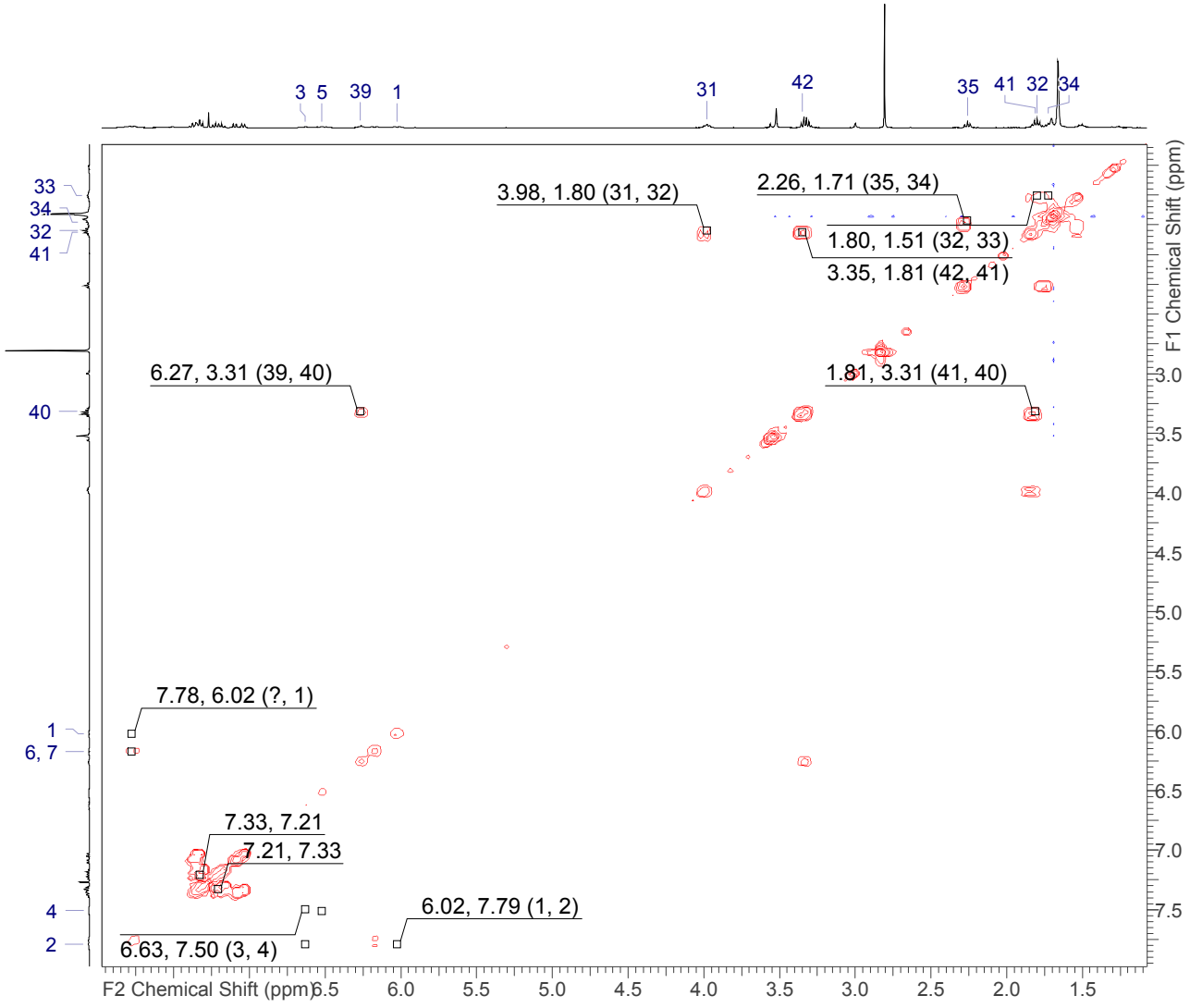


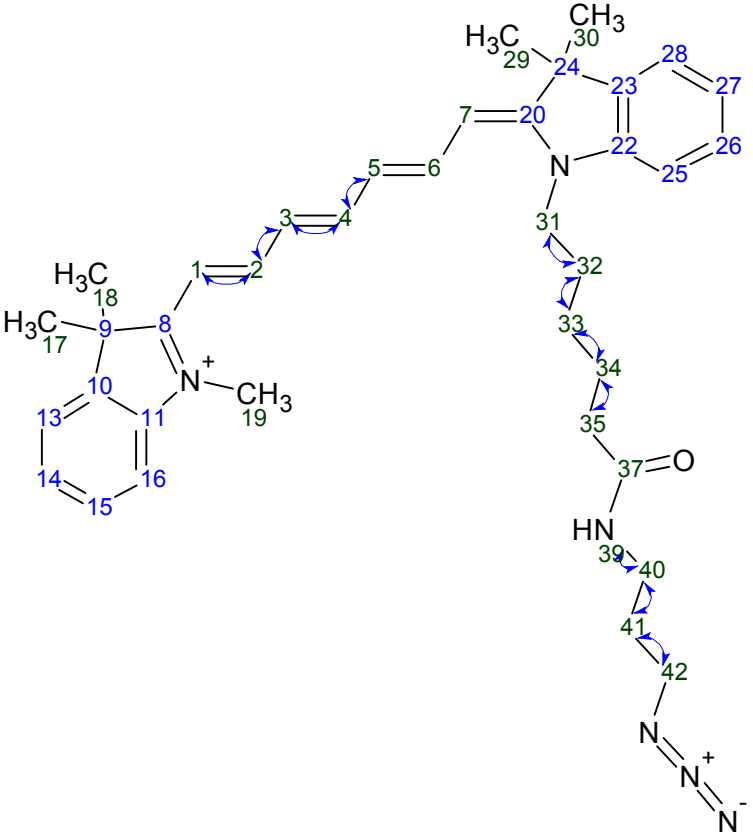
Acquisition Time (sec)	(0.1516, 0.0154)	***Comment***	mm/7500/120 Cy7-N3
Date	27 Aug 2015 03:18:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au2615mm1 Cy7-N3\16\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)	***Nucleus***	(1H, 13C)
Number of Transients	2	***Origin***	spect
Original Points Count	(512, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	hsqcetgp
Solvent	CHLOROFORM-d	***Spectrum Type***	HSQC
Sweep Width (Hz)	(3375.08, 16650.39)	***Temperature (degree C)***	25.070
Title	mm/7500/120 Cy7-N3		





Acquisition Time (sec)	(0.3031, 0.0758)	***Comment***	mm/7500/120 Cy7-N3
Date	27 Aug 2015 02:25:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\au2615mm1 Cy7-N3\13\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)		
Nucleus	(1H, 1H)	***Number of Transients***	2
Origin	spect	***Original Points Count***	(1024, 256)
Owner	nmr	***Points Count***	(1024, 1024)
Pulse Sequence	cosygpqf	***Solvent***	CHLOROFORM-d
Spectrum Type	COSY	***Sweep Width (Hz)***	(3375.08, 3375.08)
Temperature (degree C)	24.997	***Title***	mm/7500/120 Cy7-N3



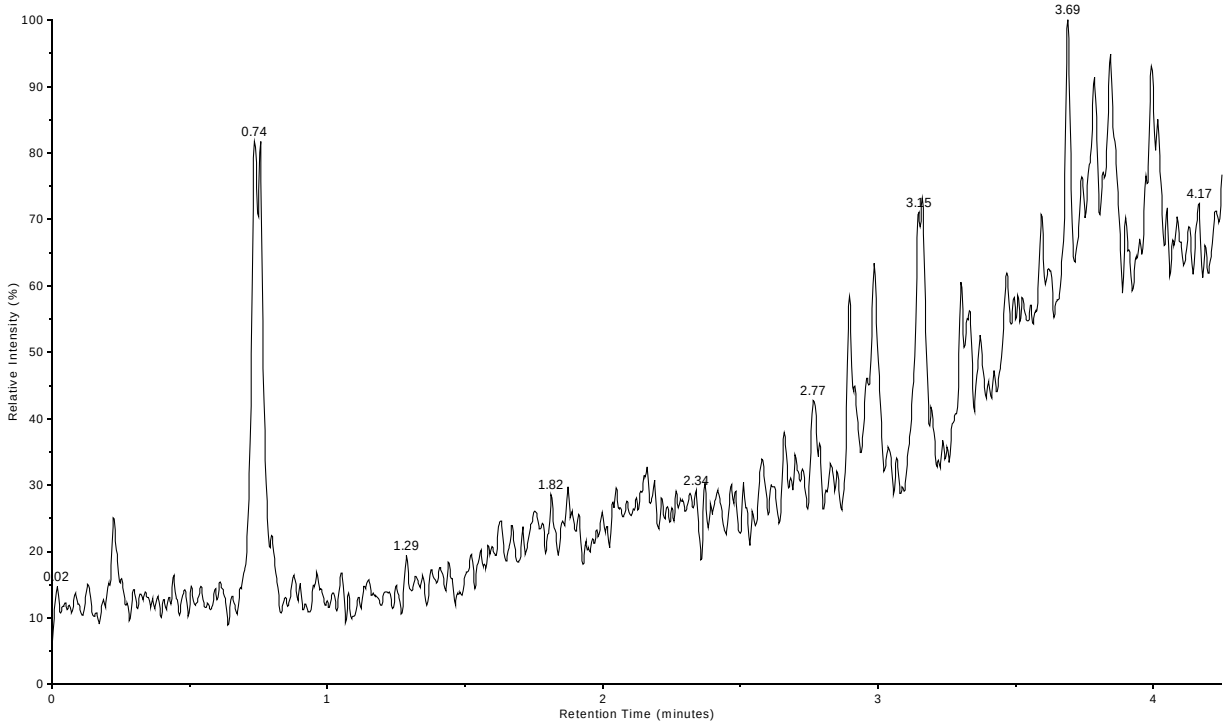


A.19 6-maleimidohexanoic acid

Top 5 Peak Report

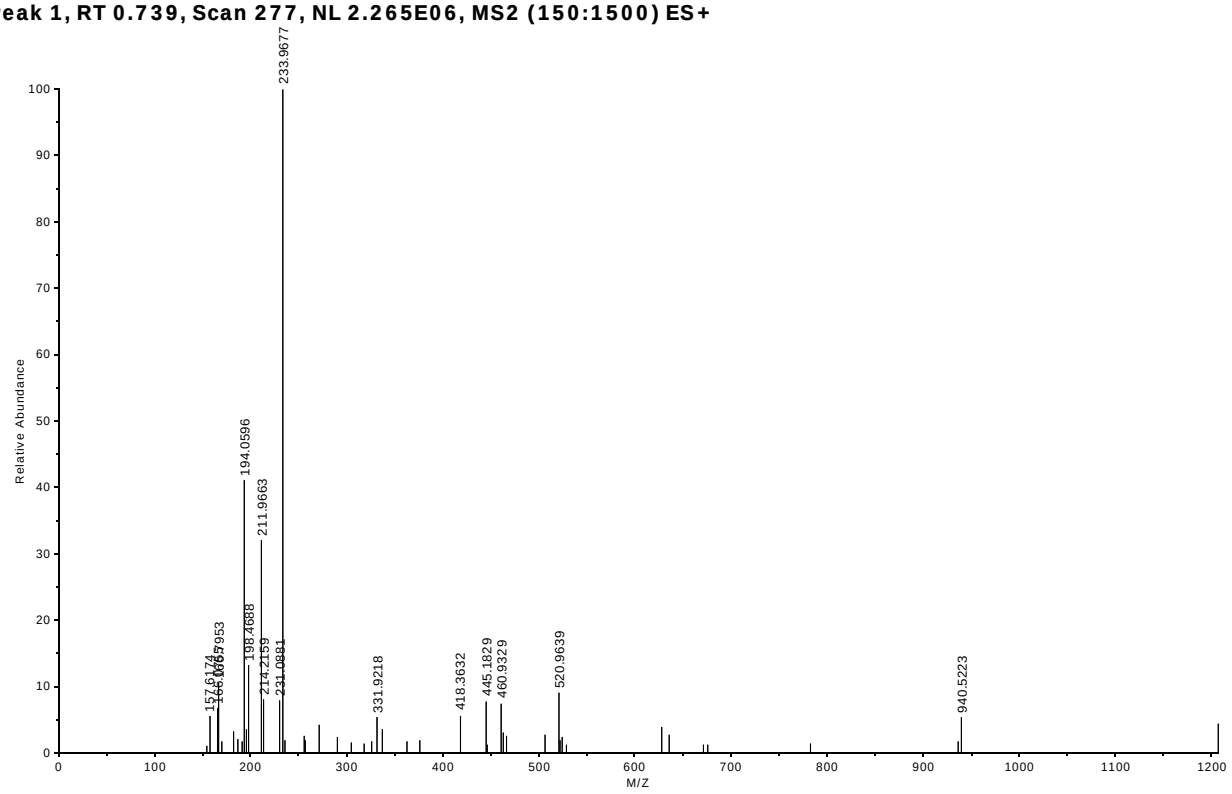
Group: Watts, J
Submitter: Michael Moazami
Project: RP-LC MW 150-1500
Sample ID: Mm/7316/53 maleimidohexanoic acid
Experiment: B ESIPOS (simple mix) C18 Acid
Filename: Watts_J_Mm731653_maleimidohexanoic_acid_M
Instrument: Blue RP UHPLC-MS (B30:1023)
Acquisition Date: 02/09/2014 12:35:27

TIC, NL 9.443E06, MS2 (150:1500) ES+

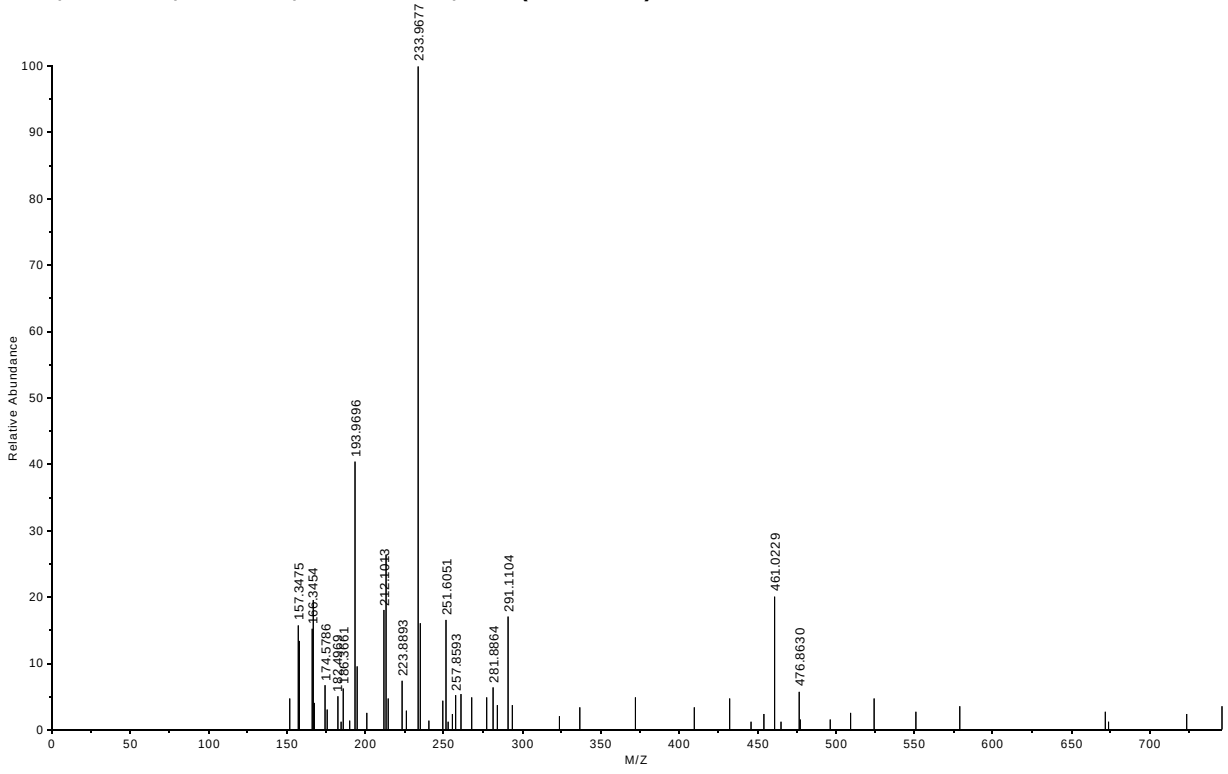


	RT Mins	Height	Height %	Area	Area %
1	0.74	6362457	100.00	10034200	100.00
2	0.76	5770082	90.69	6588592	65.66
3	3.16	3236674	50.87	3812065	37.99
4	3.69	3838867	60.34	6834588	68.11
5	3.85	2588469	40.68	7615690	75.90

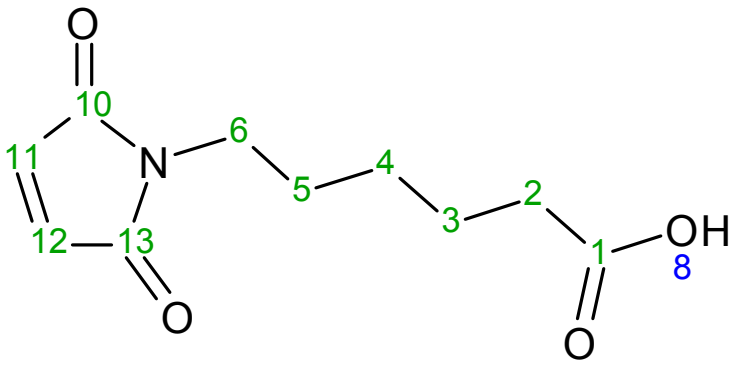
Peak 1, RT 0.739, Scan 277, NL 2.265E06, MS2 (150:1500) ES+



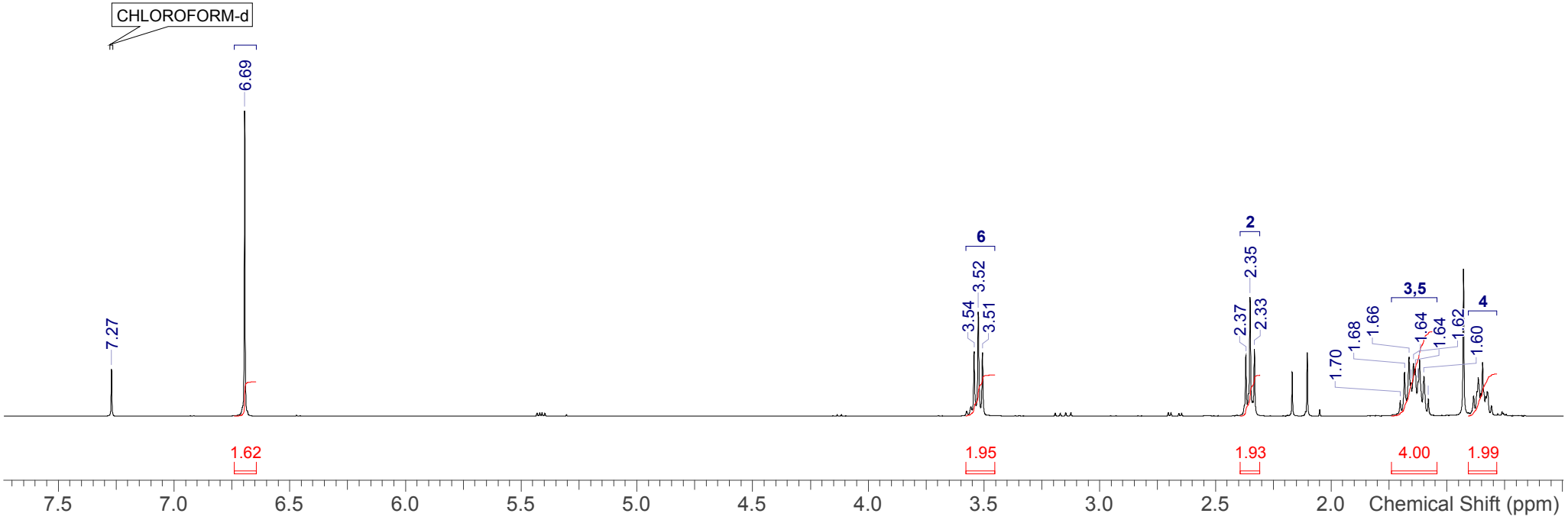
Peak 2, RT 0.760, Scan 285, NL 2.155E06, MS2 (150:1500) ES+



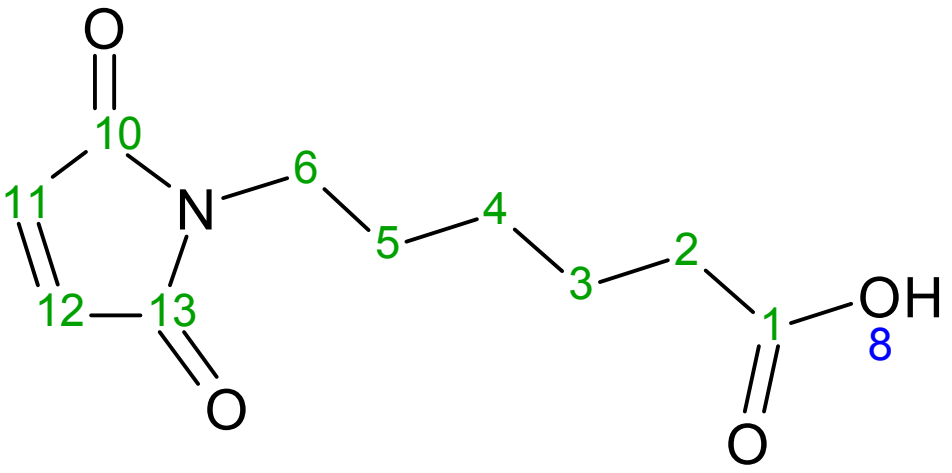
Acquisition Time (sec)	4.0894	***Comment***	mm/7316/53 Maleimidohexanoic acid
Date	02 Sep 2014 14:49:58		
Date Stamp	02 Sep 2014 14:49:58		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\se0214mm1 Maleimidohexanoic Acid\10\PDATA\1\1r		
Frequency (MHz)	400.1300	***Nucleus***	1H
Number of Transients	16	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	65536	***Pulse Sequence***	zg30
Receiver Gain	83.67	***SW(cyclical) (Hz)***	8012.82
Solvent	CHLOROFORM-d	***Spectrum Offset (Hz)***	2465.1768
Spectrum Type	standard	***Sweep Width (Hz)***	8012.70
Temperature (degree C)	25.000		



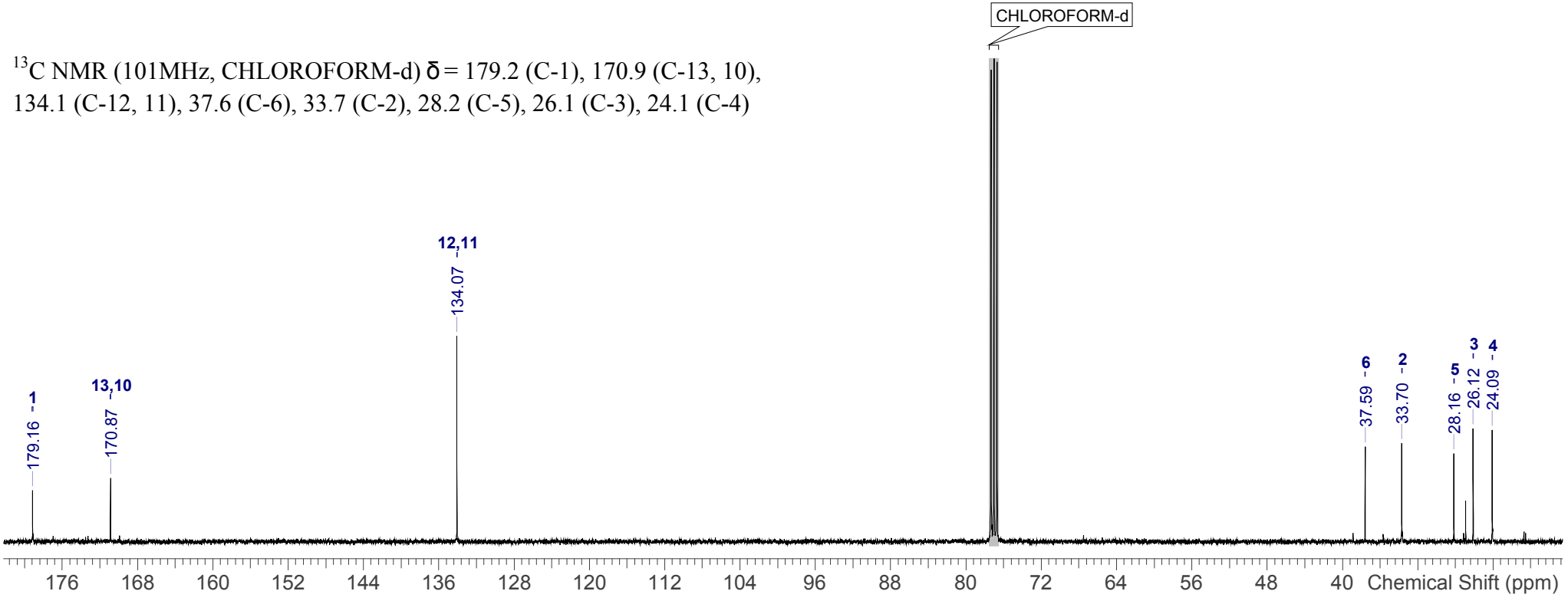
^{1}H NMR (400MHz, CHLOROFORM-d) δ = 6.69 (2H, s, H-12, 11), 3.52 (2H, t, J = 7.2 Hz, H-6), 2.35 (2H, t, J = 7.4 Hz, H-2), 1.64 (4H, ddt, J = 18.2 Hz, J = 15.3 Hz, J = 7.5 Hz, H-3, 5), 1.28 - 1.41 (2H, m, H-4)



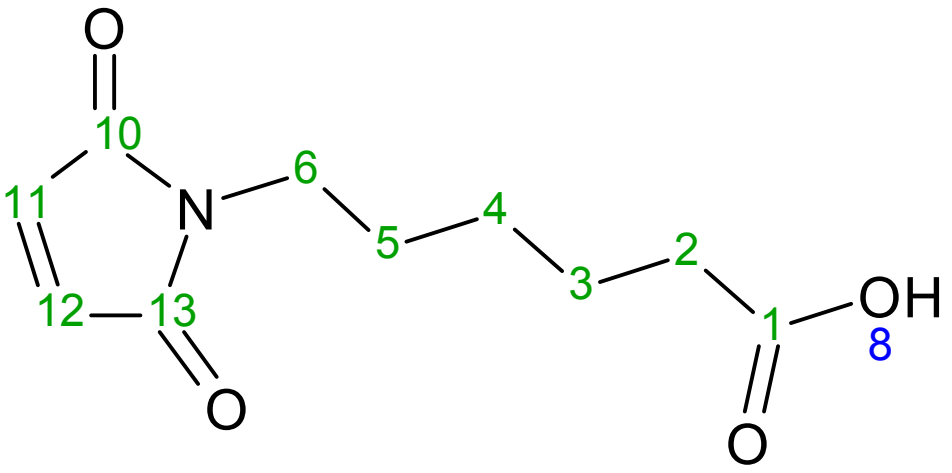
Acquisition Time (sec)	1.3631		
Comment	mm/7316/53 Maleimidohexanoic acid		
Date	02 Sep 2014 15:20:20		
Date Stamp	02 Sep 2014 15:20:20		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\se0214mm1 Maleimidohexanoic Acid\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d	***Spectrum Offset (Hz)***	10060.3320
Spectrum Type	standard	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	25.001		



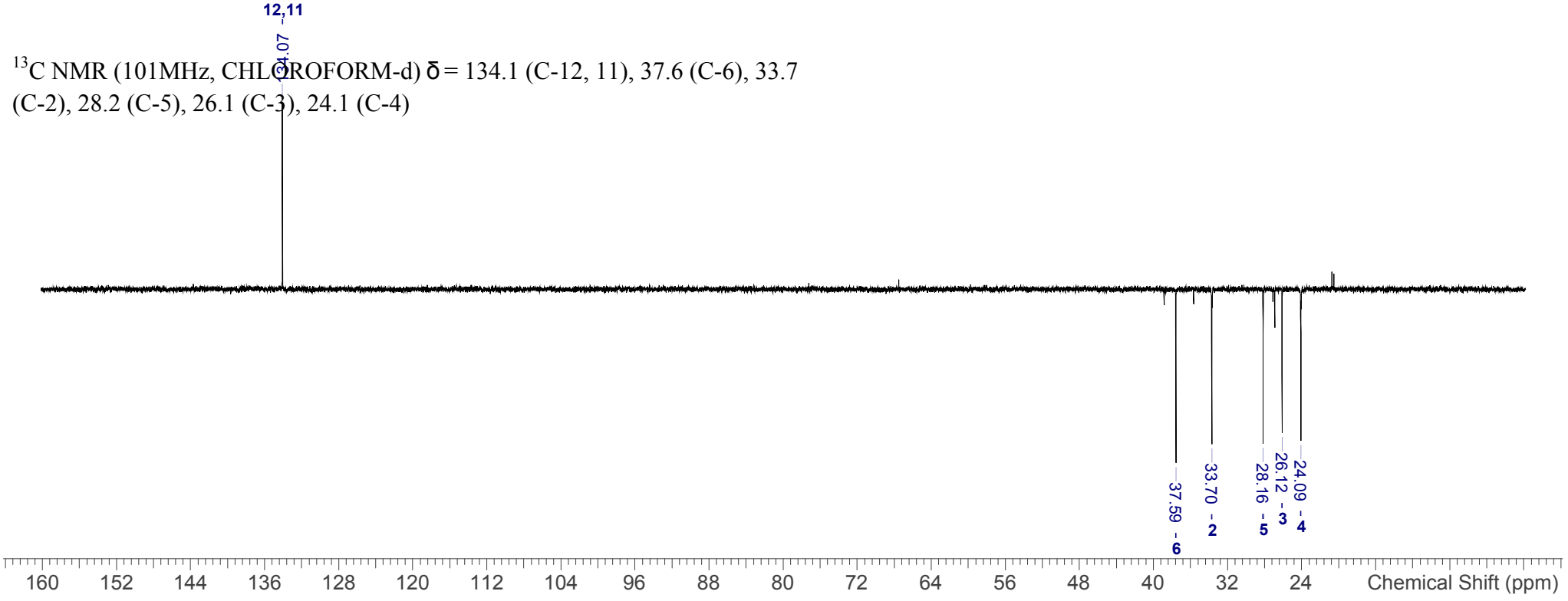
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 179.2 (C-1), 170.9 (C-13, 10), 134.1 (C-12, 11), 37.6 (C-6), 33.7 (C-2), 28.2 (C-5), 26.1 (C-3), 24.1 (C-4)



Acquisition Time (sec)	2.0316		
Comment	mm/7316/53 Maleimidohexanoic acid		
Date	02 Sep 2014 15:38:56		
Date Stamp	02 Sep 2014 15:38:56		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\se0214mm1 Maleimidohexanoic Acid\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	CHLOROFORM-d	***Spectrum Offset (Hz)***	8047.7910
Spectrum Type	DEPT135	***Sweep Width (Hz)***	16128.54
Temperature (degree C)	24.997		



^{13}C NMR (101MHz, CHLOROFORM-d) δ = 134.1 (C-12, 11), 37.6 (C-6), 33.7 (C-2), 28.2 (C-5), 26.1 (C-3), 24.1 (C-4)

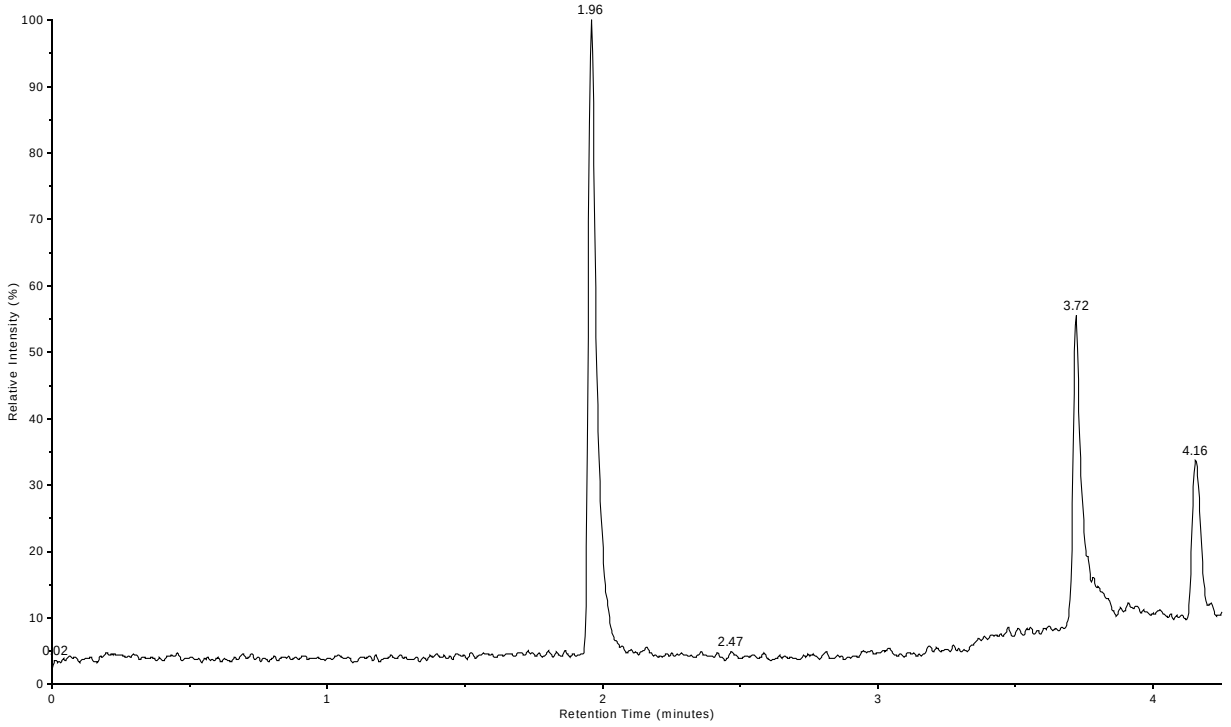


A.20 (S)-2,6-bis((tert-butoxycarbonyl)amino)hexanoic acid

Top 5 Peak Report

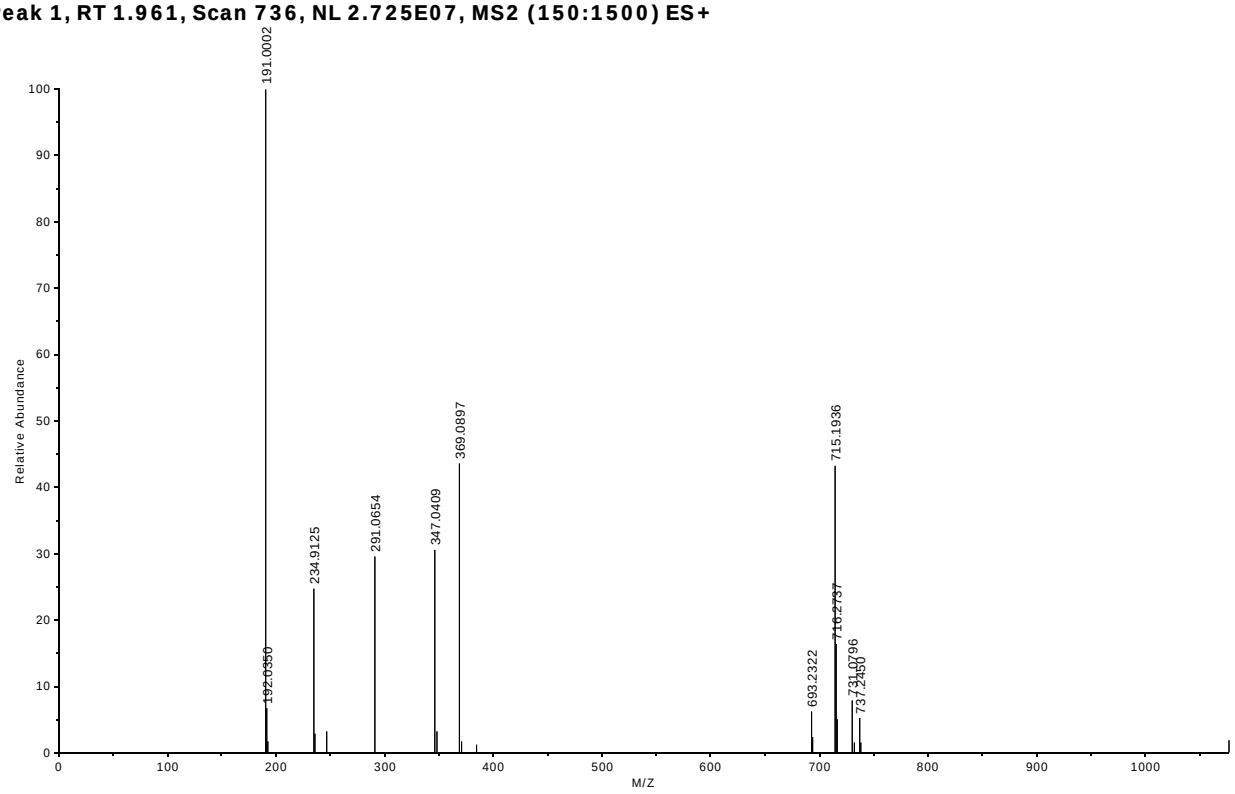
Group: Watts, J
Submitter: Michael Moazami
Project: RP LC C18 < 2 cmpds
Sample ID: Diboclys
Experiment: BLUE ESIPOS C18 5 min
Filename: Diboclys_Michael_Moazami__Watts_J_4266.pdf
Instrument: Blue RP UHPLC-MS (B30:1023)
Acquisition Date: 27/01/2015 15:45:42

TIC, NL 9.227E07, MS2 (150:1500) ES+

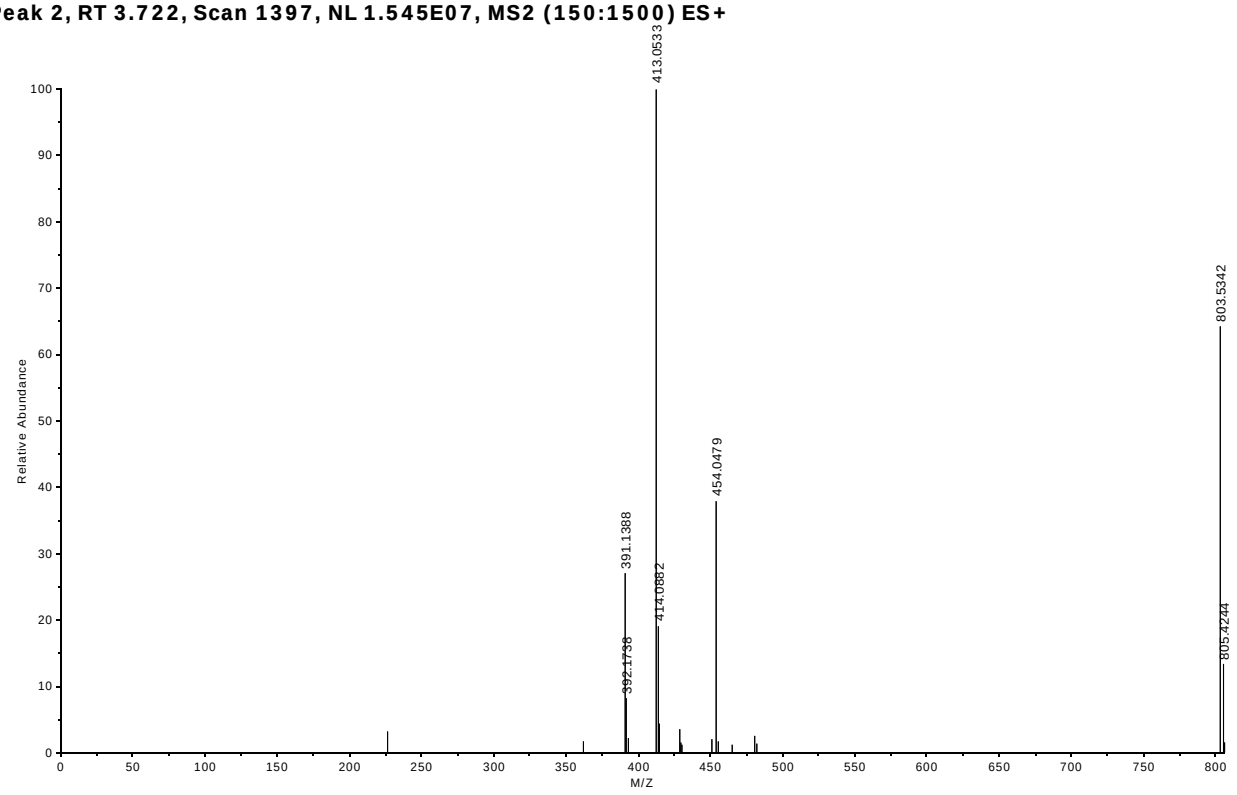


	RT Mins	Height	Height %	Area	Area %
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2	3.72	40300219	45.96	95850158	49.43
3	4.16	21090426	24.05	48298250	24.91

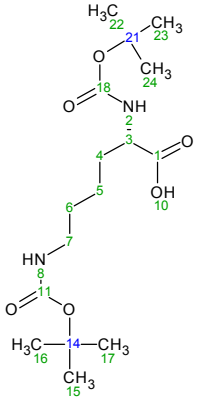
Peak 1, RT 1.961, Scan 736, NL 2.725E07, MS2 (150:1500) ES+



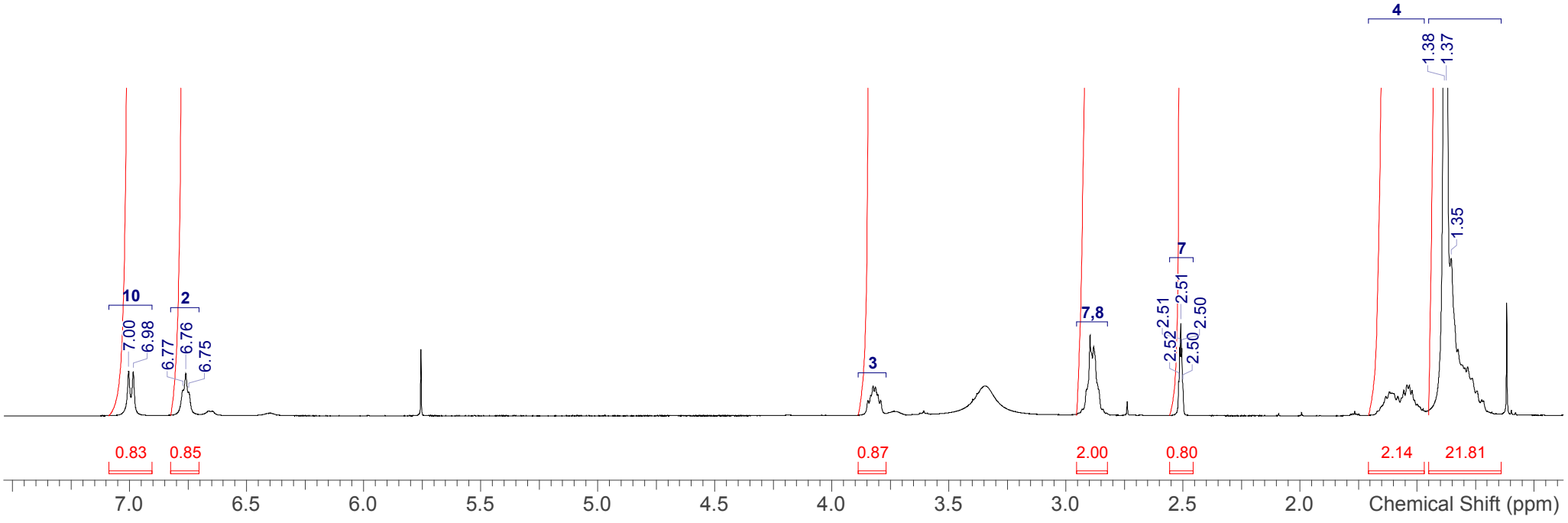
Peak 2, RT 3.722, Scan 1397, NL 1.545E07, MS2 (150:1500) ES+



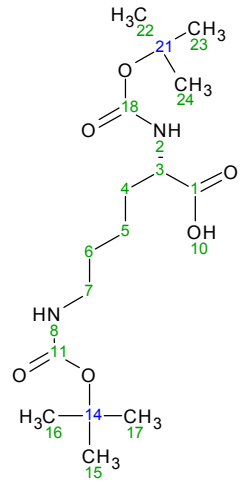
Acquisition Time (sec)	4.0894	***Comment***	mm/7316/15		
Date	15 Jul 2014 16:47:41				
Date Stamp	15 Jul 2014 16:47:41				
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\jy1514mm1 Boc-lys(Boc)-OH\10\PDATA\1\1r				
Frequency (MHz)	400.1300	***Nucleus***	1H	***Number of Transients***	16
Origin	spect	***Original Points Count***	32768	***Owner***	nmr
Points Count	131072	***Pulse Sequence***	zg30	***Receiver Gain***	31.69
SW(cyclical) (Hz)	8012.82	***Solvent***	DMSO-d6	***Spectrum Offset (Hz)***	2470.9973
Spectrum Type	standard	***Sweep Width (Hz)***	8012.76	***Temperature (degree C)***	25.005



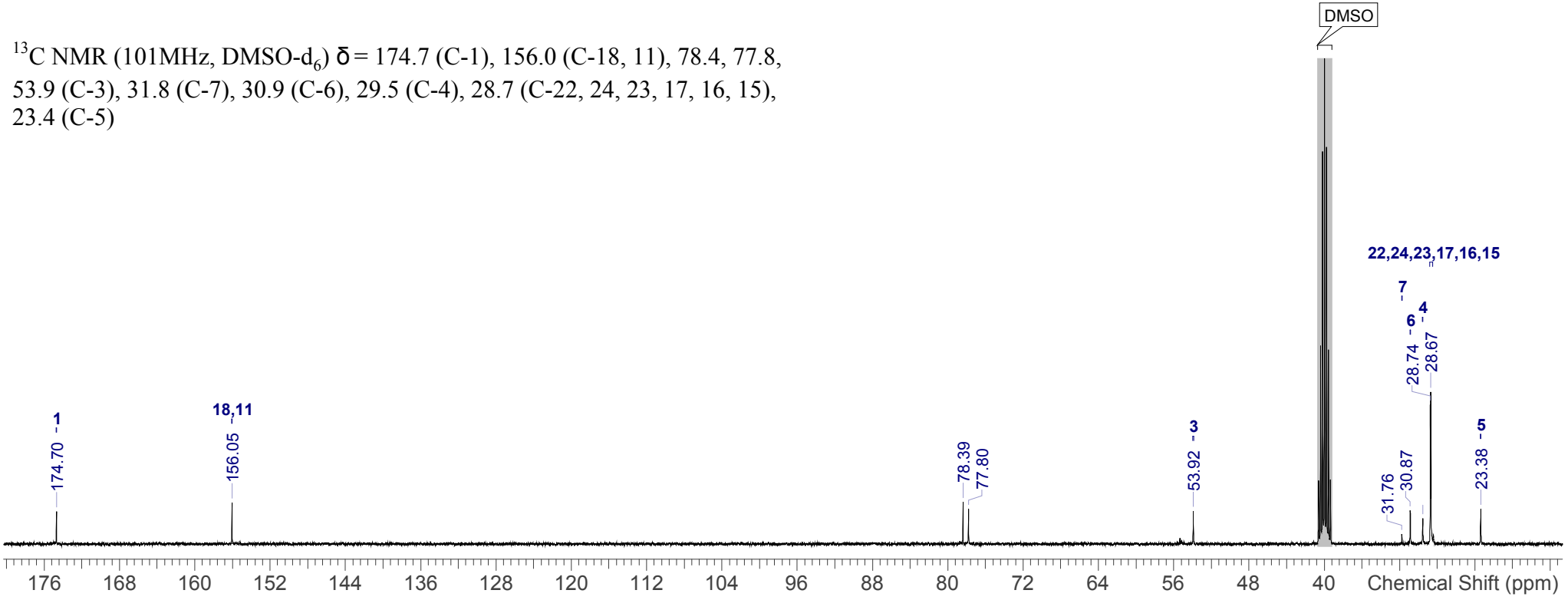
^{1}H NMR (400MHz, DMSO-d_6) δ = 6.99 (1H, br d, J = 7.9 Hz, H-10), 6.76 (1H, br t, J = 5.2 Hz, H-2), 3.77 - 3.89 (1H, m, H-3), 2.82 - 2.95 (2H, m, H-7, 8), 2.51 (1H, dt, J = 3.5 Hz, J = 1.7 Hz, H-7), 1.47 - 1.71 (2H, m, H-4), 1.38 (21H, d, J = 2.9 Hz, H-17, 16, 15, 22, 24, 23, 6, 5)



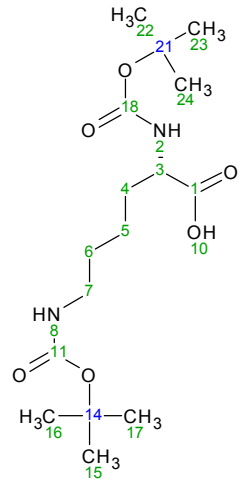
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/15
Date	15 Jul 2014 17:18:10		
Date Stamp	15 Jul 2014 17:18:10		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\jy1514mm1 Boc-lys(Boc)-OH\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	10060.3320
Spectrum Type	standard	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	25.005		



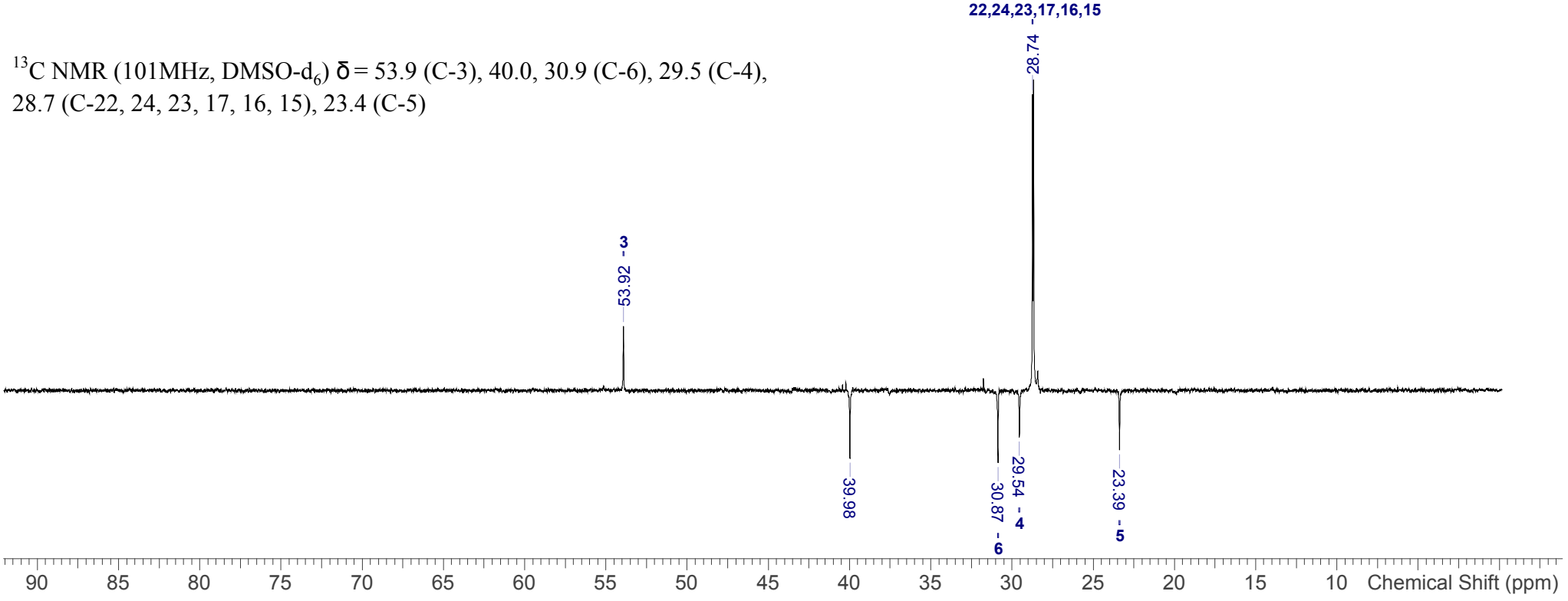
^{13}C NMR (101MHz, DMSO-d$_6$) δ = 174.7 (C-1), 156.0 (C-18, 11), 78.4, 77.8, 53.9 (C-3), 31.8 (C-7), 30.9 (C-6), 29.5 (C-4), 28.7 (C-22, 24, 23, 17, 16, 15), 23.4 (C-5)



Acquisition Time (sec)	2.0316	***Comment***	mm/7316/15
Date	15 Jul 2014 17:36:46		
Date Stamp	15 Jul 2014 17:36:46		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\jy1514mm1 Boc-lys(Boc)-OH\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	8048.0767
Spectrum Type	DEPT135	***Sweep Width (Hz)***	16128.54
Temperature (degree C)	24.999		



^{13}C NMR (101MHz, DMSO-d$_6$) δ = 53.9 (C-3), 40.0, 30.9 (C-6), 29.5 (C-4), 28.7 (C-22, 24, 23, 17, 16, 15), 23.4 (C-5)

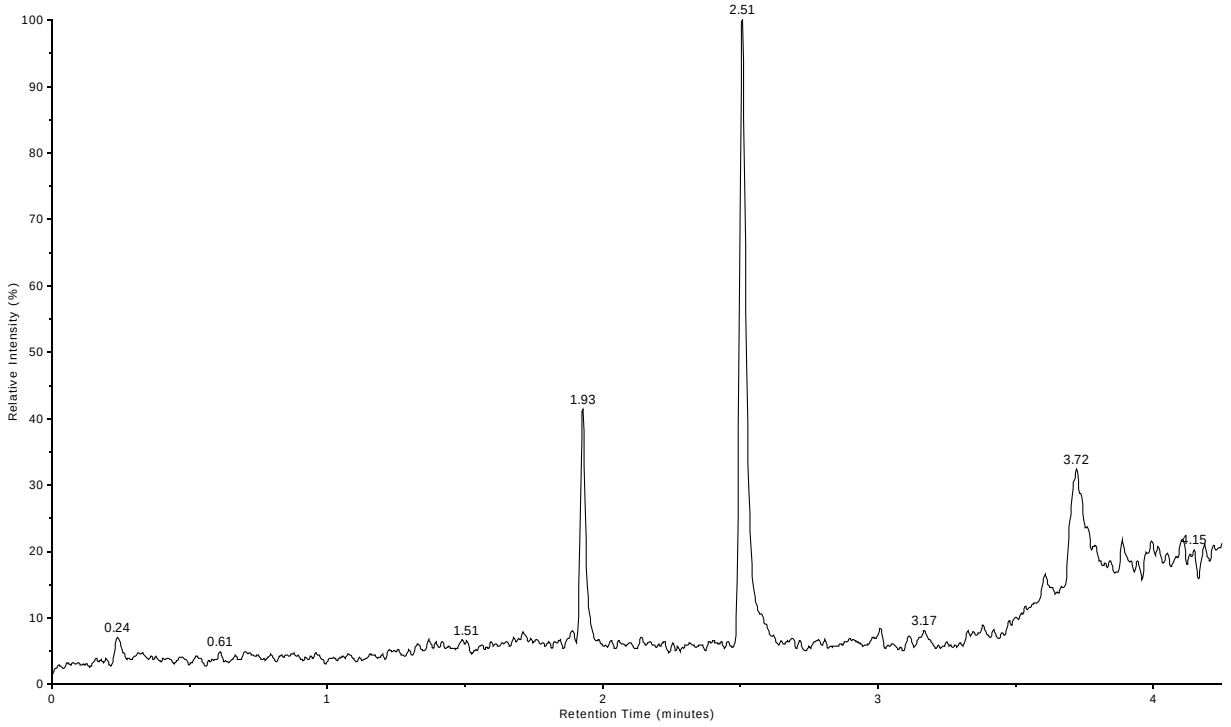


A.21 Tetra-tert-butyl ((5S,5'S)-(hexane-1,6-diylbis(azanediyl))bis(6-oxohexane-6,5,1-triyl))tetracarbamate

Top 5 Peak Report

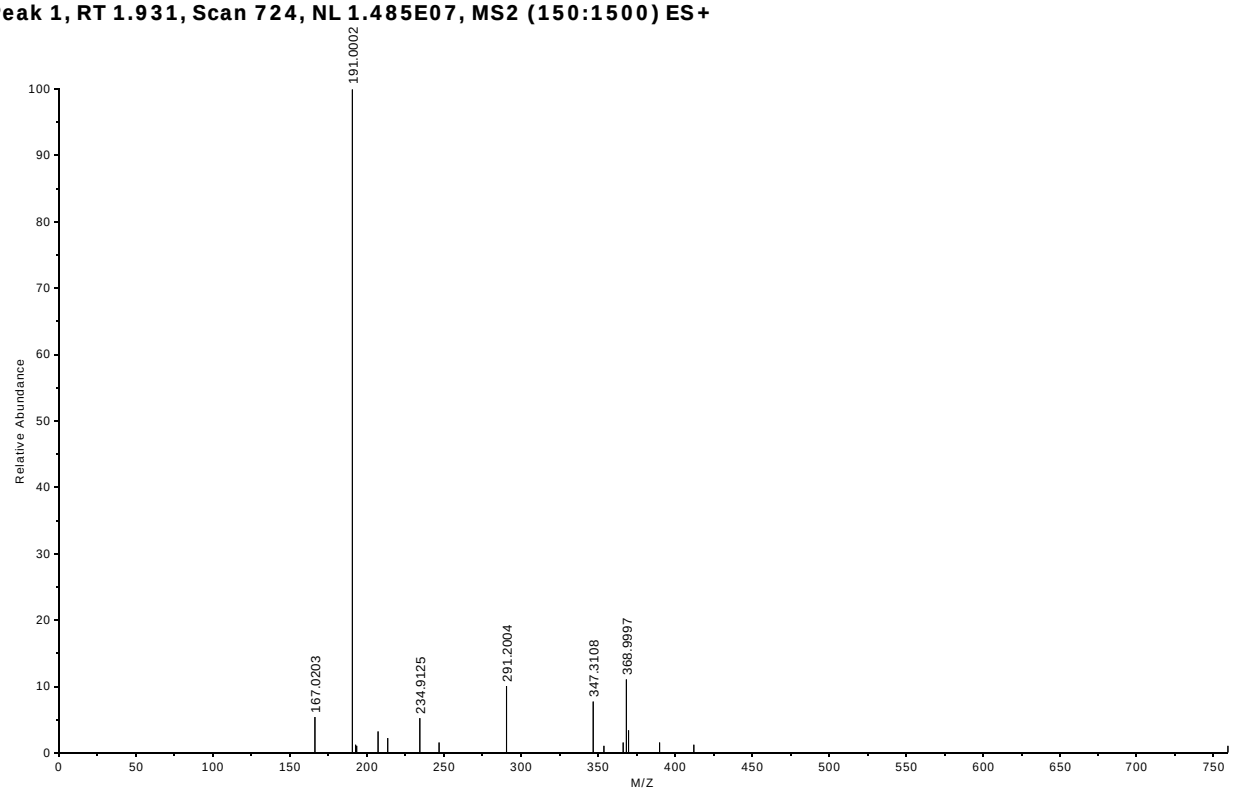
Group: Watts, J
Submitter: Michael Moazami
Project: RP-LC MW 150-1500
Sample ID: mm/7316/37
Experiment: B ESIPOS (simple mix) C18 Acid
Filename: Watts_J_mm731637_MM_370.pdf
Instrument: Blue RP UHPLC-MS (B30:1023)
Acquisition Date: 08/08/2014 15:51:20

TIC, NL 5.287E07, MS2 (150:1500) ES+

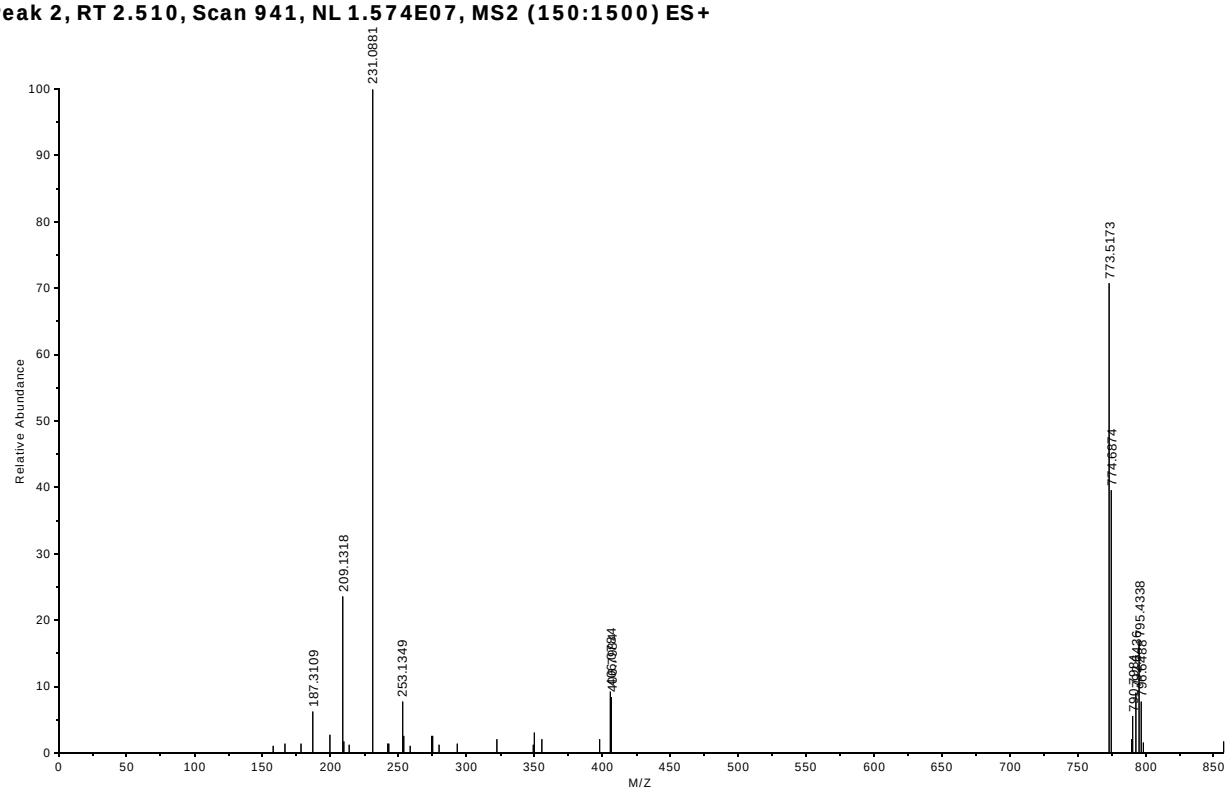


	RT Mins	Height	Height %	Area	Area %
1	1.93	18465712	37.97	25231610	27.60
2	2.51	48631432	100.00	91403764	100.00
3	3.61	2757865	5.67	5699455	6.24
4	3.72	7056888	14.51	27661526	30.26

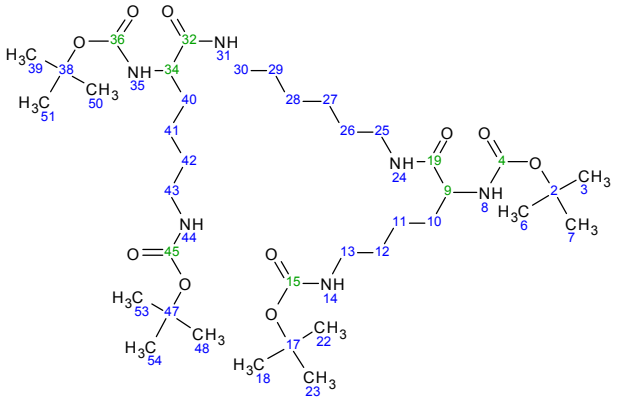
Peak 1, RT 1.931, Scan 724, NL 1.485E07, MS2 (150:1500) ES+



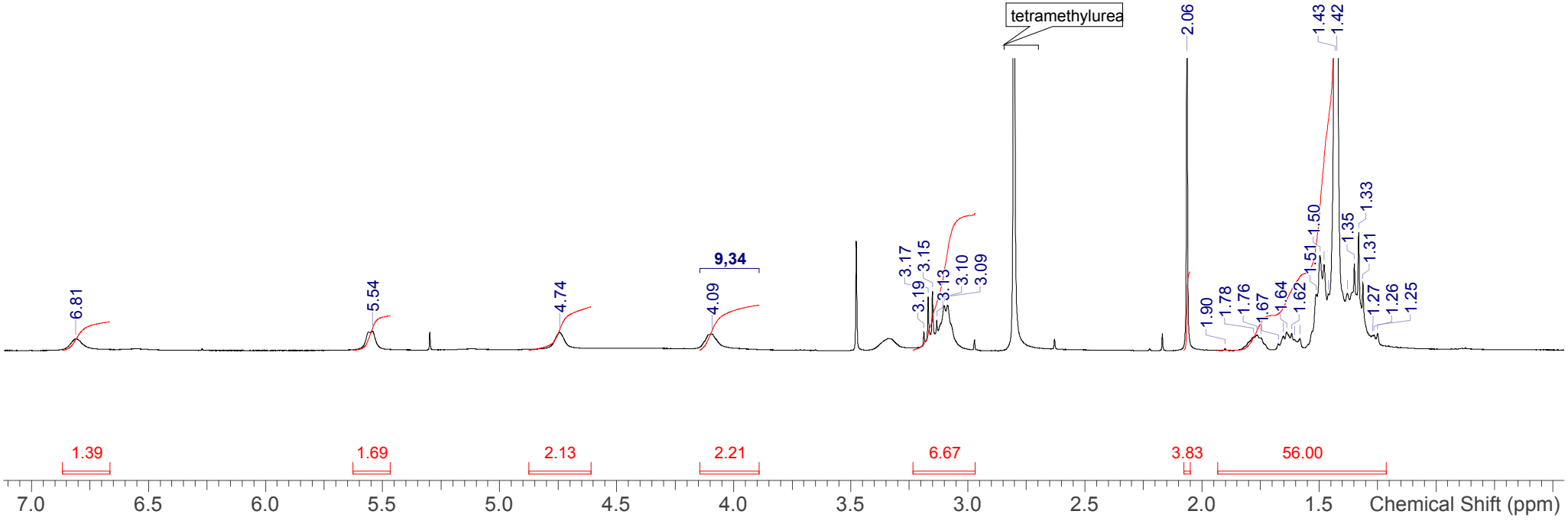
Peak 2, RT 2.510, Scan 941, NL 1.574E07, MS2 (150:1500) ES+



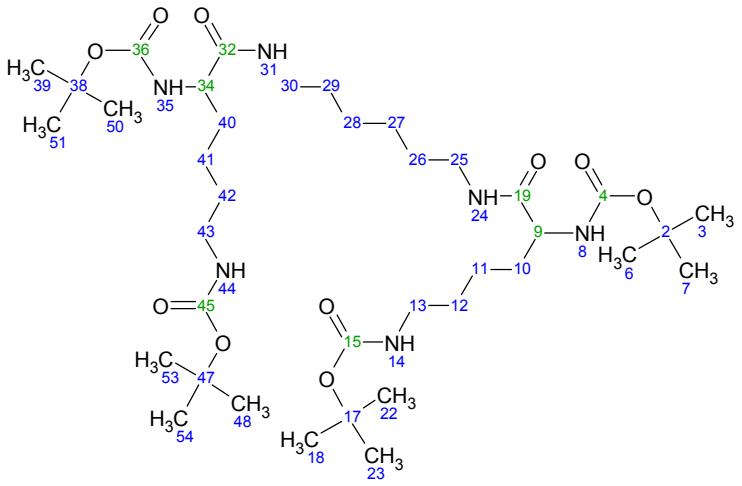
Acquisition Time (sec)	4.0894	***Comment***	mm/7316/37	***Date***	11 Aug 2014 17:30:52
Date Stamp	11 Aug 2014 17:30:52				
File Name	C:\Users\Mike Moazami\Desktop\au1114mm2 Boc4Ly2Hexanediamine\10\PDATA\1\1r				
Frequency (MHz)	400.1300	***Nucleus***	1H	***Number of Transients***	16
Origin	spect	***Original Points Count***	32768	***Owner***	nmr
Points Count	65536	***Pulse Sequence***	zg30	***Receiver Gain***	50.16
SW(cyclical) (Hz)	8012.82	***Solvent***	CHLOROFORM-d		
Spectrum Offset (Hz)	2465.1812	***Spectrum Type***	standard	***Sweep Width (Hz)***	8012.70
Temperature (degree C)	24.993				



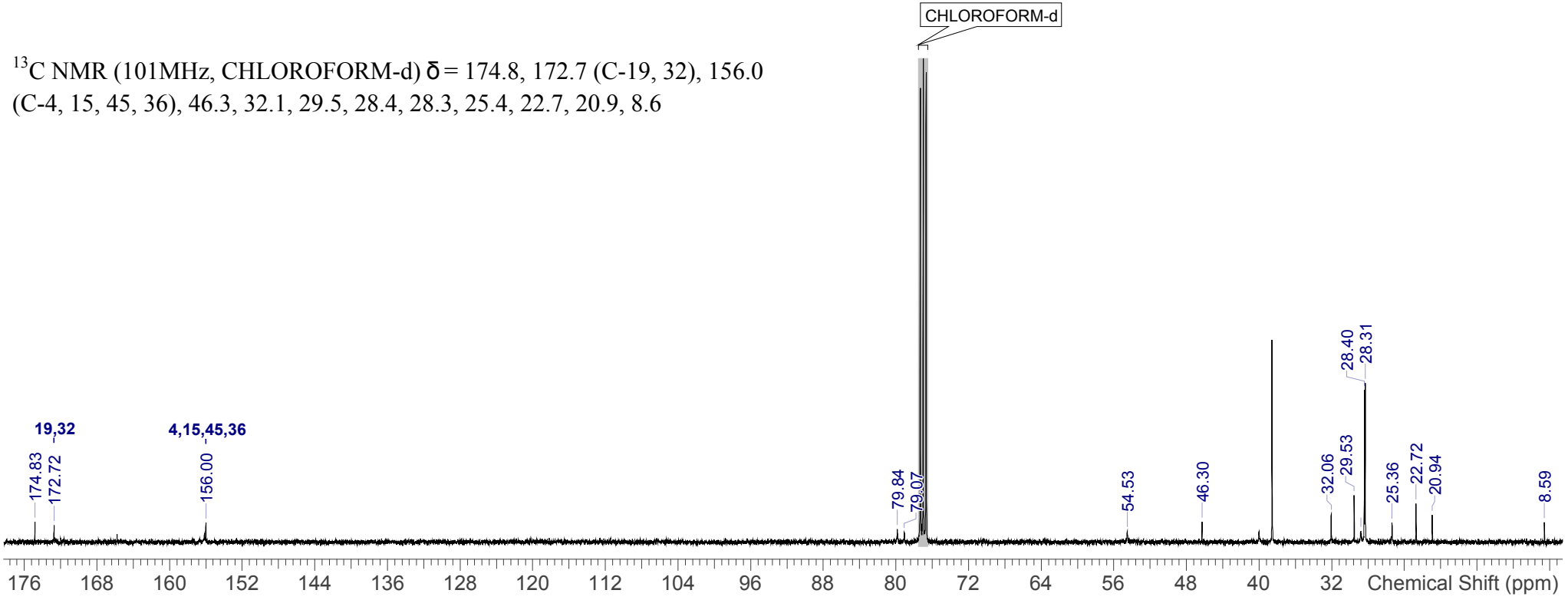
^{1}H NMR (400MHz, CHLOROFORM-d) δ = 6.81 (1H, br s, M15), 5.47 - 5.63 (2H, m, M14), 4.74 (2H, br s, M12), 4.09 (2H, br s, H-9, 34), 2.97 - 3.23 (7H, m, M10), 2.06 (4H, s, M08), 1.21 - 1.93 (49H, m, M03)



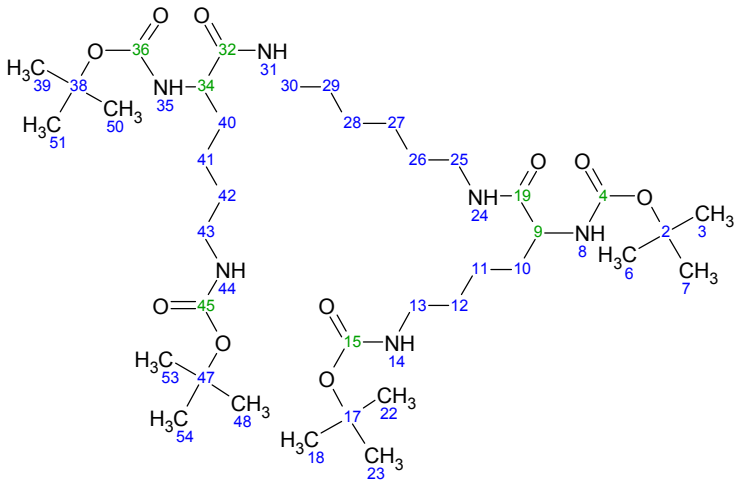
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/37
Date	11 Aug 2014 18:01:15		
Date Stamp	11 Aug 2014 18:01:15		
File Name	C:\Users\Mike Moazami\Desktop\au1114mm2 Boc4Ly2Hexanediamine\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	10056.9111	***Spectrum Type***	standard
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	25.002



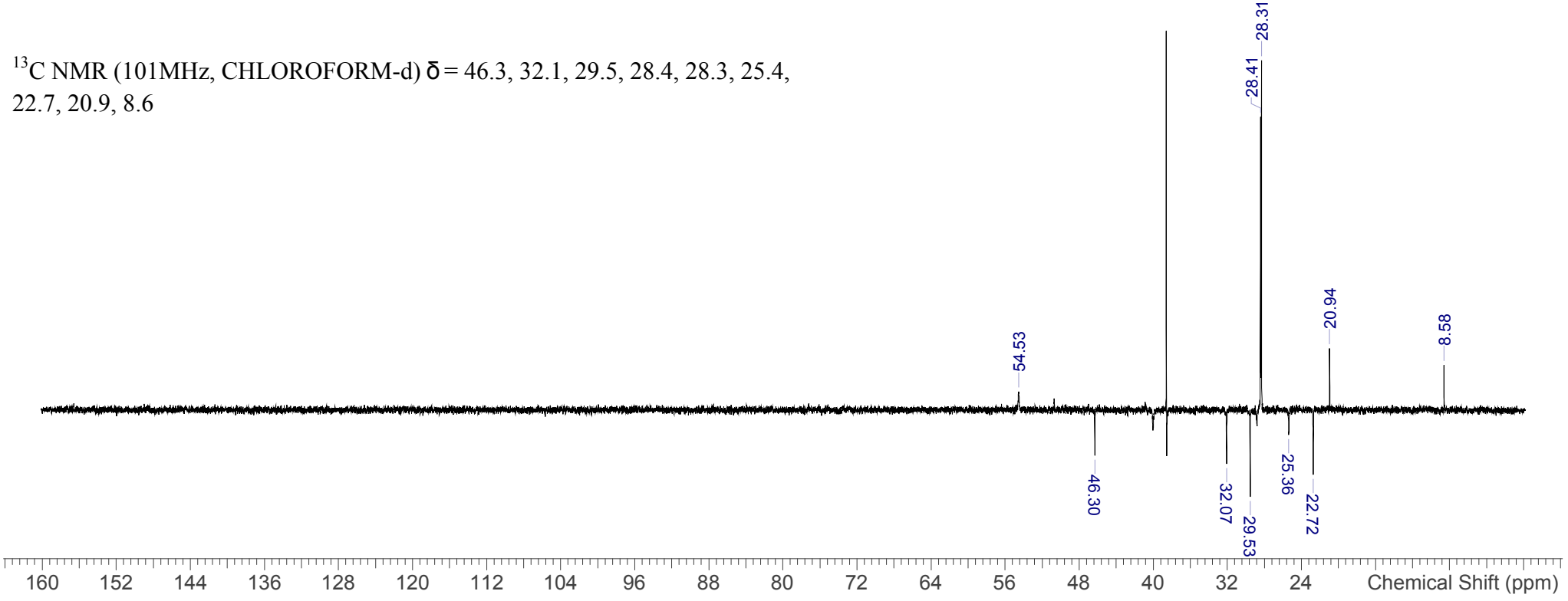
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 174.8, 172.7 (C-19, 32), 156.0 (C-4, 15, 45, 36), 46.3, 32.1, 29.5, 28.4, 28.3, 25.4, 22.7, 20.9, 8.6



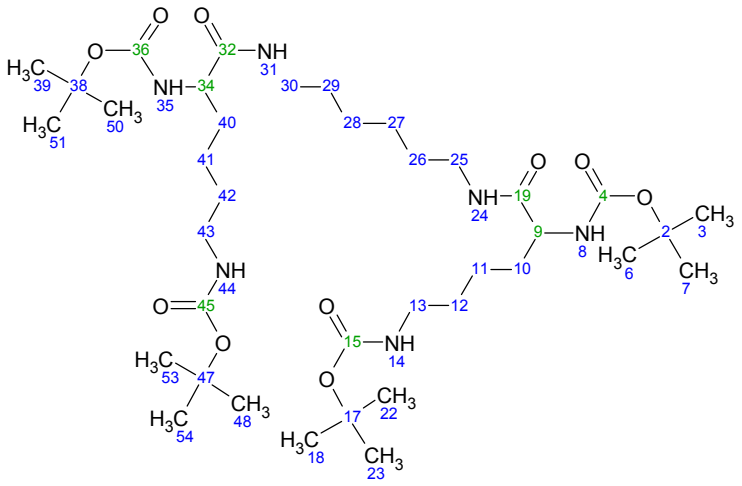
Acquisition Time (sec)	2.0316	***Comment***	mm/7316/37
Date	11 Aug 2014 18:19:55		
Date Stamp	11 Aug 2014 18:19:55		
File Name	C:\Users\Mike Moazami\Desktop\au1114mm2 Boc4Ly2Hexanediamine\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	8044.8184	***Spectrum Type***	DEPT135
Sweep Width (Hz)	16128.54	***Temperature (degree C)***	24.992



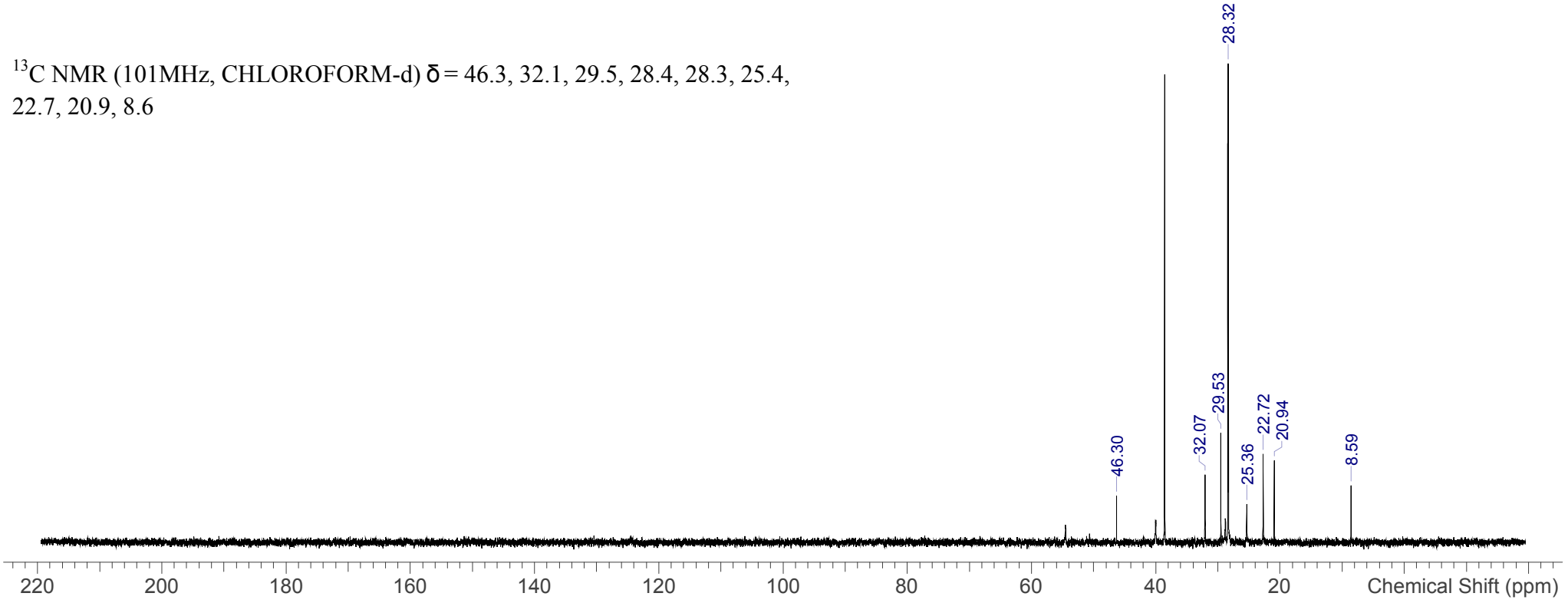
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 46.3, 32.1, 29.5, 28.4, 28.3, 25.4, 22.7, 20.9, 8.6



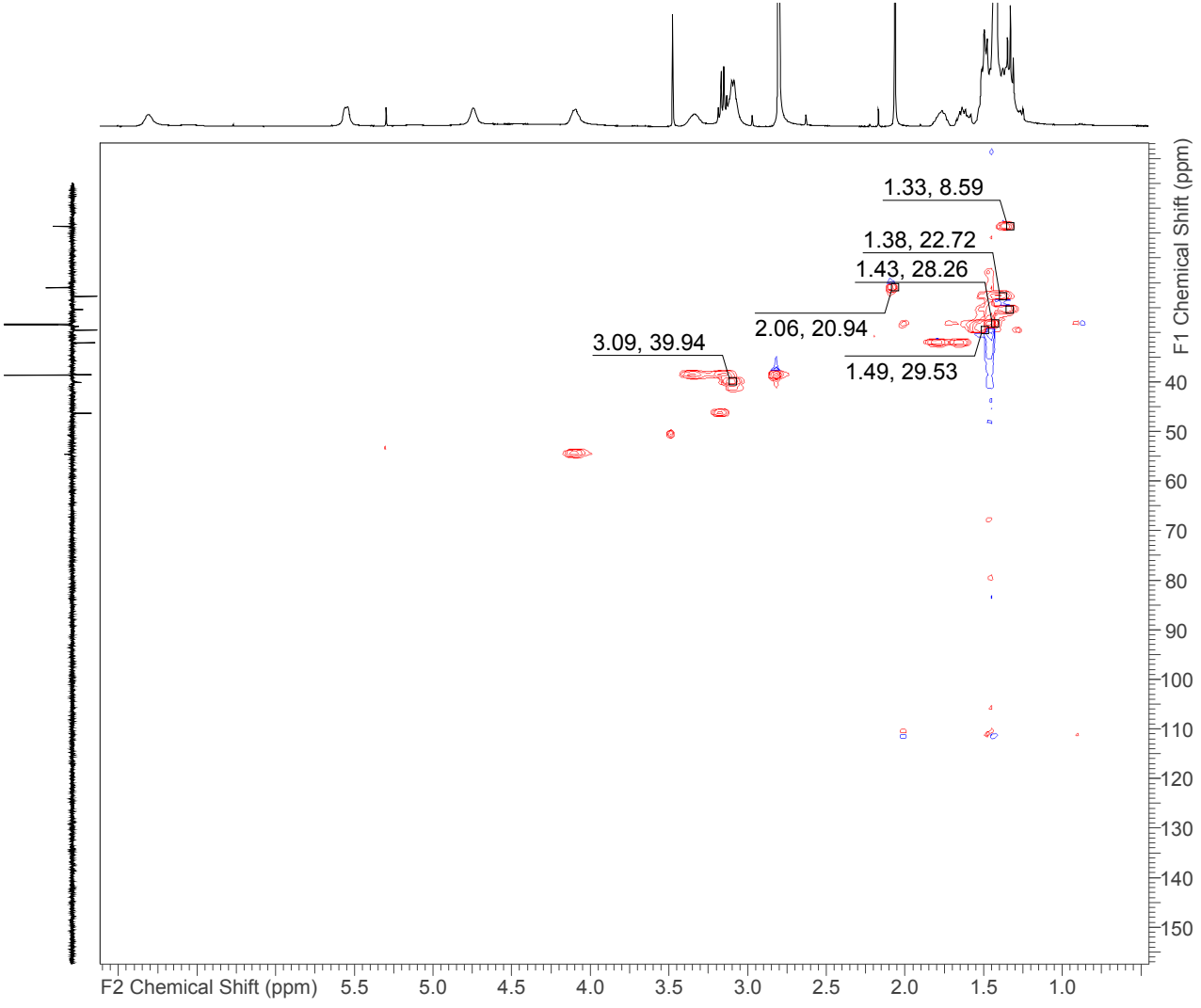
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/37
Date	11 Aug 2014 18:51:49		
Date Stamp	11 Aug 2014 18:51:49		
File Name	C:\Users\Mike Moazami\Desktop\au1114mm2 Boc4Ly2Hexanediamine\14\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp45
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d		
Spectrum Offset (Hz)	10057.6475	***Spectrum Type***	DEPT45
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	24.995

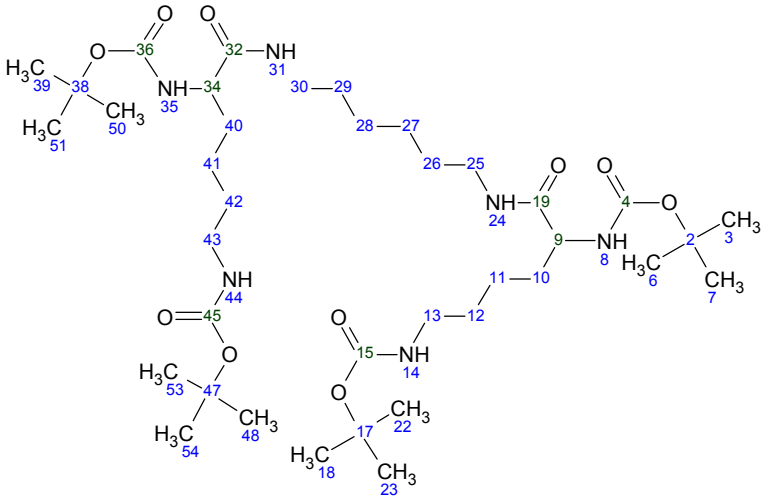


^{13}C NMR (101MHz, CHLOROFORM-d) δ = 46.3, 32.1, 29.5, 28.4, 28.3, 25.4, 22.7, 20.9, 8.6

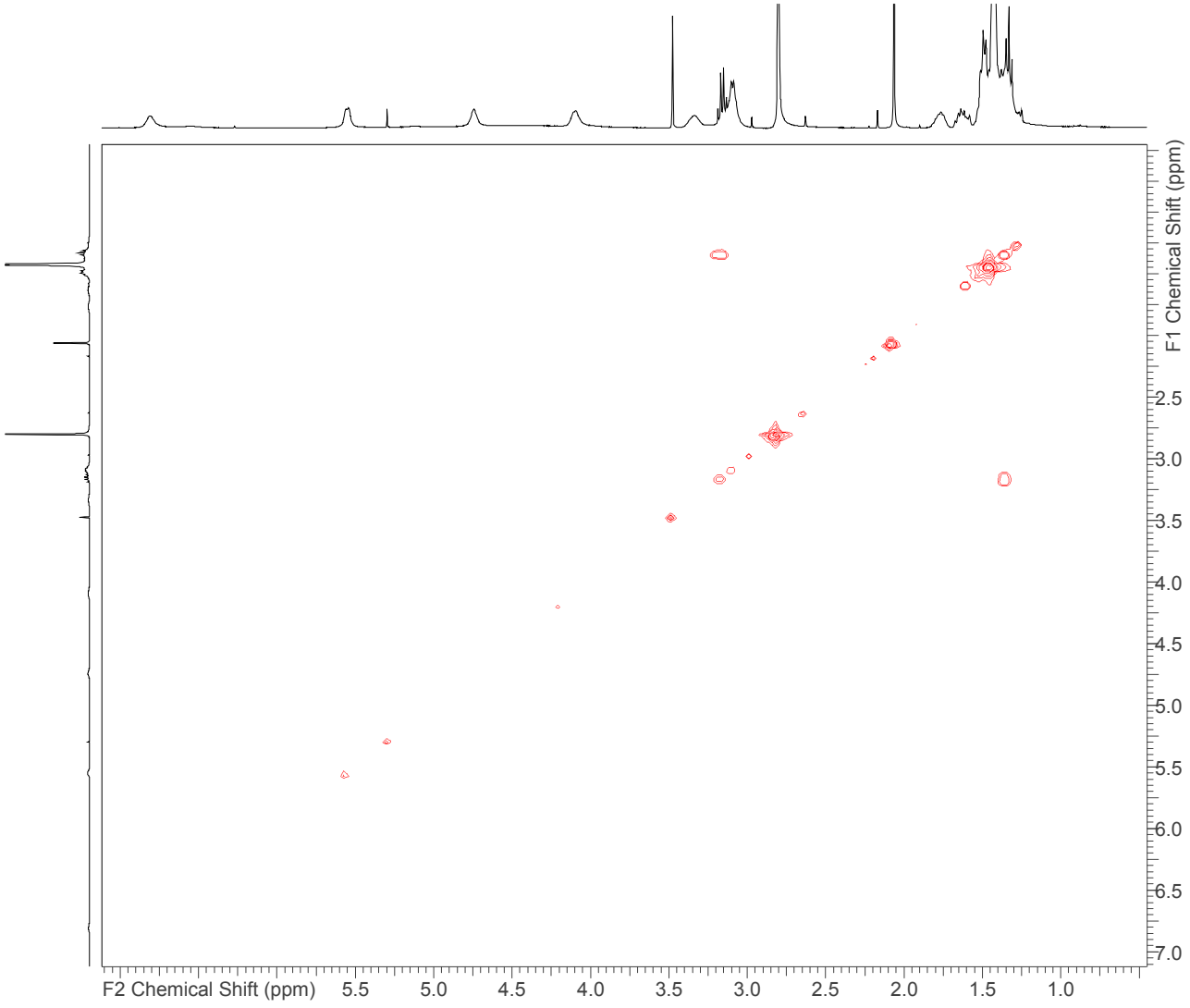


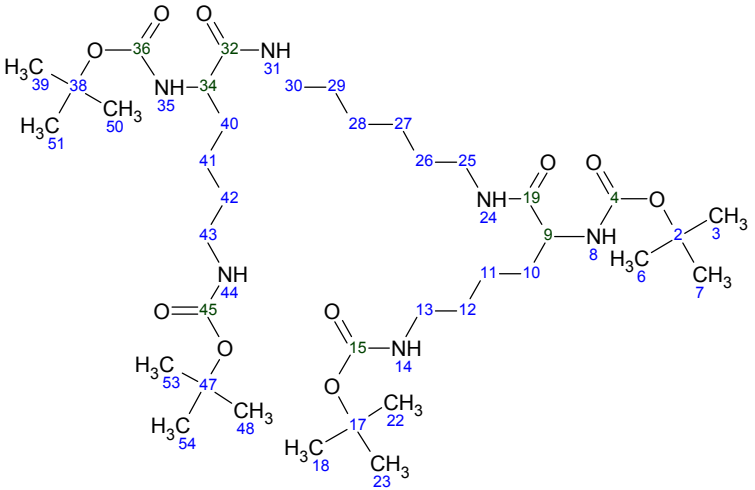
Acquisition Time (sec)	(0.1495, 0.0154)	***Comment***	5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218
Date	11 Aug 2014 19:07:00		
File Name	C:\Users\Mike Moazami\Desktop\au1114mm2 Boc4Ly2Hexanediamine\15\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)	***Nucleus***	(1H, 13C)
Number of Transients	2	***Origin***	spect
Original Points Count	(512, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	hsqcetgp
Solvent	CHLOROFORM-d	***Spectrum Type***	HSQC
Sweep Width (Hz)	(3421.31, 16650.39)	***Temperature (degree C)***	25.079
Title	mm/		





Acquisition Time (sec)	(0.2990, 0.0748)	***Comment***	5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218
Date	11 Aug 2014 18:36:00		
File Name	C:\Users\Mike Moazami\Desktop\au1114mm2 Boc4Ly2Hexanediamine\13\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)	***Nucleus***	(1H, 1H)
Number of Transients	2	***Origin***	spect
Original Points Count	(1024, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	cosygpqf
Solvent	CHLOROFORM-d	***Spectrum Type***	COSY
Sweep Width (Hz)	(3421.31, 3421.31)	***Temperature (degree C)***	24.993
Title	mm/		



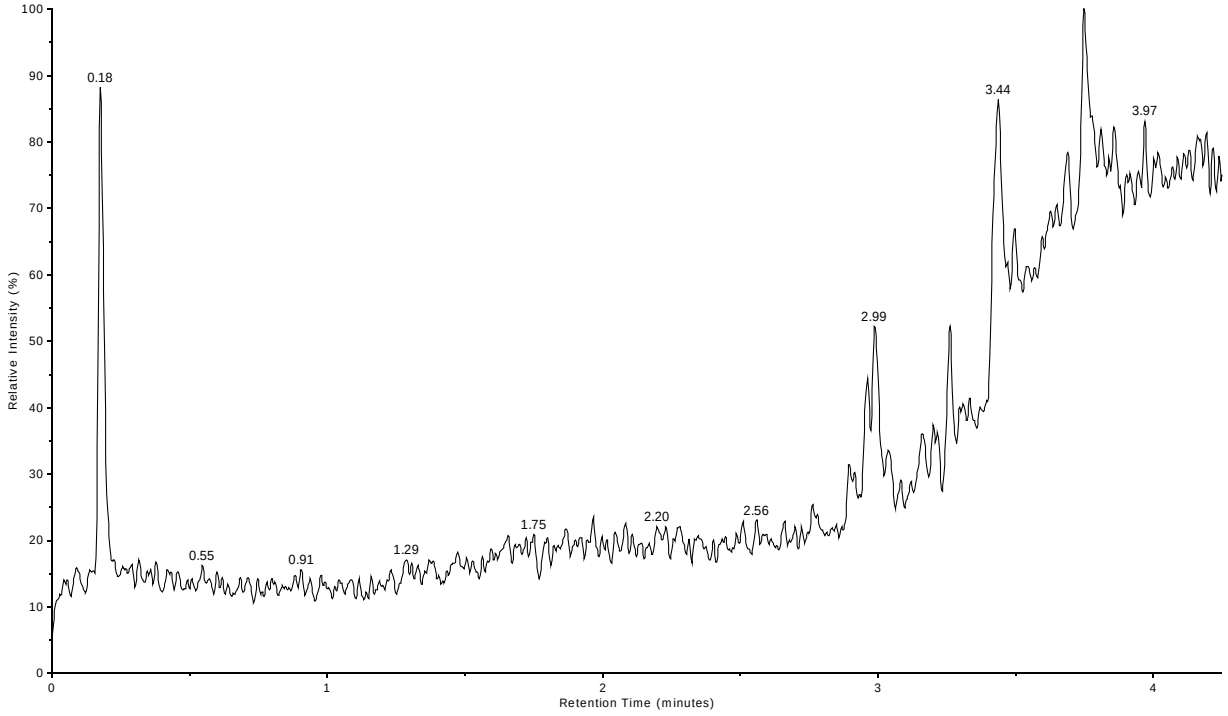


A.22 (2S,2'S)-N,N'-(hexane-1,6-diyl)bis(2,6-diaminohexanamide)

Top 5 Peak Report

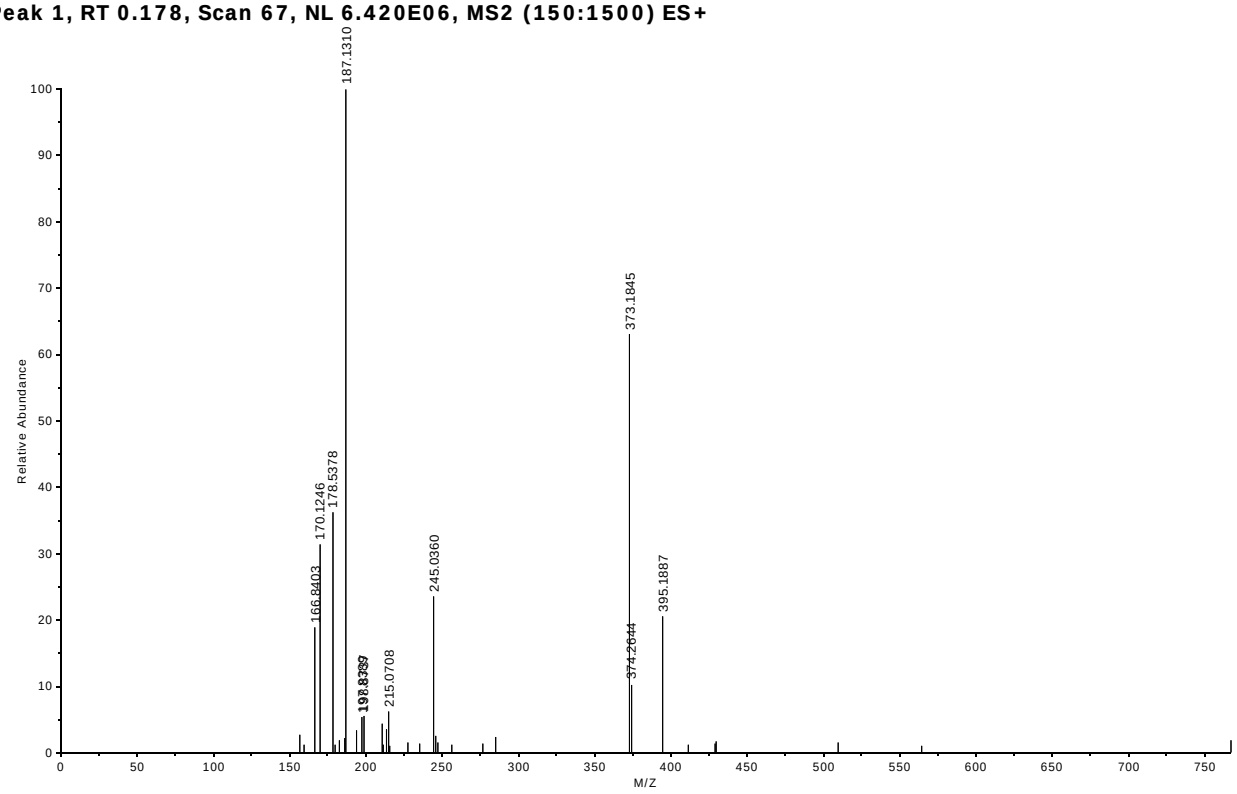
Group: Watts, J
Submitter: Michael Moazami
Project: RP-LC MW 150-1500
Sample ID: mm/7316/38
Experiment: B ESIPOS (simple mix) C18 Acid
Filename: Watts_J_mm731638_MM_381.pdf
Instrument: Blue RP UHPLC-MS (B30:1023)
Acquisition Date: 13/08/2014 12:25:06

TIC, NL 2.449E07, MS2 (150:1500) ES+

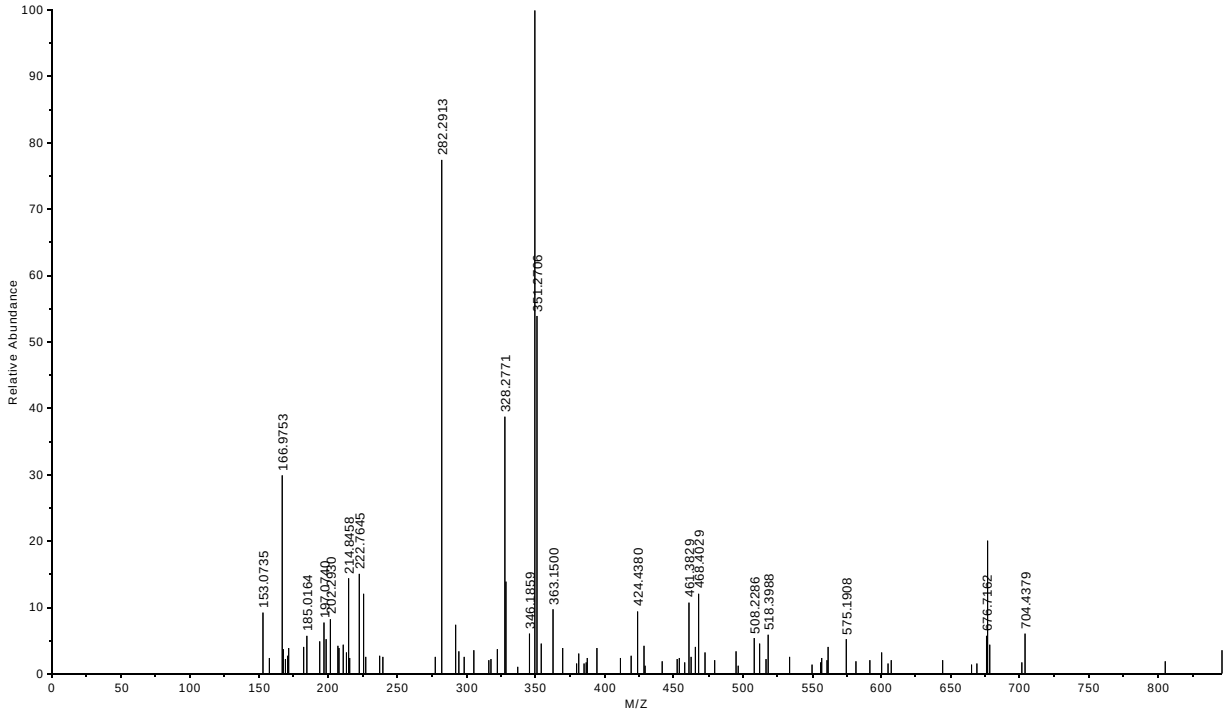


	RT Mins	Height	Height %	Area	Area %
1	0.18	17681380	100.00	25733686	95.03
2	2.99	4700146	26.58	8339579	30.80
3	3.26	5223633	29.54	9602561	35.46
4	3.44	8637096	48.85	27079551	100.00
5	3.75	6066243	34.31	14552222	53.74

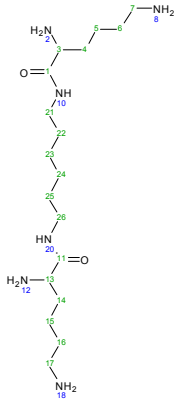
Peak 1, RT 0.178, Scan 67, NL 6.420E06, MS2 (150:1500) ES+



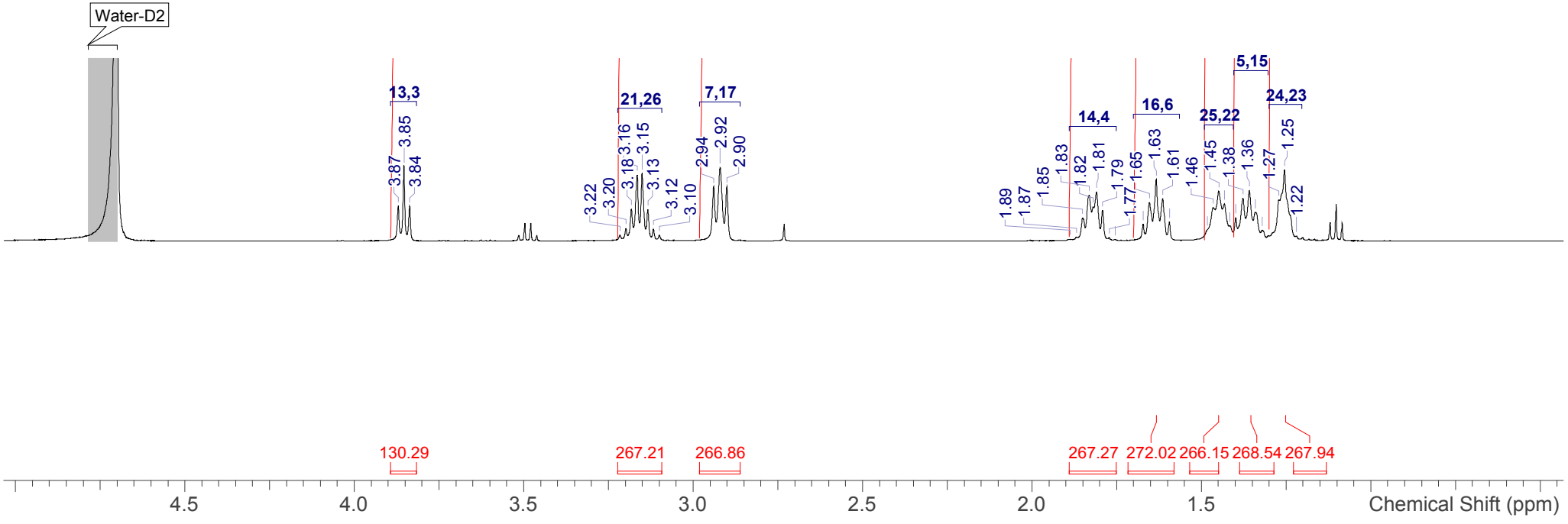
Peak 2, RT 2.990, Scan 1121, NL 2.112E06, MS2 (150:1500) ES+



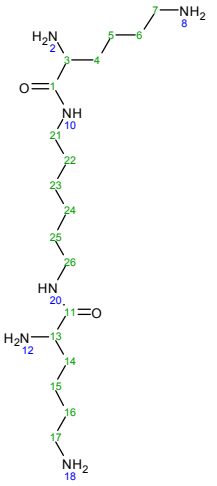
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Date Stamp	12 Aug 2014 18:40:43				
File Name	C:\Users\Mike Moazami\Desktop\au1214mm2\10\PDATA\1\1r				
Frequency (MHz)	400.1300	***Nucleus***	1H	***Number of Transients***	16
Origin	spect	***Original Points Count***	32768	***Owner***	nmr
Points Count	131072	***Pulse Sequence***	zg30	***Receiver Gain***	64.46
SW(cyclical) (Hz)	8012.82	***Solvent***	DEUTERIUM OXIDE		
Spectrum Offset (Hz)	2470.9973	***Spectrum Type***	standard	***Sweep Width (Hz)***	8012.76
Temperature (degree C)	25.002				



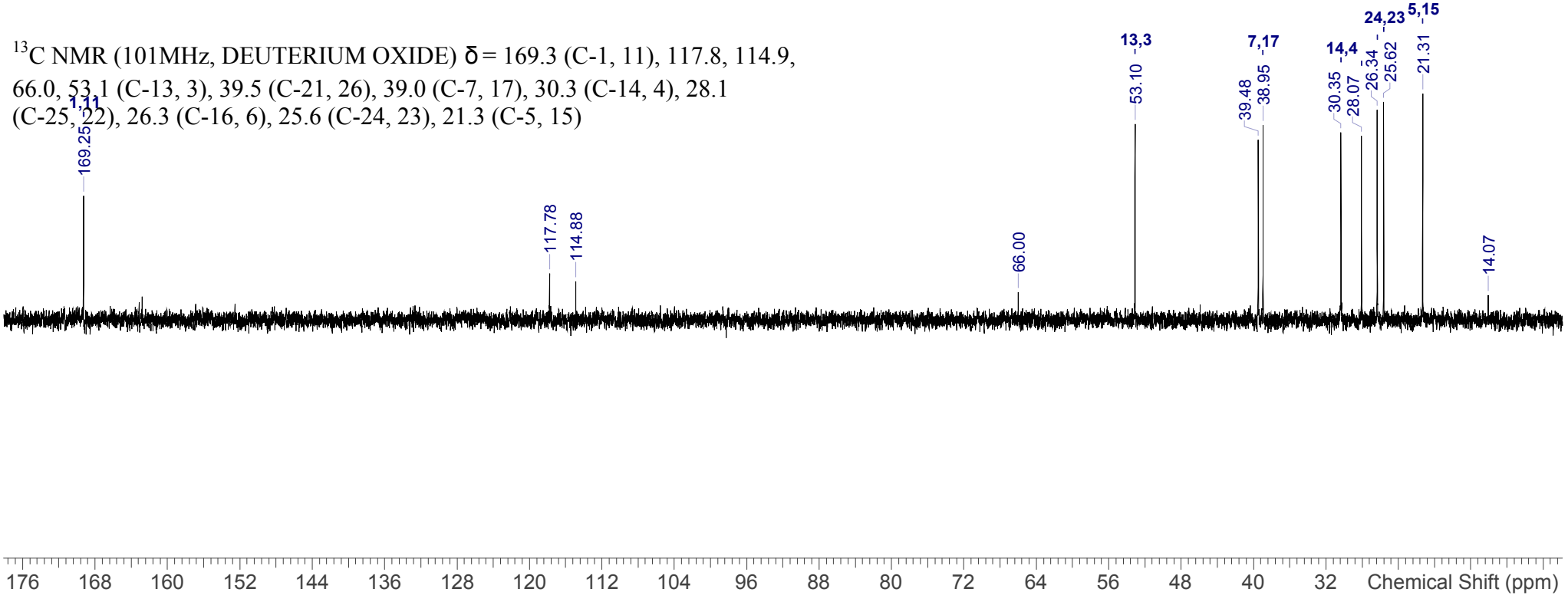
^{1}H NMR (400MHz, DEUTERIUM OXIDE) δ = 3.85 (2H, t, J = 6.7 Hz, H-13, 3), 3.09 - 3.22 (4H, m, H-21, 26), 2.92 (4H, t, J = 8.4 Hz, H-7, 17), 1.75 - 1.89 (4H, m, H-14, 4), 1.63 (4H, quin, J = 7.8 Hz, H-16, 6), 1.44 (21H, dq, J = 7.1 Hz, J = 6.2 Hz, H-25, 22), 1.36 (4H, tt, J = 8.8 Hz, J = 7.5 Hz, H-5, 15), 1.20 - 1.30 (4H, m, H-24, 23)



Acquisition Time (sec)	1.3631	***Comment***	mm/7316/38
Date	12 Aug 2014 19:11:08		
Date Stamp	12 Aug 2014 19:11:08		
File Name	C:\Users\Mike Moazami\Desktop\au1214mm2\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	DEUTERIUM OXIDE		
Spectrum Offset (Hz)	10060.3320	***Spectrum Type***	standard
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	24.999

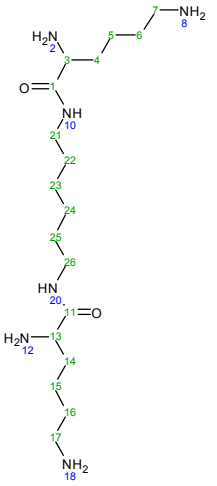


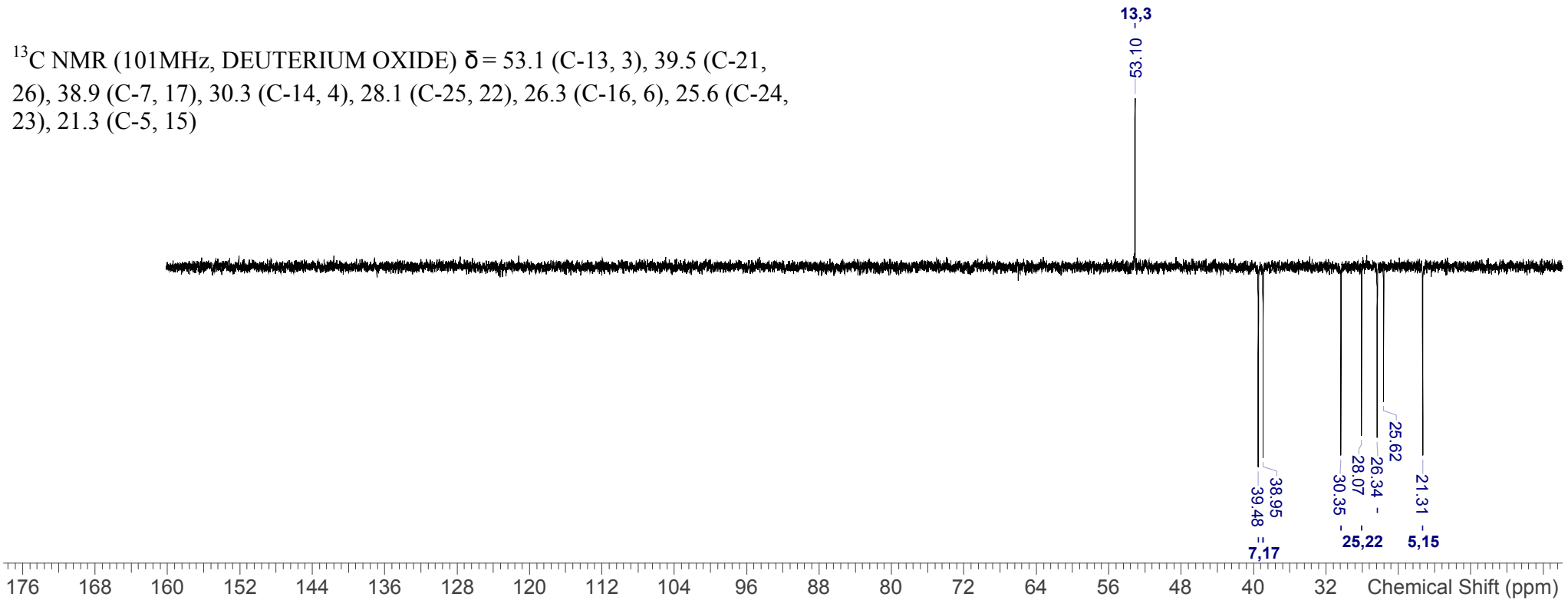
^{13}C NMR (101MHz, DEUTERIUM OXIDE) δ = 169.3 (C-1, 11), 117.8, 114.9, 66.0, 53.1 (C-13, 3), 39.5 (C-21, 26), 39.0 (C-7, 17), 30.3 (C-14, 4), 28.1 (C-25, 22), 26.3 (C-16, 6), 25.6 (C-24, 23), 21.3 (C-5, 15)



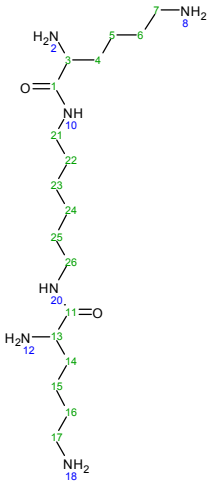
Acquisition Time (sec)	2.0316	***Comment***	mm/7316/38
Date	12 Aug 2014 19:29:44		
Date Stamp	12 Aug 2014 19:29:44		
File Name	C:\Users\Mike Moazami\Desktop\au1214mm2\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	DEUTERIUM OXIDE		
Spectrum Offset (Hz)	8048.1914	***Spectrum Type***	DEPT135
Sweep Width (Hz)	16128.54	***Temperature (degree C)***	25.001

^{13}C NMR (101MHz, DEUTERIUM OXIDE) δ = 53.1 (C-13, 3), 39.5 (C-21, 26), 38.9 (C-7, 17), 30.3 (C-14, 4), 28.1 (C-25, 22), 26.3 (C-16, 6), 25.6 (C-24, 23), 21.3 (C-5, 15)

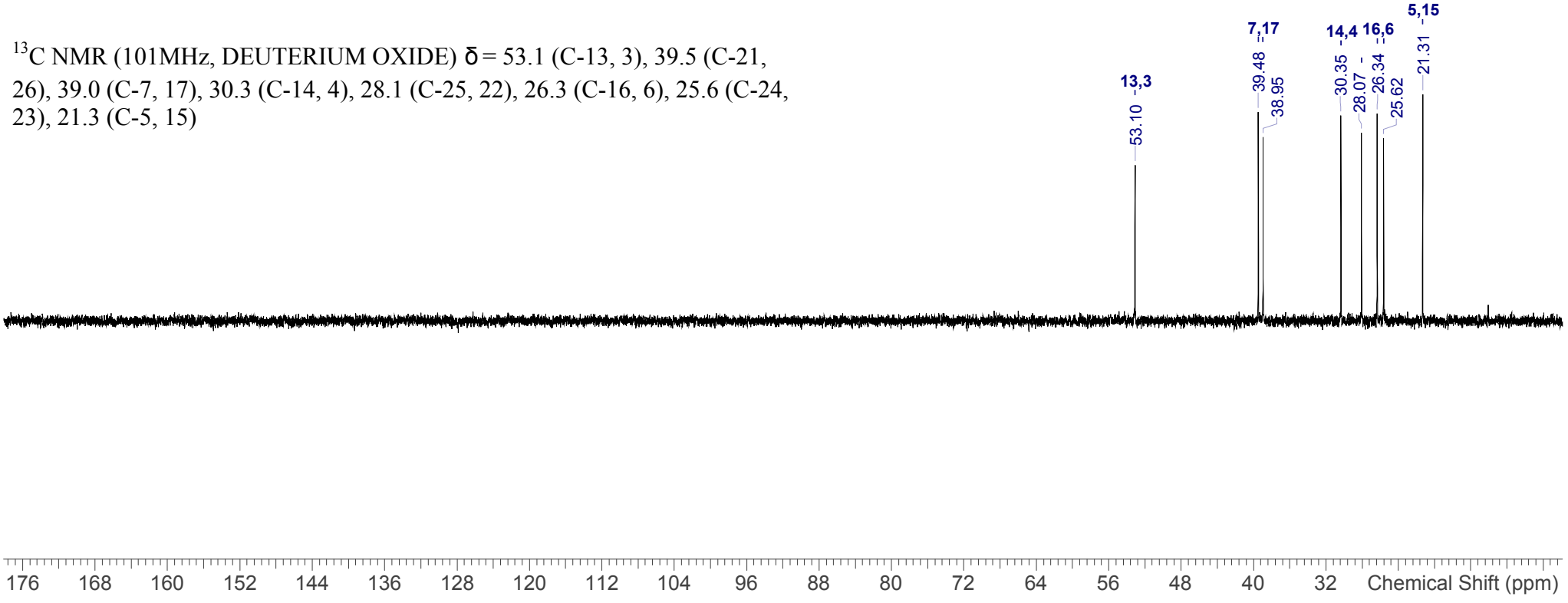




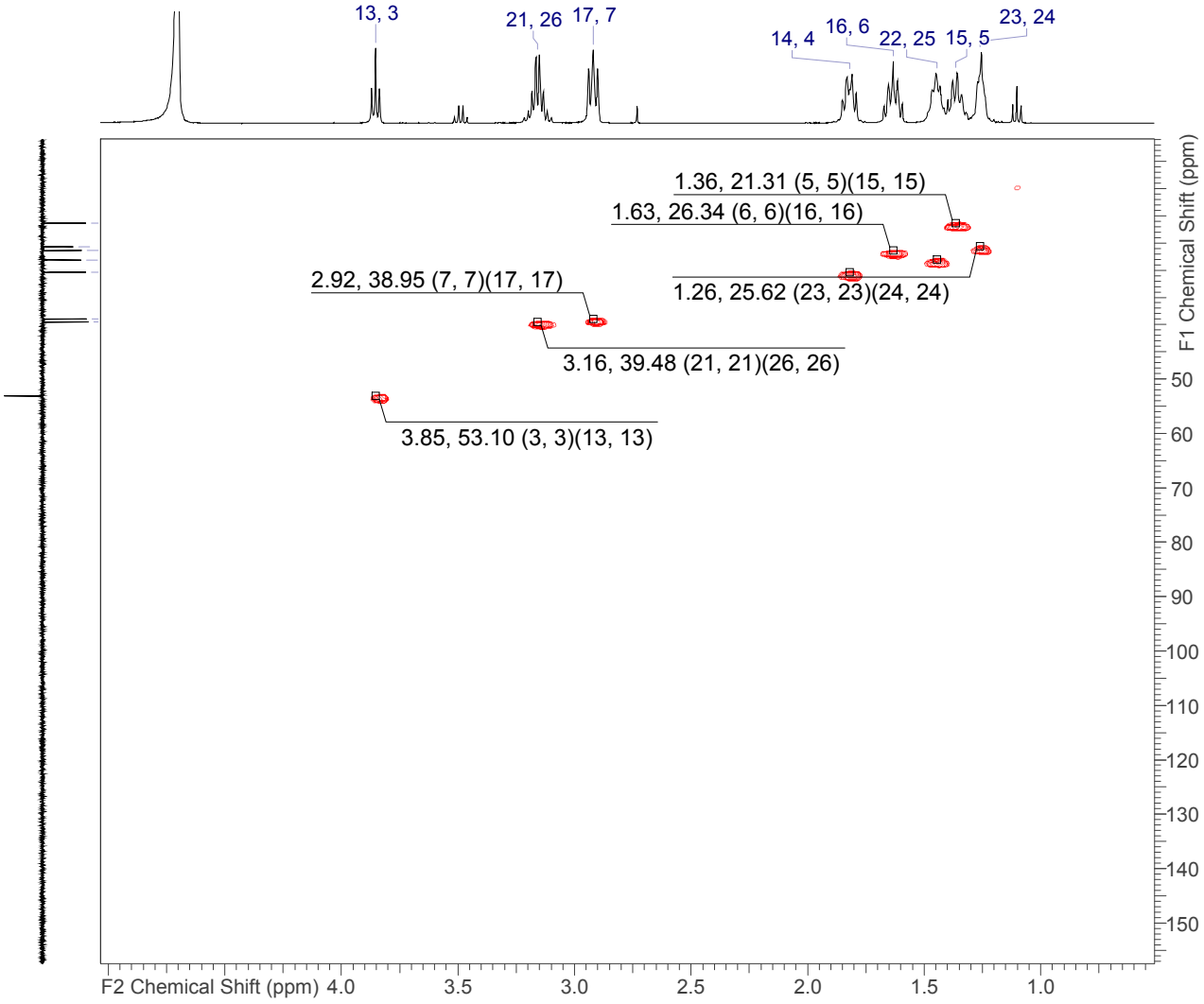
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/38
Date	12 Aug 2014 20:01:52		
Date Stamp	12 Aug 2014 20:01:52		
File Name	C:\Users\Mike Moazami\Desktop\au1214mm2\14\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp45
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	DEUTERIUM OXIDE		
Spectrum Offset (Hz)	10061.0684	***Spectrum Type***	DEPT45
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	24.999

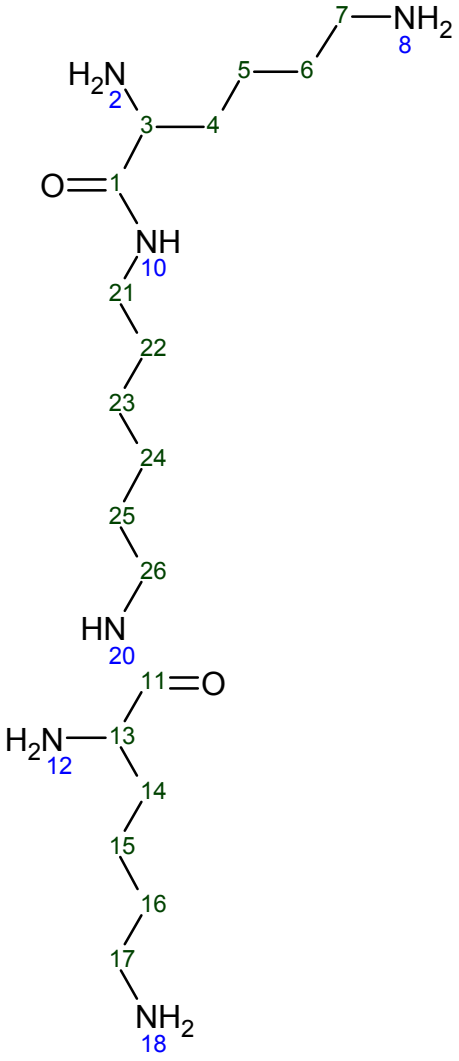


^{13}C NMR (101MHz, DEUTERIUM OXIDE) δ = 53.1 (C-13, 3), 39.5 (C-21, 26), 39.0 (C-7, 17), 30.3 (C-14, 4), 28.1 (C-25, 22), 26.3 (C-16, 6), 25.6 (C-24, 23), 21.3 (C-5, 15)

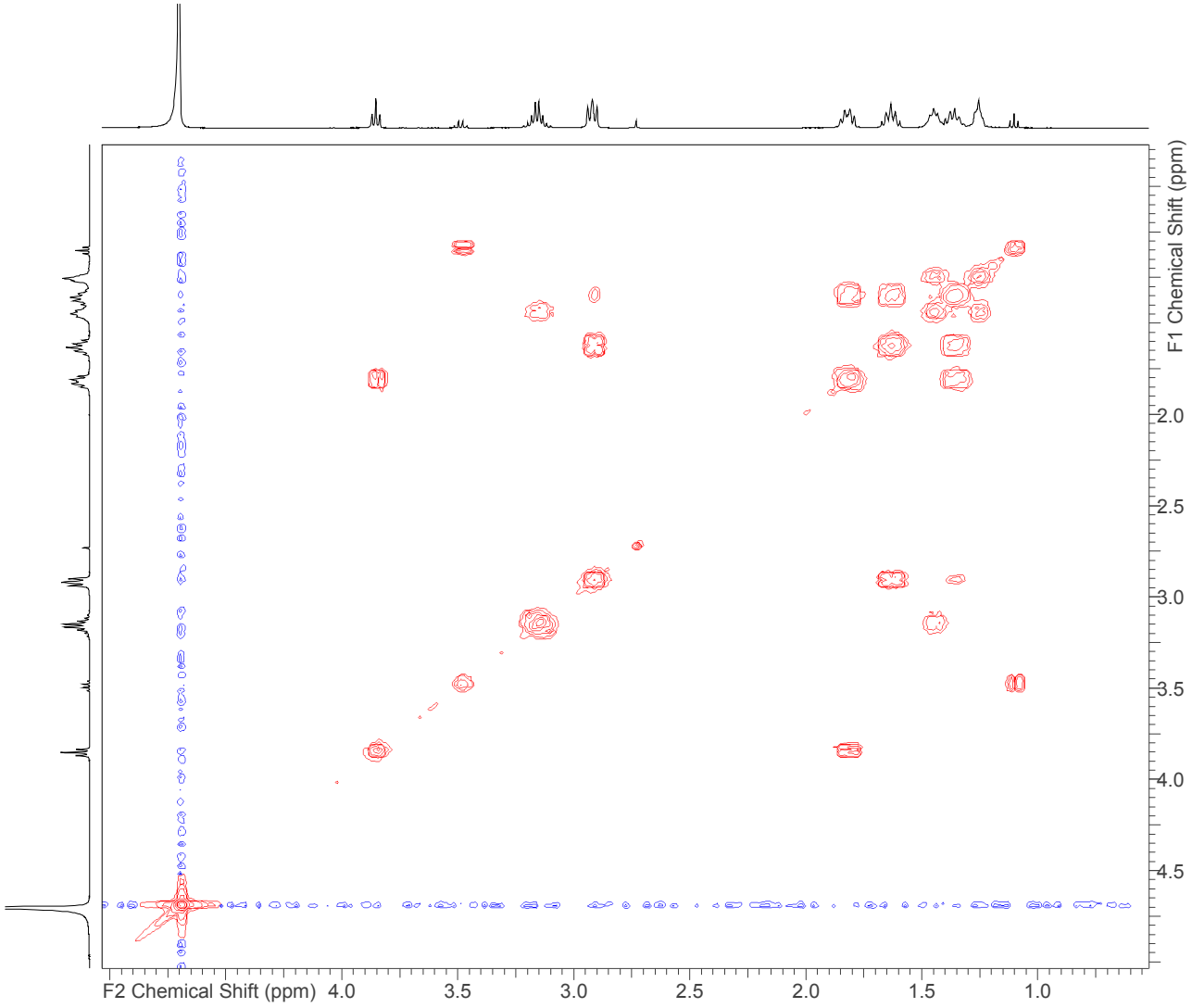


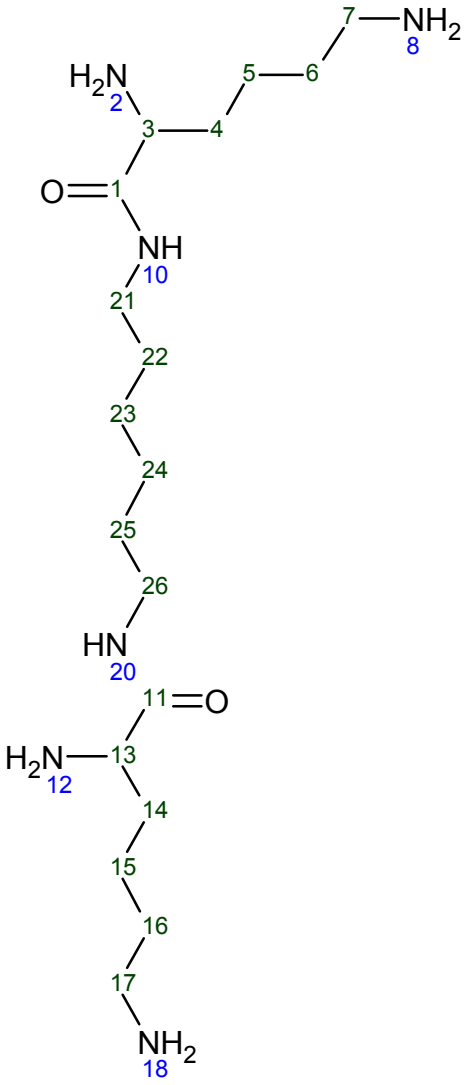
Acquisition Time (sec)	(0.2540, 0.0154)	***Comment***	5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218
Date	12 Aug 2014 20:17:00		
File Name	C:\Users\Mike Moazami\Desktop\au1214mm2\15\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)		
Nucleus	(1H, 13C)	***Number of Transients***	2
Origin	spect	***Original Points Count***	(512, 256)
Owner	nmr	***Points Count***	(1024, 1024)
Pulse Sequence	hsqcetgp	***Solvent***	DEUTERIUM OXIDE
Spectrum Type	HSQC	***Sweep Width (Hz)***	(2014.16, 16650.39)
Temperature (degree C)	25.188	***Title***	mm/



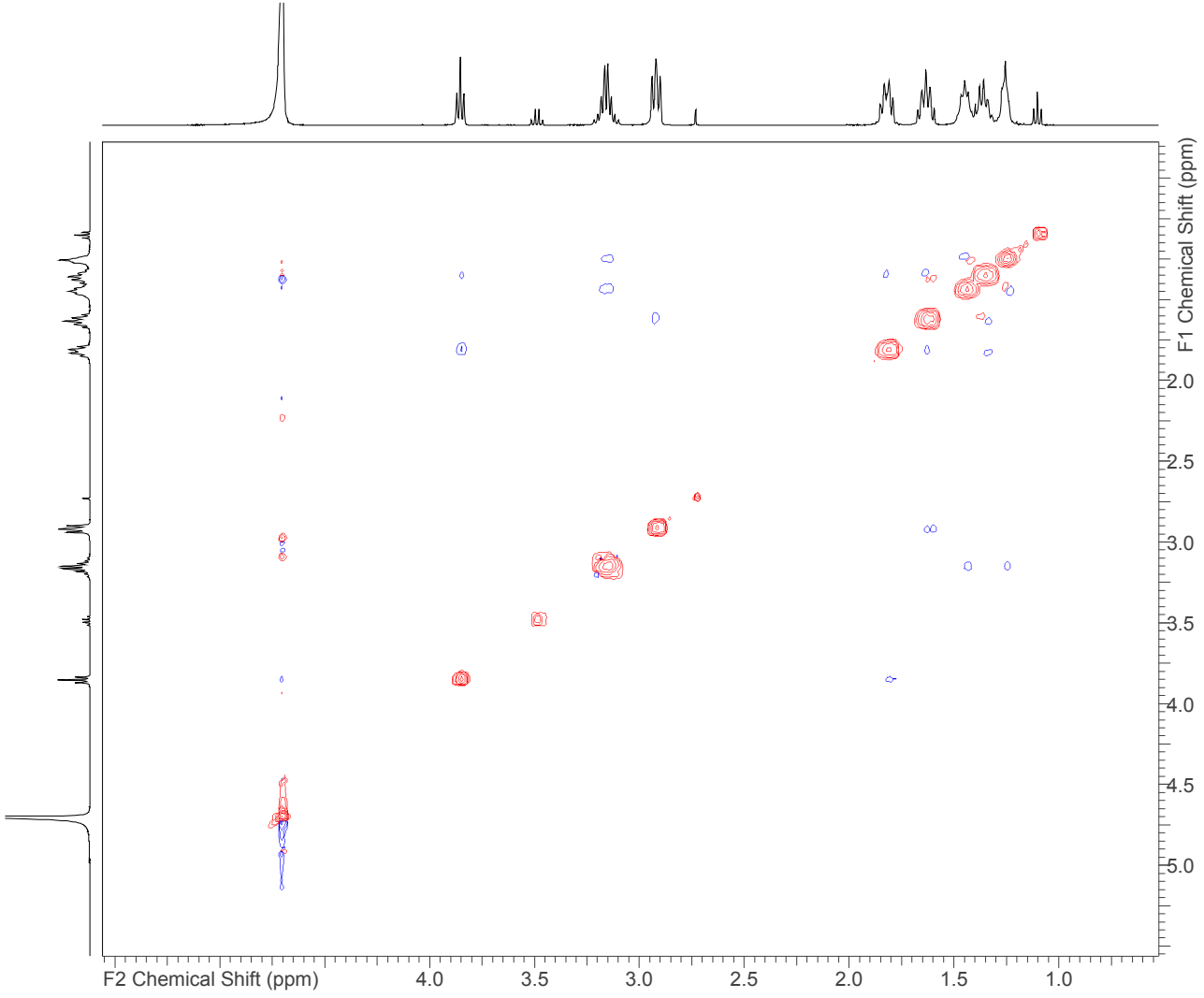


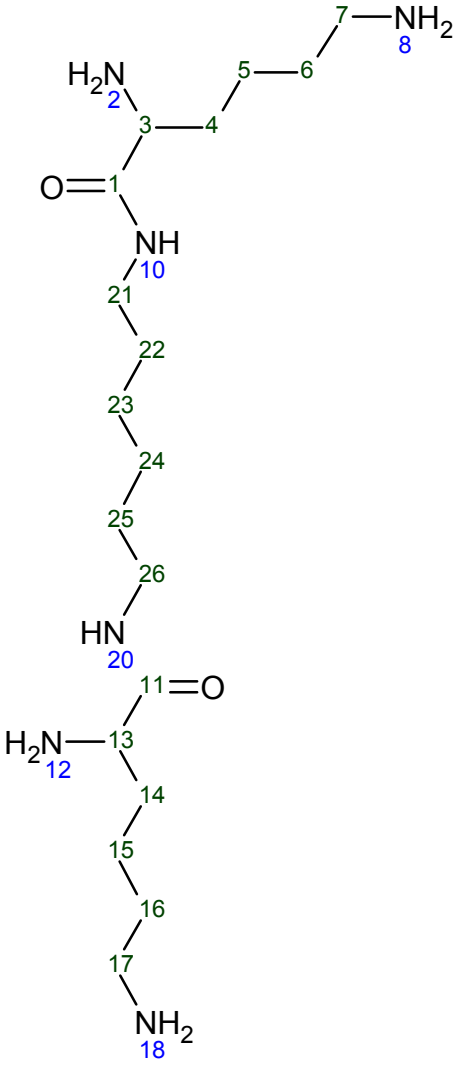
Acquisition Time (sec)	(0.5079, 0.1270)	***Comment***	5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218
Date	12 Aug 2014 19:46:00		
File Name	C:\Users\Mike Moazami\Desktop\au1214mm2\13\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)		
Nucleus	(1H, 1H)	***Number of Transients***	2
Origin	spect	***Original Points Count***	(1024, 256)
Owner	nmr	***Points Count***	(1024, 1024)
Pulse Sequence	cosygpqf	***Solvent***	DEUTERIUM OXIDE
Spectrum Type	COSY	***Sweep Width (Hz)***	(2014.16, 2014.16)
Temperature (degree C)	24.999	***Title***	mm/





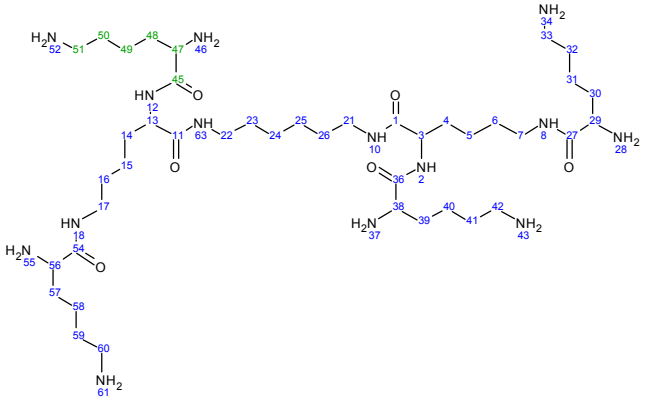
Acquisition Time (sec)	(0.5079, 0.1270)	***Comment***	5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218
Date	12 Aug 2014 21:54:00		
File Name	C:\Users\Mike Moazami\Desktop\au1214mm2\16\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)		
Nucleus	(1H, 1H)	***Number of Transients***	8
Origin	spect	***Original Points Count***	(1024, 256)
Owner	nmr	***Points Count***	(1024, 1024)
Pulse Sequence	noesygpphpp	***Solvent***	DEUTERIUM OXIDE
Spectrum Type	NOESY	***Sweep Width (Hz)***	(2014.16, 2014.16)
Temperature (degree C)	24.985	***Title***	mm/



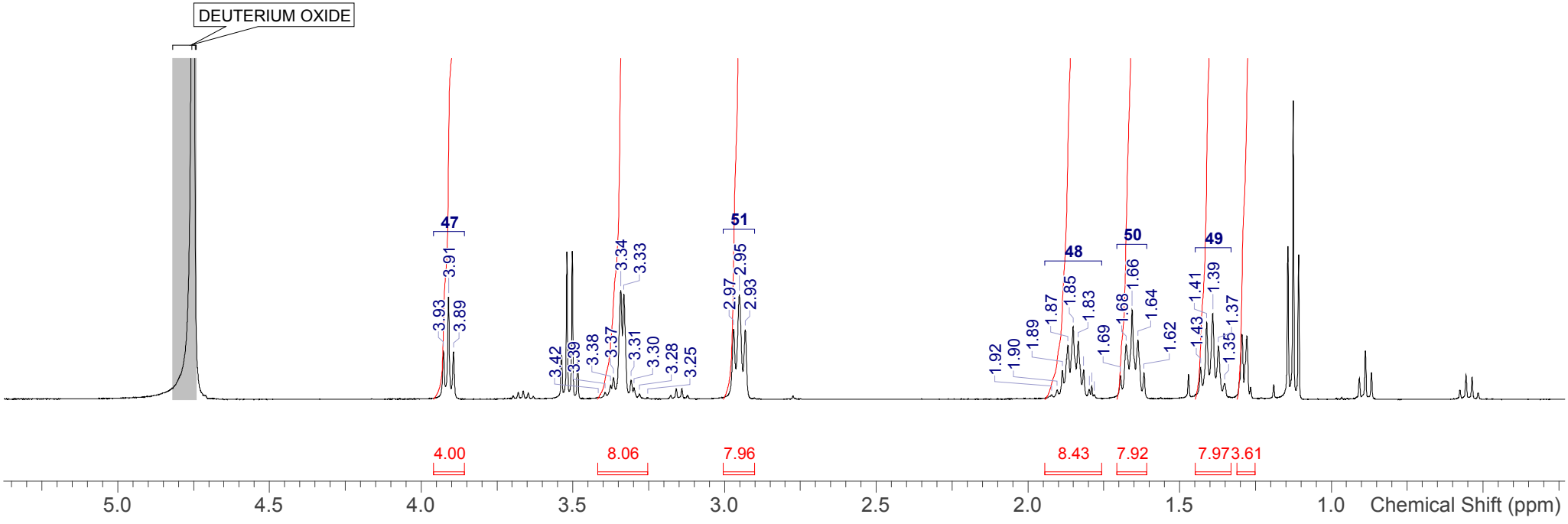


A.23 OctaAmine dendrimer

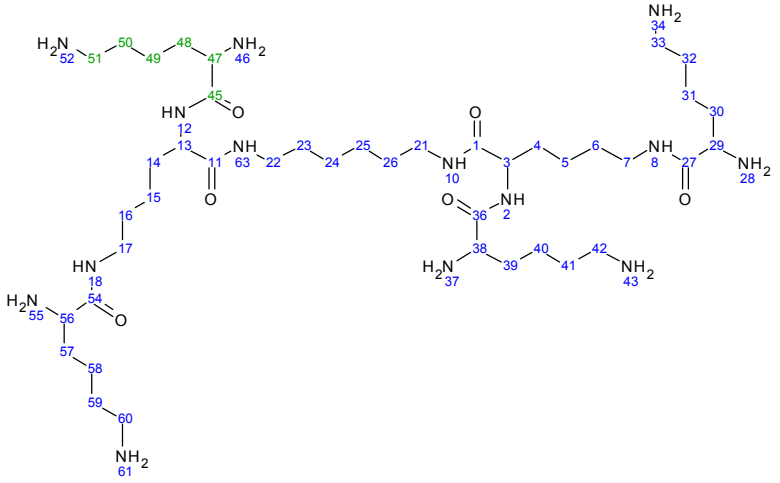
Acquisition Time (sec)	4.0894	***Comment***	mm/7316/31
Date	31 Jul 2014 18:34:09		
Date Stamp	31 Jul 2014 18:34:09		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\jy3114mm3 G2 Lysine deprotected\10\PDATA\1\1r		
Frequency (MHz)	400.1300	***Nucleus***	1H
Number of Transients	16	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	262144	***Pulse Sequence***	zg30
Receiver Gain	72.67	***SW(cyclical) (Hz)***	8012.82
Solvent	DEUTERIUM OXIDE		
Spectrum Offset (Hz)	2487.3779	***Spectrum Type***	standard
Sweep Width (Hz)	8012.79	***Temperature (degree C)***	25.004



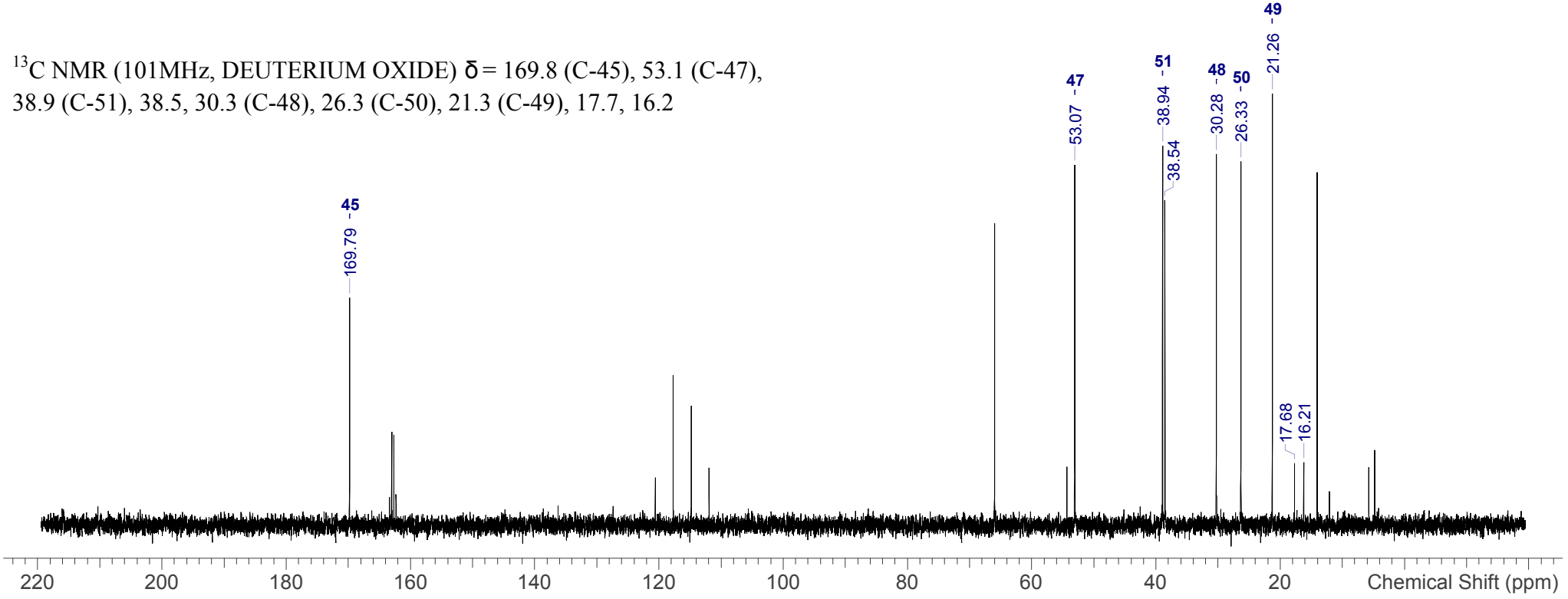
^{1}H NMR (400MHz, DEUTERIUM OXIDE) δ = 3.91 (4H, t, J = 6.6 Hz, H-47), 3.25 - 3.42 (8H, m, M02), 2.95 (8H, t, J = 7.7 Hz, H-51), 1.76 - 1.94 (8H, m, H-48), 1.66 (8H, quin, J = 7.7 Hz, H-50), 1.33 - 1.45 (8H, m, H-49), 1.25 - 1.31 (4H, m, M07)



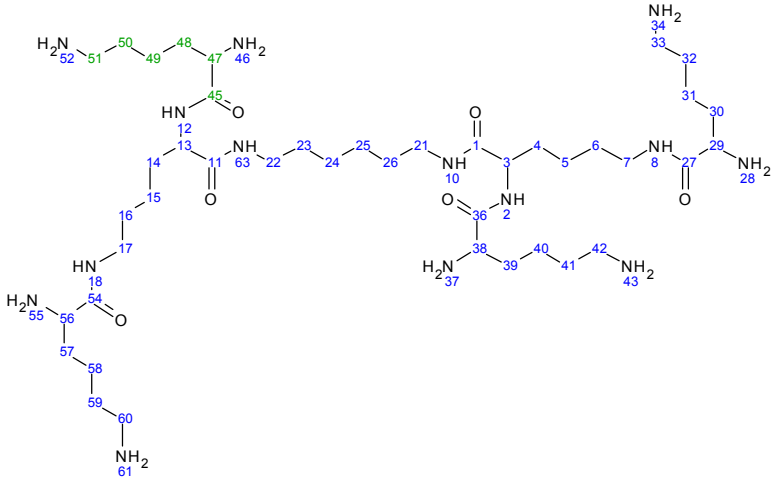
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/31
Date	31 Jul 2014 20:32:19		
Date Stamp	31 Jul 2014 20:32:19		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\jy3114mm3 G2 Lysine deprotected\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	2048	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	DEUTERIUM OXIDE		
Spectrum Offset (Hz)	10060.3320	***Spectrum Type***	standard
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	25.006



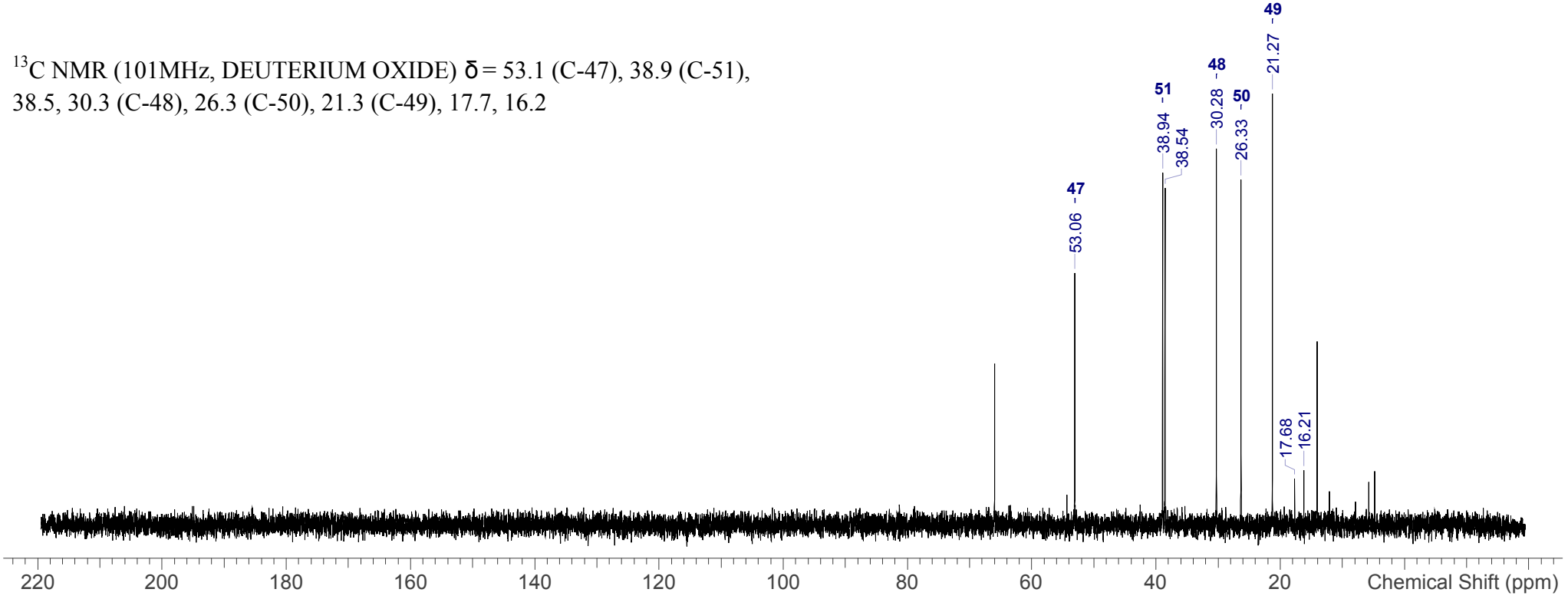
^{13}C NMR (101MHz, DEUTERIUM OXIDE) δ = 169.8 (C-45), 53.1 (C-47), 38.9 (C-51), 38.5, 30.3 (C-48), 26.3 (C-50), 21.3 (C-49), 17.7, 16.2



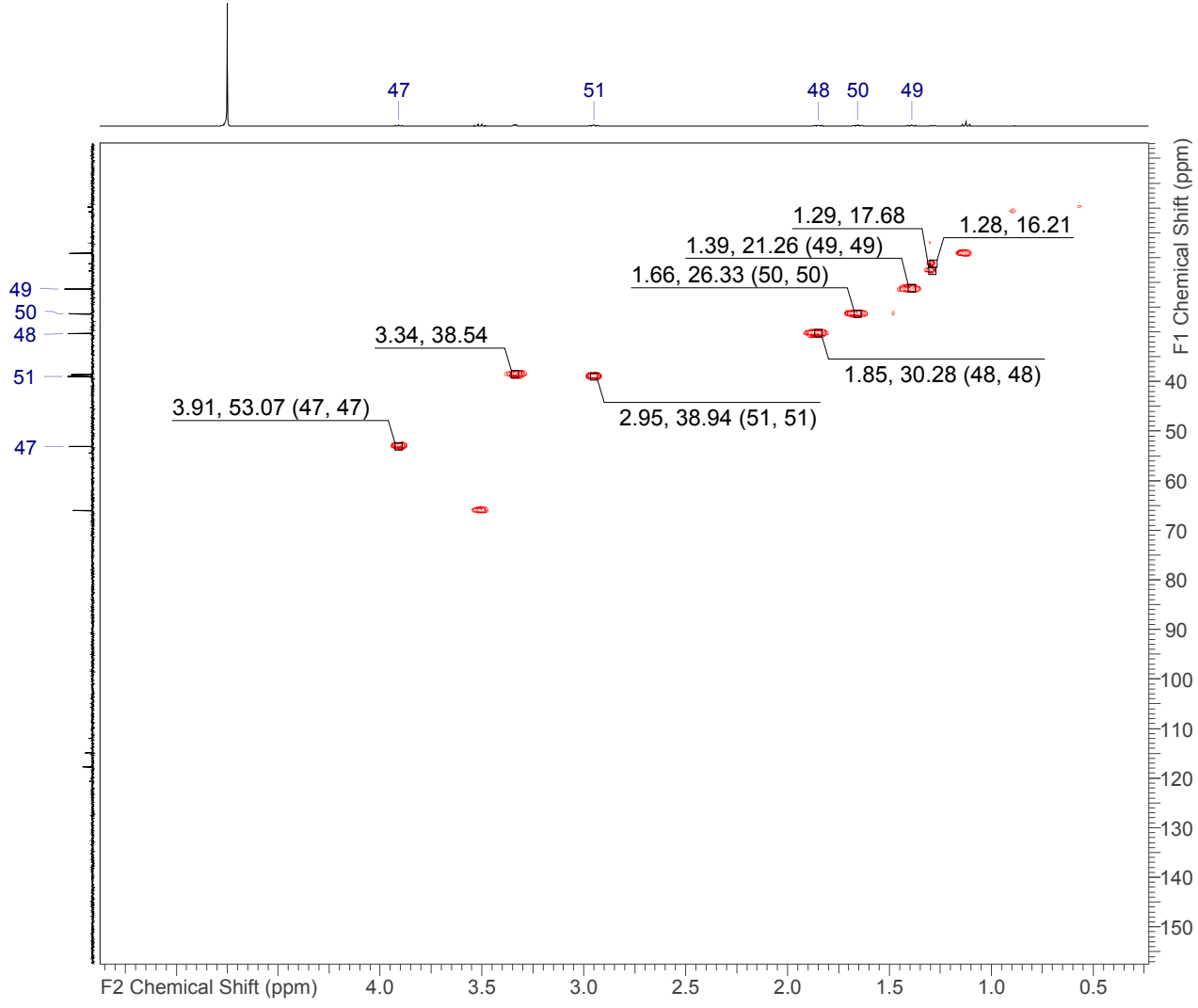
Acquisition Time (sec)	1.3631	***Comment***	mm/7316/31
Date	31 Jul 2014 20:48:02		
Date Stamp	31 Jul 2014 20:48:02		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\jy3114mm3 G2 Lysine deprotected\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp45
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	DEUTERIUM OXIDE		
Spectrum Offset (Hz)	10061.2959	***Spectrum Type***	DEPT45
Sweep Width (Hz)	24037.73	***Temperature (degree C)***	24.994

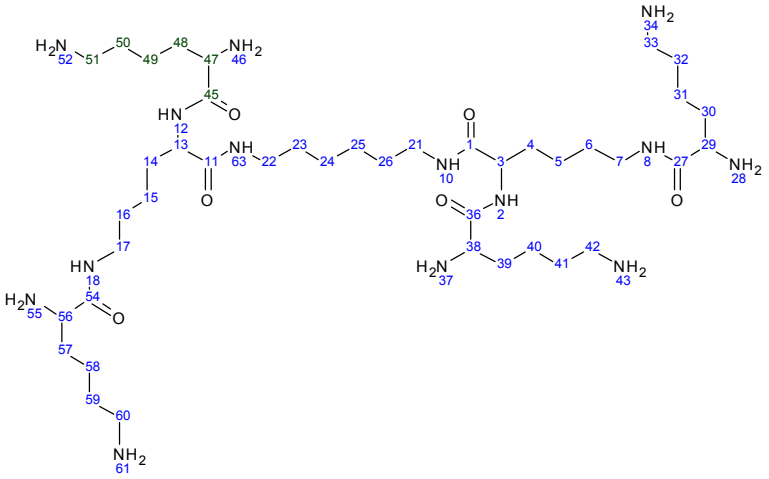


^{13}C NMR (101MHz, DEUTERIUM OXIDE) δ = 53.1 (C-47), 38.9 (C-51), 38.5, 30.3 (C-48), 26.3 (C-50), 21.3 (C-49), 17.7, 16.2



Acquisition Time (sec)	(0.2294, 0.0154)	***Comment***	mm/7316/31
Date	31 Jul 2014 20:03:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\PhD NMR Data RAW\jy3114mm3 G2 Lysine deprotected\13\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)	***Nucleus***	(1H, 13C)
Number of Transients	2	***Origin***	spect
Original Points Count	(512, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	hsqcetgp
Solvent	DEUTERIUM OXIDE	***Spectrum Type***	HSQC
Sweep Width (Hz)	(2229.96, 16650.39)	***Temperature (degree C)***	25.102
Title	mm/7316/31		



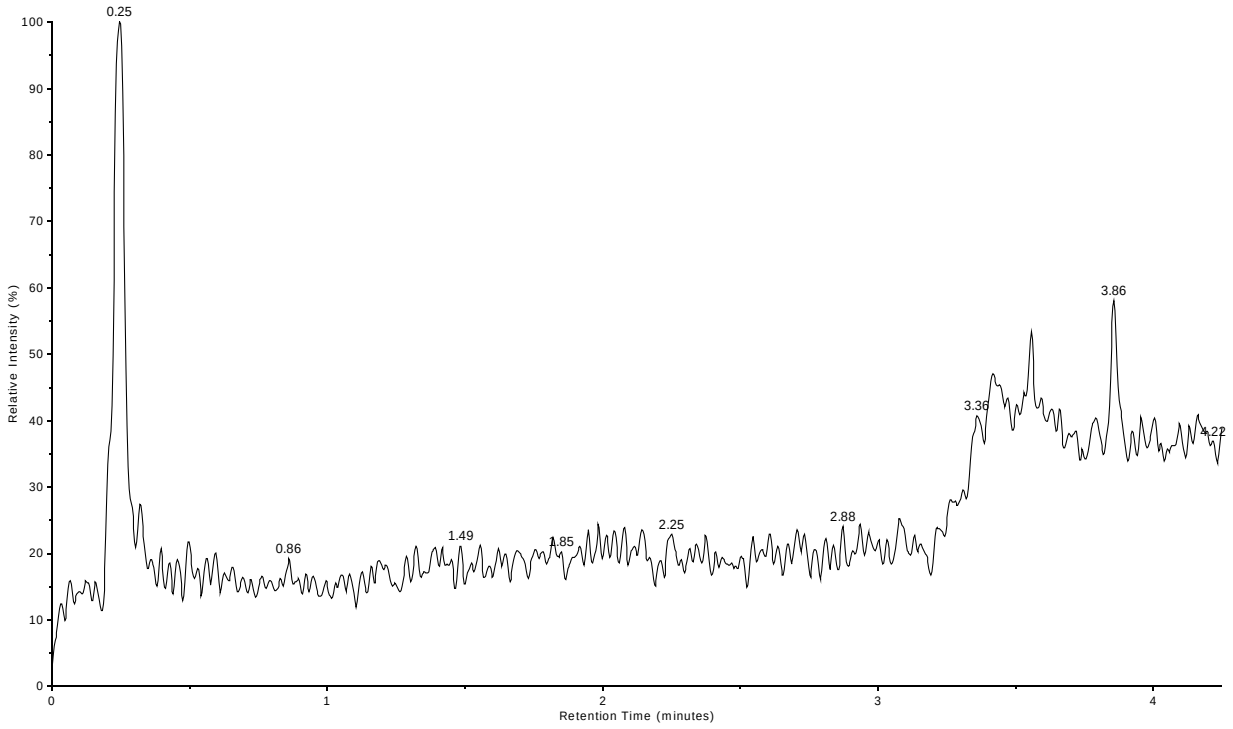


A.24 Doubler Alcohol

Top 5 Peak Report

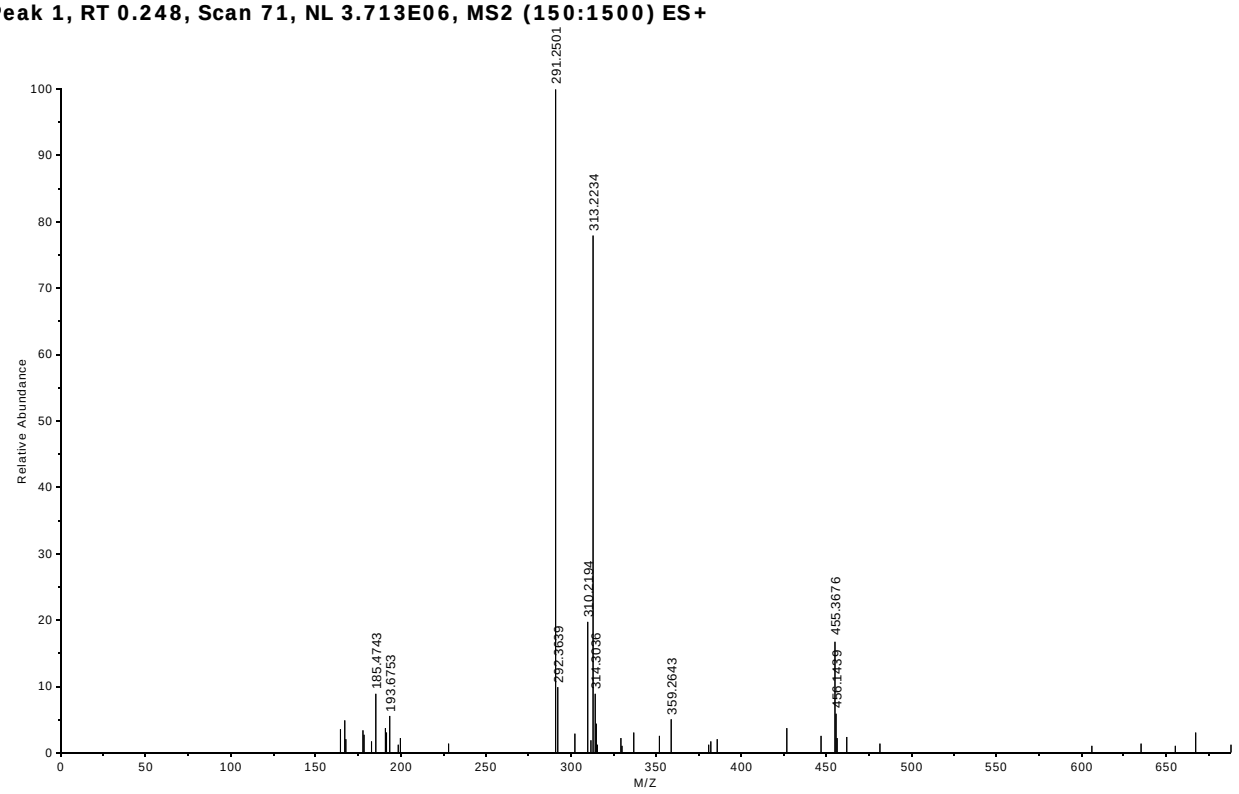
Group: Watts, J
Submitter: Michael Moazami
Project: RP LC C18 < 2 cmpds
Sample ID: mm/7500/38 alcohol doubler
Experiment: BLUE ESIPOS C18 5 min
Filename: mm750038_alcohol_doubler_Michael_Moazami_
Instrument: Blue RP UHPLC-MS (B30:1023)
Acquisition Date: 28/04/2015 12:22:52

TIC, NL 1.199E07, MS2 (150:1500) ES+

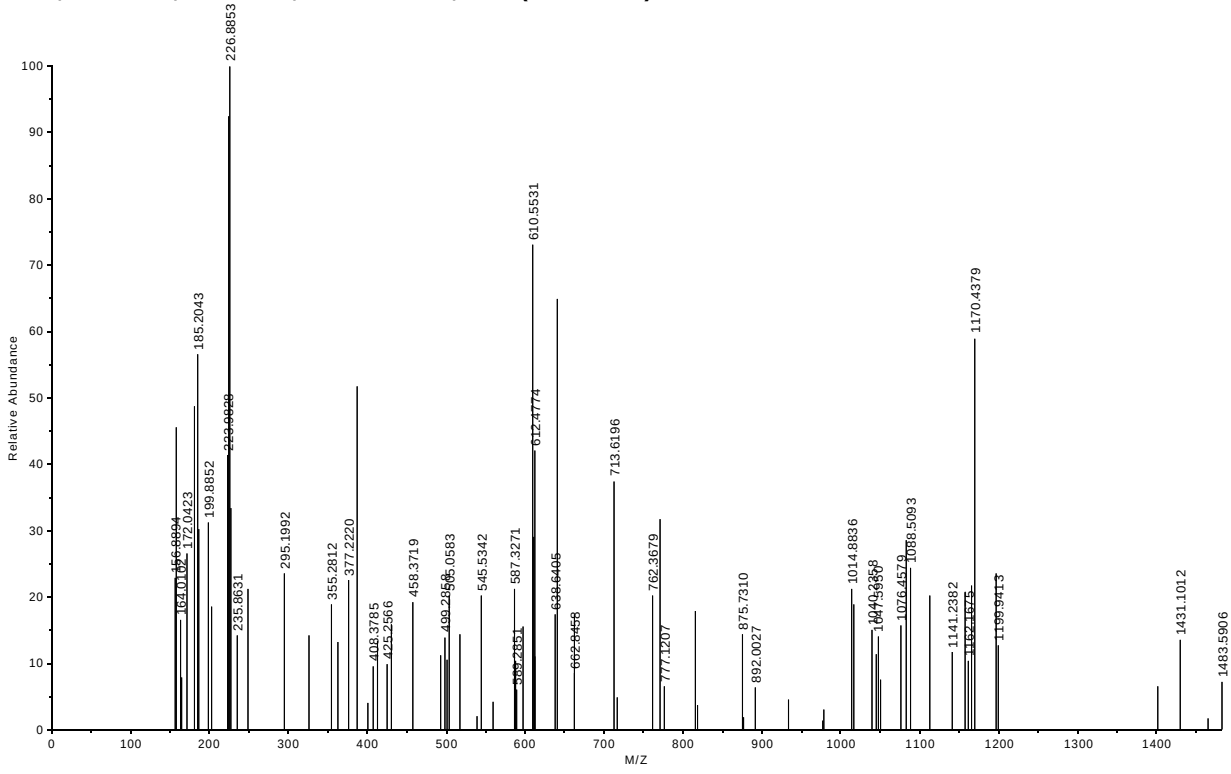


	RT Mins	Height	Height %	Area	Area %
1	0.25	10057141	100.00	34206889	100.00
2	3.36	1507106	14.99	3892684	11.38
3	3.42	1258402	12.51	2725157	7.97
4	3.56	1261828	12.55	1511862	4.42
5	3.86	2838836	28.23	5950853	17.40

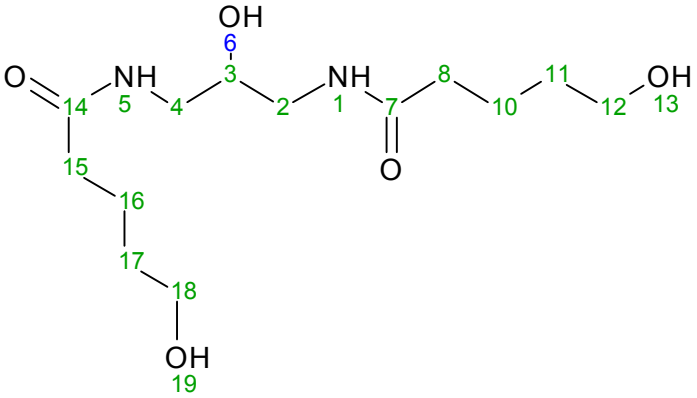
Peak 1, RT 0.248, Scan 71, NL 3.713E06, MS2 (150:1500) ES+



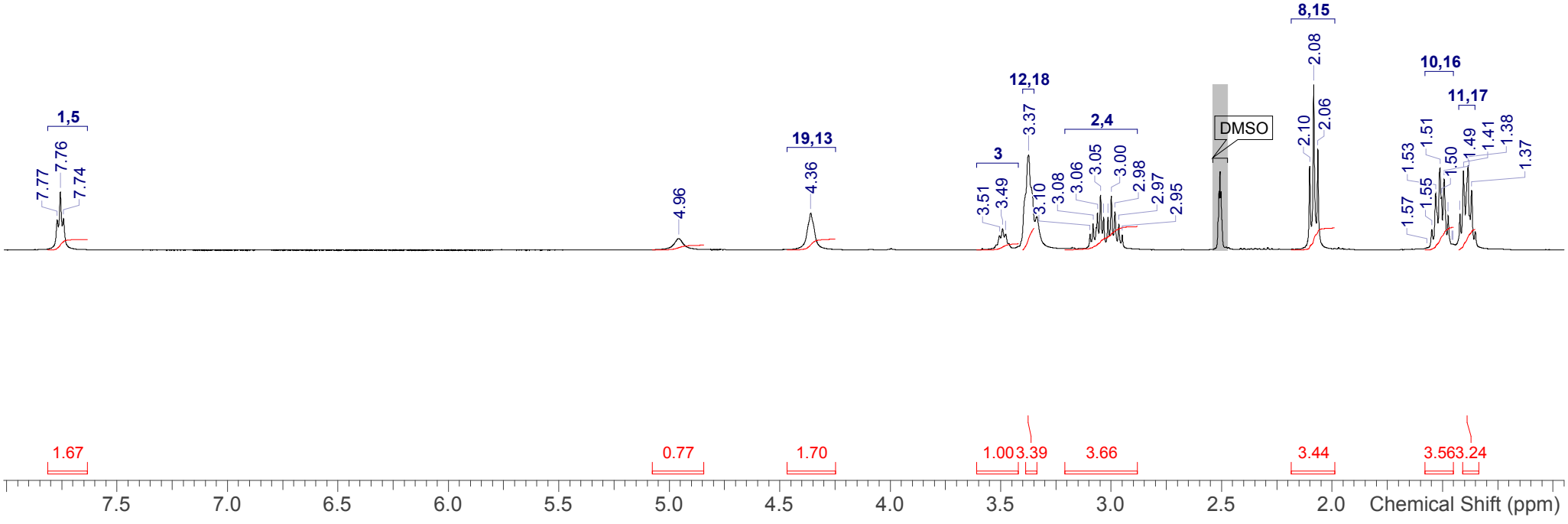
Peak 2, RT 3.362, Scan 961, NL 2.792E05, MS2 (150:1500) ES+



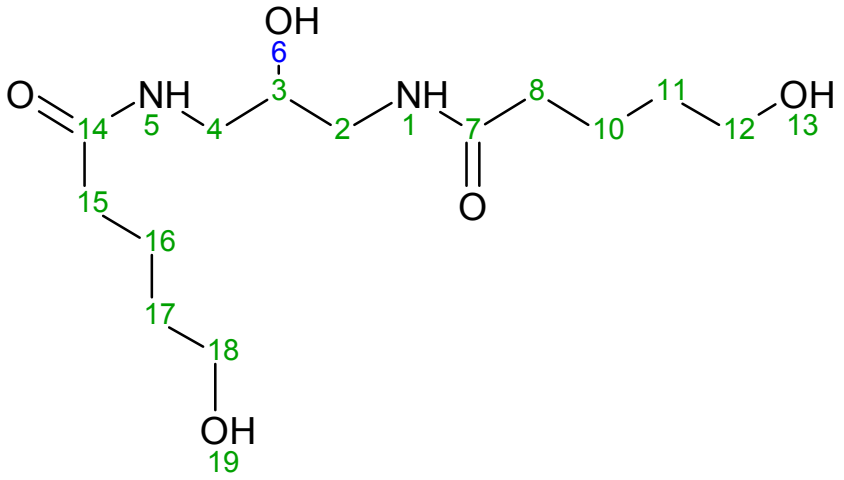
Acquisition Time (sec)	4.0894	***Comment***	mm/7500/1 Doubler amidite dialcohol		
Date	06 Jan 2015 15:21:58				
Date Stamp	06 Jan 2015 15:21:58				
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\ja0615mm1 Alcohol doubler\10\PDATA\1\1r				
Frequency (MHz)	400.1300	***Nucleus***	1H	***Number of Transients***	16
Origin	spect	***Original Points Count***	32768	***Owner***	nmr
Points Count	65536	***Pulse Sequence***	zg30	***Receiver Gain***	64.46
SW(cyclical) (Hz)	8012.82	***Solvent***	DMSO-d6	***Spectrum Offset (Hz)***	2470.9668
Spectrum Type	standard	***Sweep Width (Hz)***	8012.70	***Temperature (degree C)***	25.001



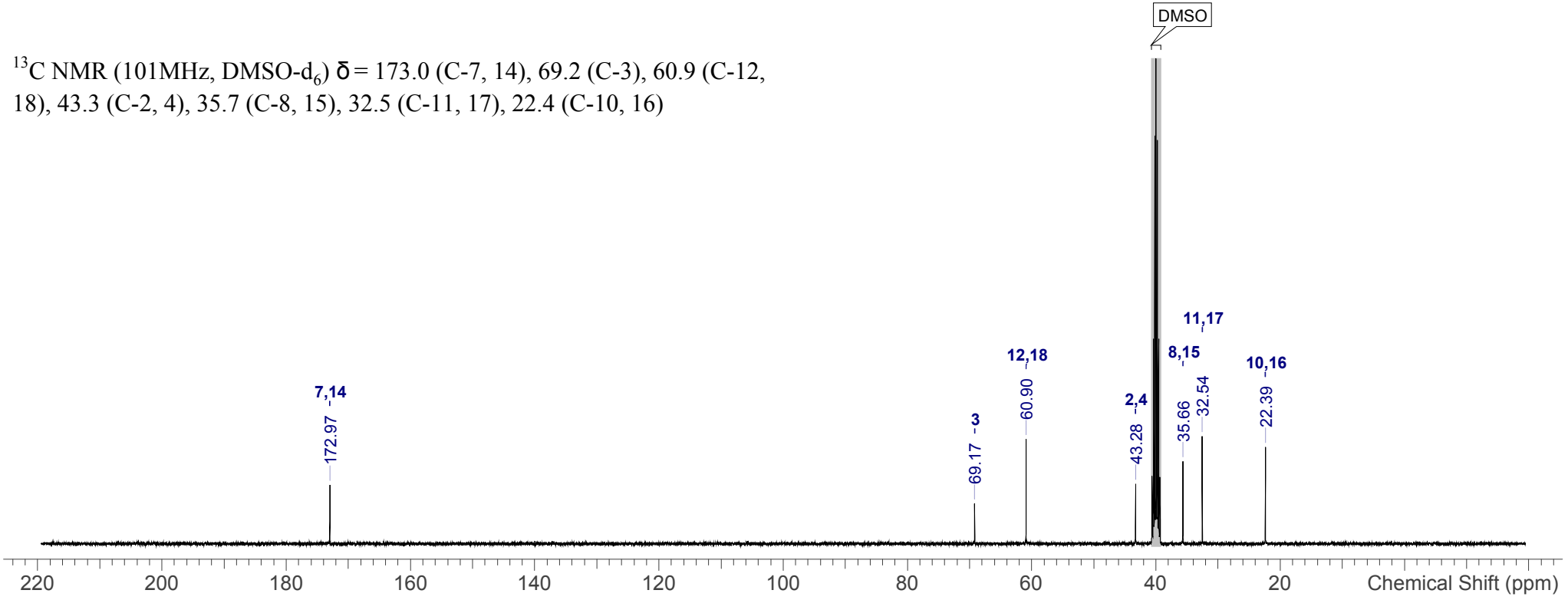
^{1}H NMR (400MHz, DMSO-d_6) δ = 7.76 (2H, br t, J = 5.7 Hz, H-1, 5), 4.96 (1H, br s, M09), 4.36 (2H, br s, H-19, 13), 3.49 (1H, t, J = 5.5 Hz, H-3), 3.37 (3H, br s, H-12, 18), 3.02 (4H, tdd, J = 19.5 Hz, J = 13.4 Hz, J = 5.8 Hz, H-2, 4), 2.08 (3H, t, J = 7.4 Hz, H-8, 15), 1.45 - 1.58 (4H, m, H-10, 16), 1.35 - 1.43 (3H, m, H-11, 17)



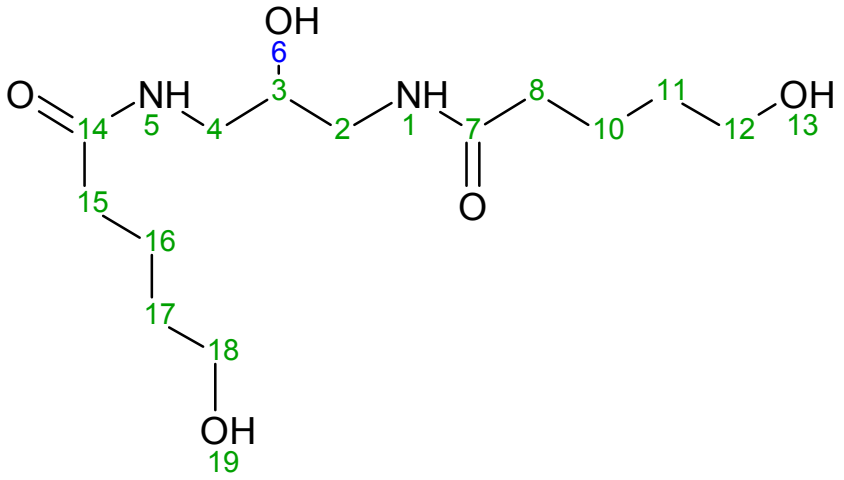
Acquisition Time (sec)	1.3631	***Comment***	mm/7500/1 Doubler amidite dialcohol
Date	06 Jan 2015 15:52:28		
Date Stamp	06 Jan 2015 15:52:28		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\ja0615mm1 Alcohol doubler\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	10060.3320
Spectrum Type	standard	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	25.005		



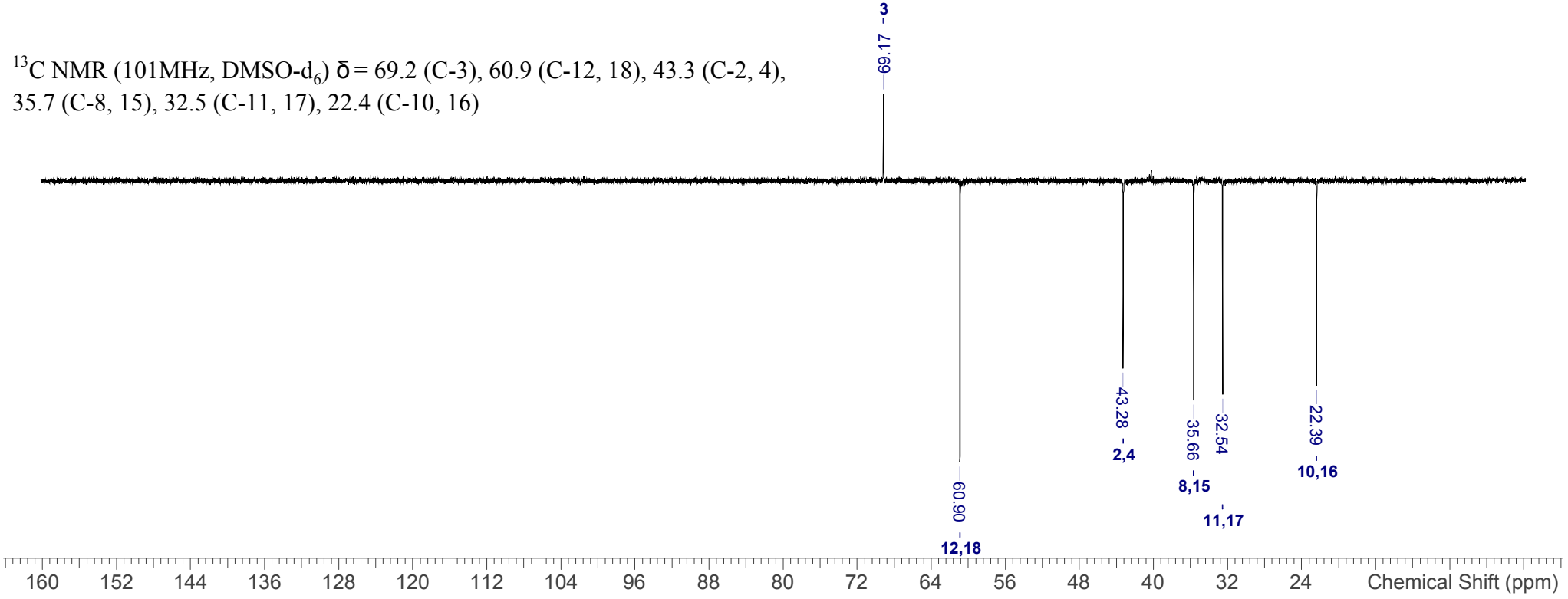
^{13}C NMR (101MHz, DMSO-d_6) δ = 173.0 (C-7, 14), 69.2 (C-3), 60.9 (C-12, 18), 43.3 (C-2, 4), 35.7 (C-8, 15), 32.5 (C-11, 17), 22.4 (C-10, 16)



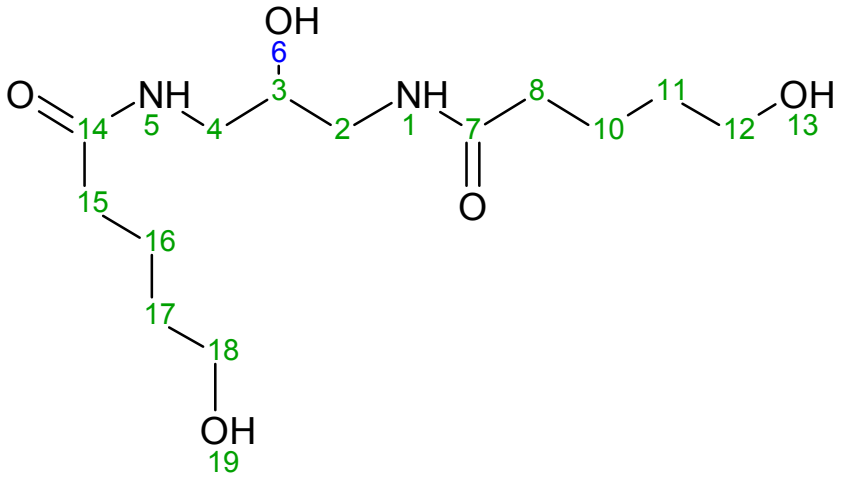
Acquisition Time (sec)	2.0316	***Comment***	mm/7500/1 Doubler amidite dialcohol
Date	06 Jan 2015 16:11:03		
Date Stamp	06 Jan 2015 16:11:03		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\ja0615mm1 Alcohol doubler\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	8047.9146
Spectrum Type	DEPT135	***Sweep Width (Hz)***	16128.54
Temperature (degree C)	25.004		



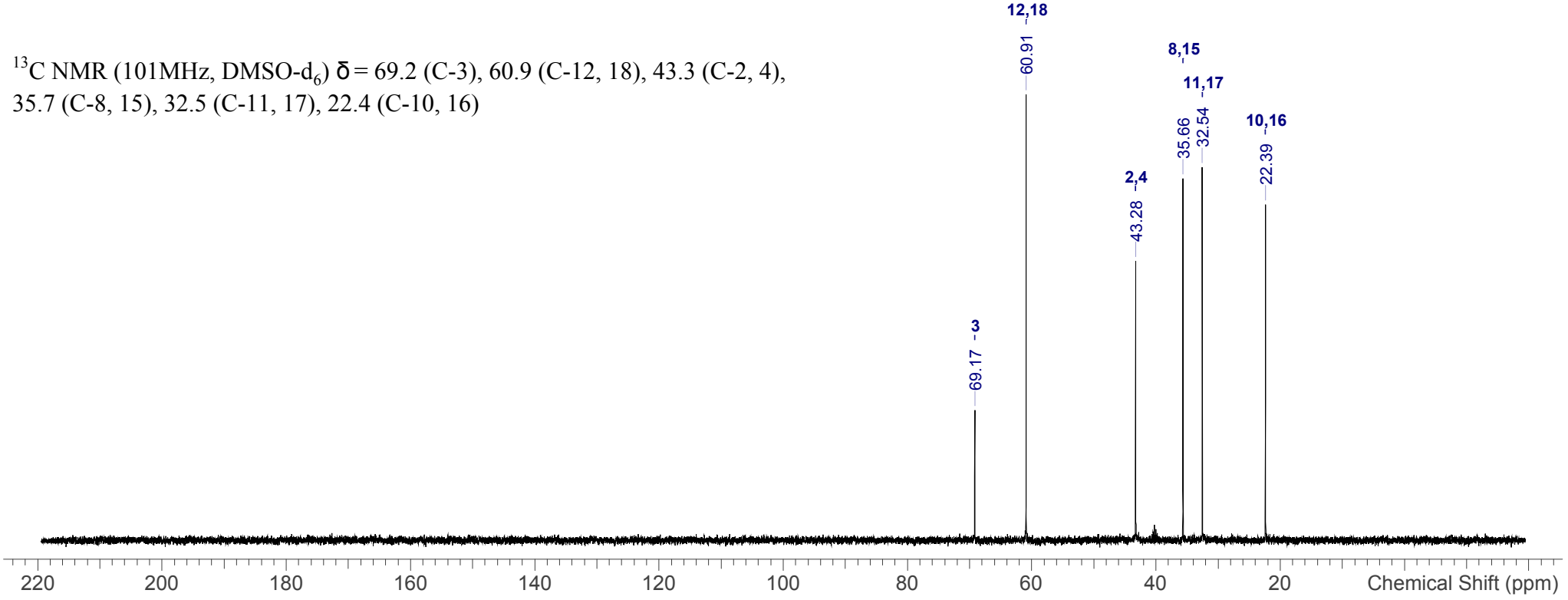
^{13}C NMR (101MHz, DMSO-d_6) δ = 69.2 (C-3), 60.9 (C-12, 18), 43.3 (C-2, 4), 35.7 (C-8, 15), 32.5 (C-11, 17), 22.4 (C-10, 16)



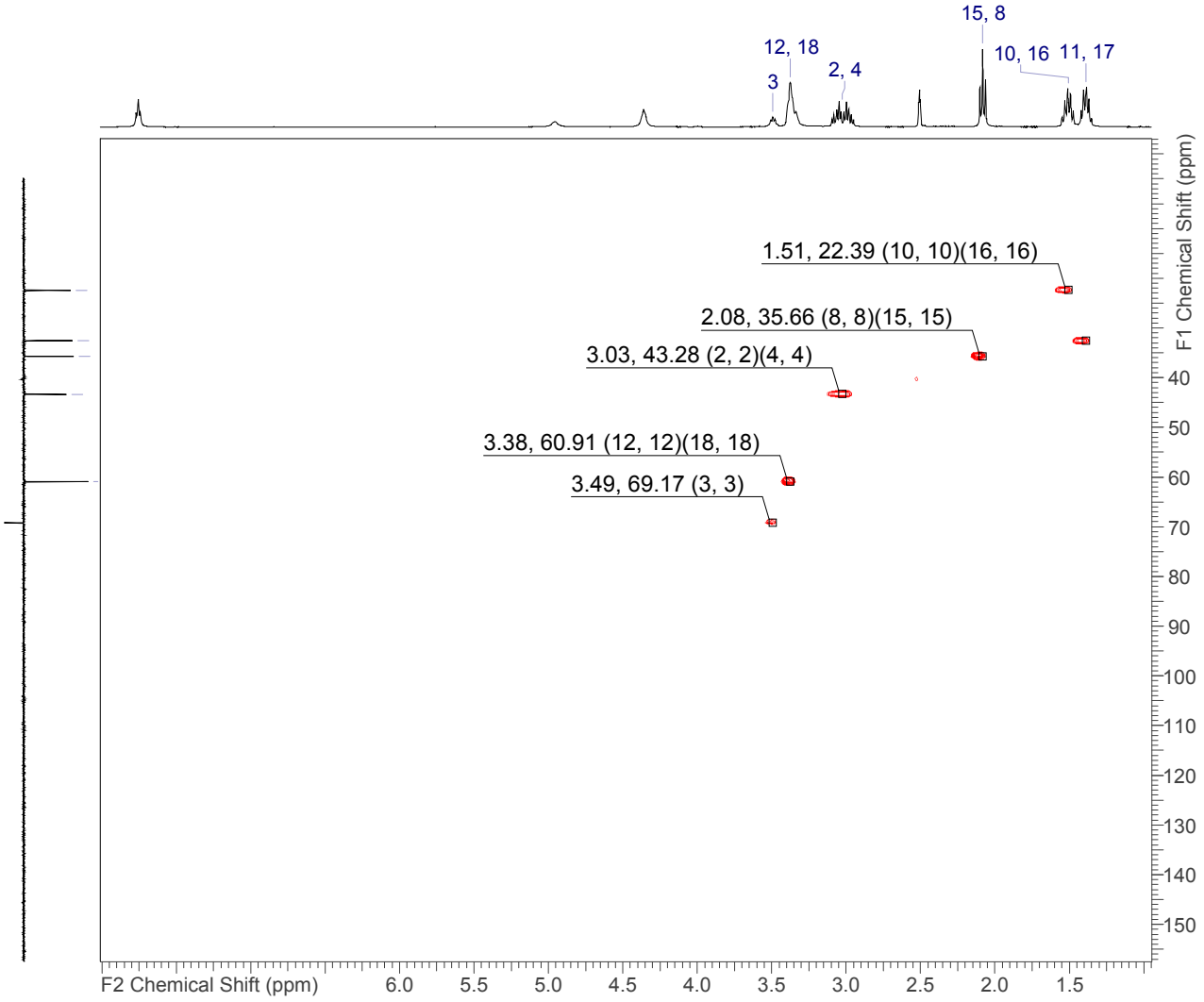
Acquisition Time (sec)	1.3631	***Comment***	mm/7500/1 Doubler amidite dialcohol
Date	06 Jan 2015 16:43:00		
Date Stamp	06 Jan 2015 16:43:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\ja0615mm1 Alcohol doubler\14\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp45
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	10061.0684
Spectrum Type	DEPT45	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	25.002		

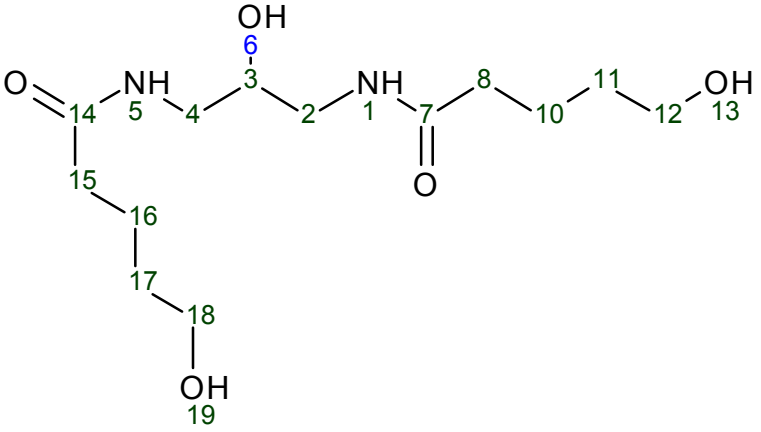


^{13}C NMR (101MHz, DMSO-d_6) δ = 69.2 (C-3), 60.9 (C-12, 18), 43.3 (C-2, 4), 35.7 (C-8, 15), 32.5 (C-11, 17), 22.4 (C-10, 16)

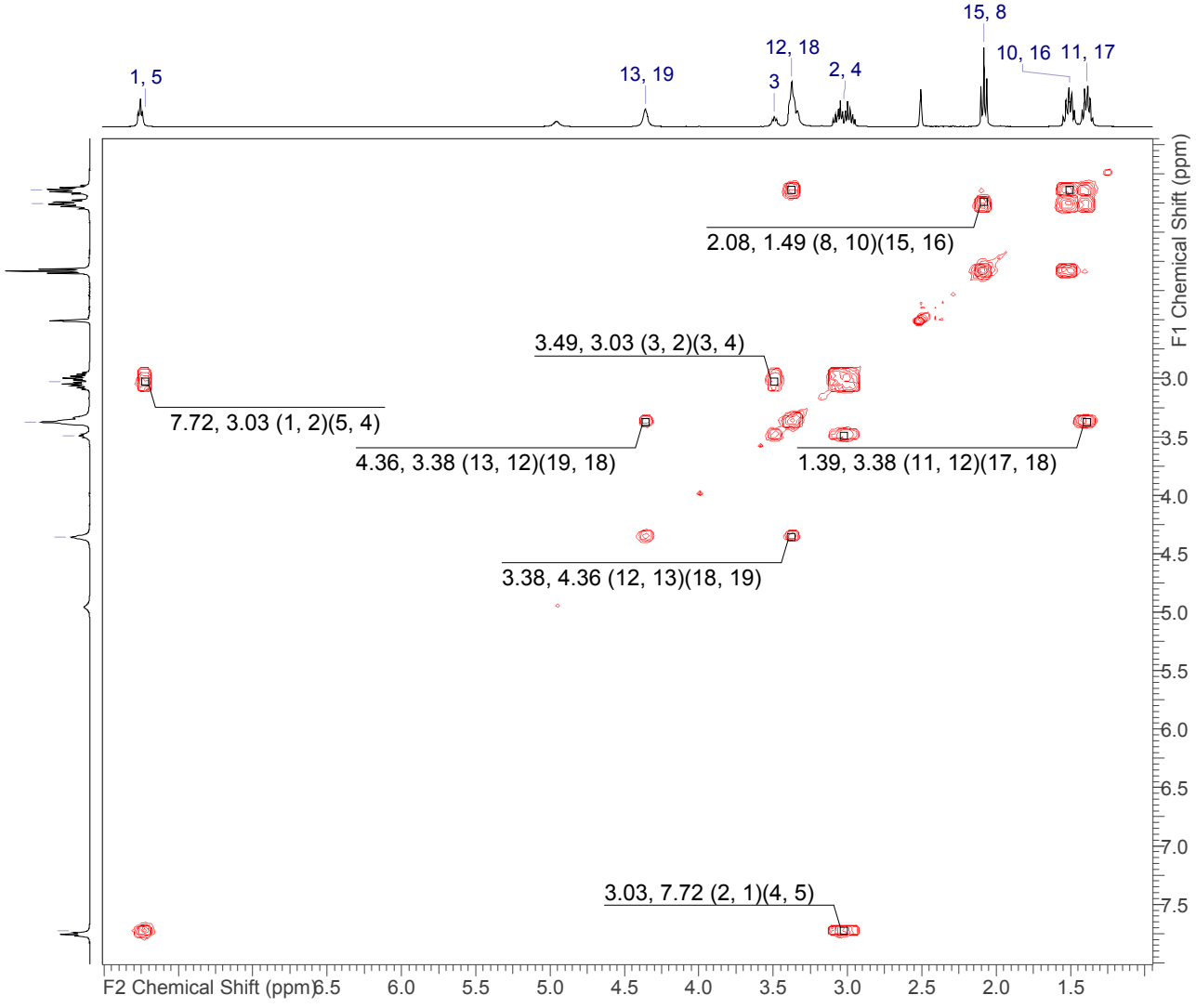


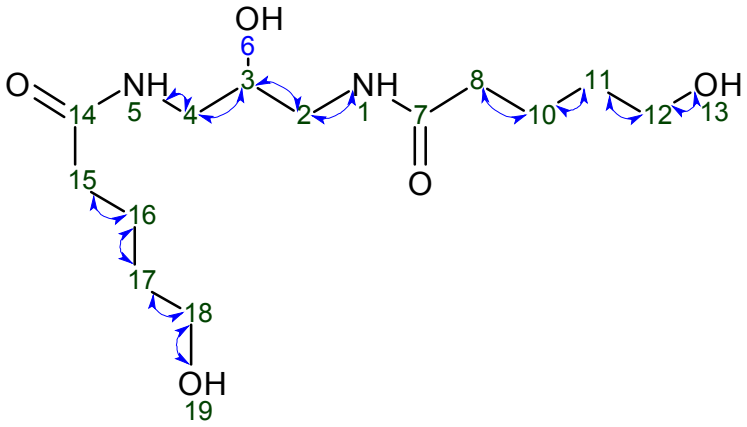
Acquisition Time (sec)	(0.1556, 0.0154)	***Comment***	mm/7500/1 Doubler amidite dialcohol
Date	06 Jan 2015 16:58:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\ja0615mm1 Alcohol doubler\15\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)	***Nucleus***	(1H, 13C)
Number of Transients	2	***Origin***	spect
Original Points Count	(512, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	hsqcetgp
Solvent	DMSO-d6	***Spectrum Type***	HSQC
Sweep Width (Hz)	(3286.26, 16650.39)	***Temperature (degree C)***	25.072
Title	mm/7500/1 Doubler amidite dialcohol		





Acquisition Time (sec)	(0.3113, 0.0778)	***Comment***	mm/7500/1 Doubler amidite dialcohol
Date	06 Jan 2015 16:27:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\ja0615mm1 Alcohol doubler\13\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)	***Nucleus***	(1H, 1H)
Number of Transients	2	***Origin***	spect
Original Points Count	(1024, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	cosygpqf
Solvent	DMSO-d6	***Spectrum Type***	COSY
Sweep Width (Hz)	(3286.26, 3286.26)	***Temperature (degree C)***	25.000
Title	mm/7500/1 Doubler amidite dialcohol		



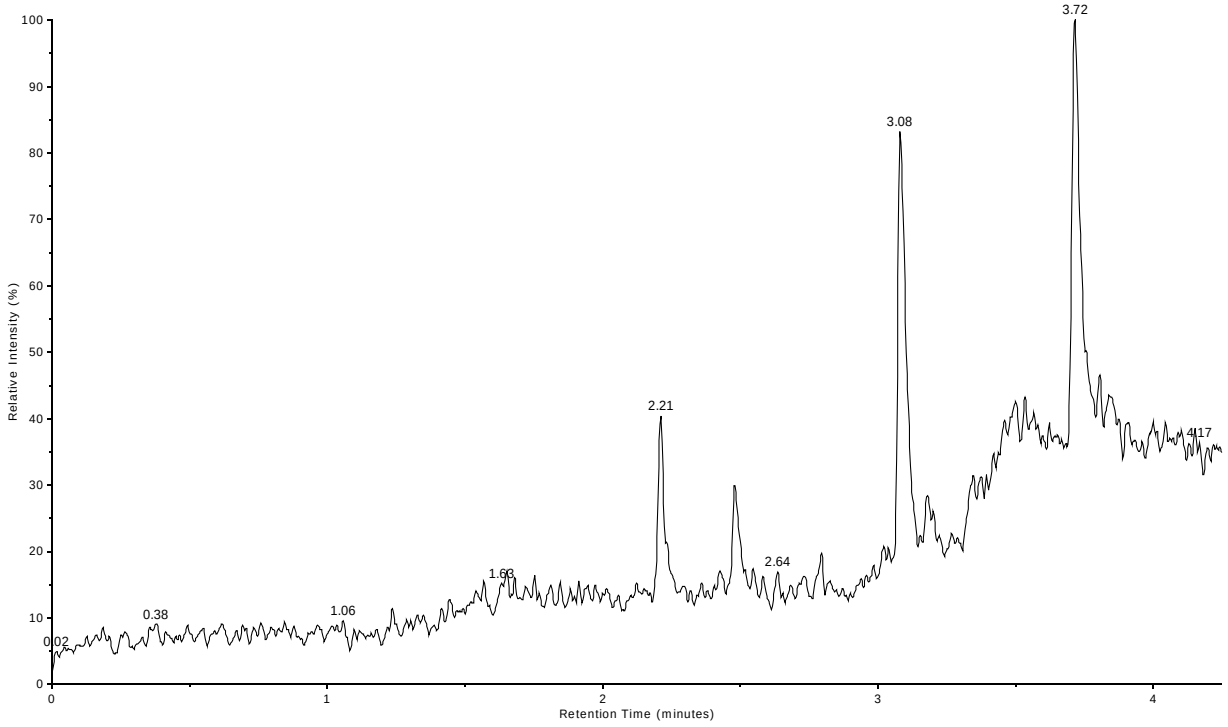


A.25 DMT Doubler

Top 5 Peak Report

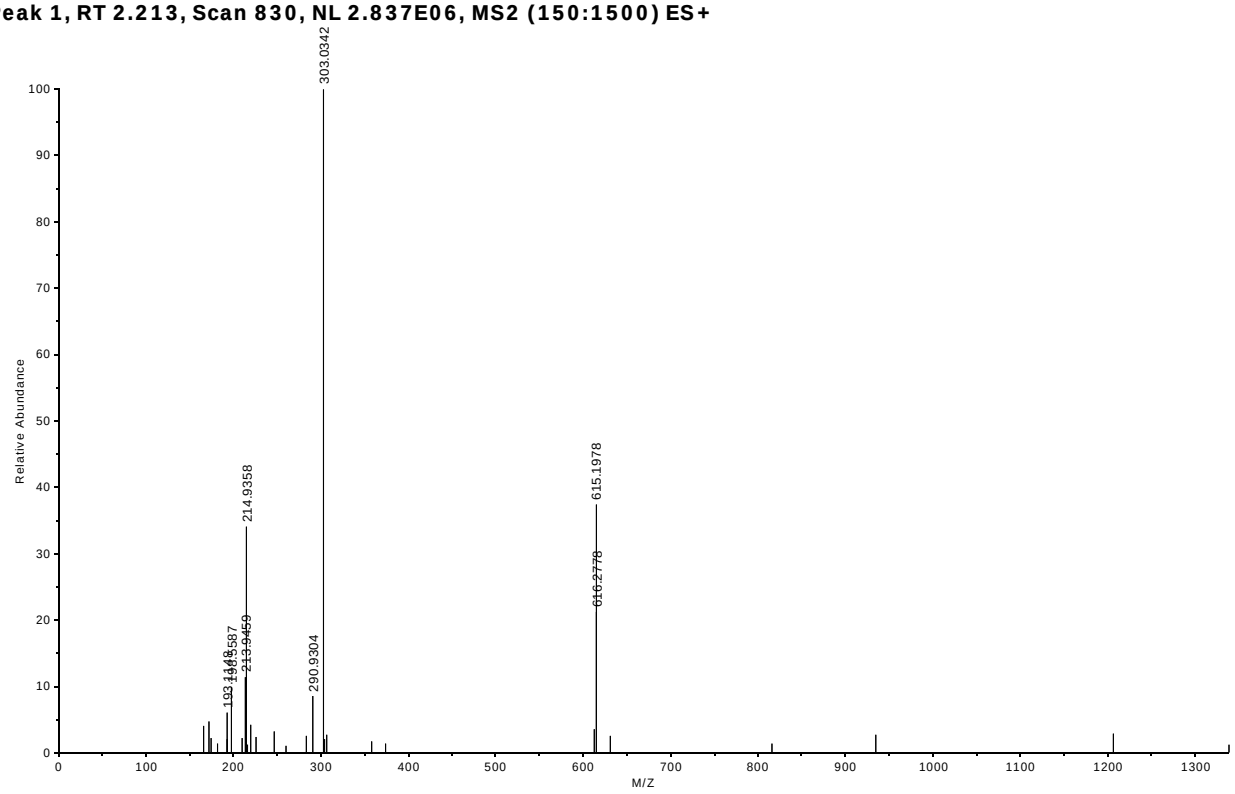
Group: Watts, J
Submitter: Michael Moazami
Project: RP LC C18 < 2 cmpds
Sample ID: mm/7500/3 DMT Doubler pure
Experiment: BLUE ESIPOS C18 5 min
Filename: mm75003_DMT_Doubler_pure_Michael_Moazam
Instrument: Blue RP UHPLC-MS (B30:1023)
Acquisition Date: 08/01/2015 13:25:40

TIC, NL 1.880E07, MS2 (150:1500) ES+

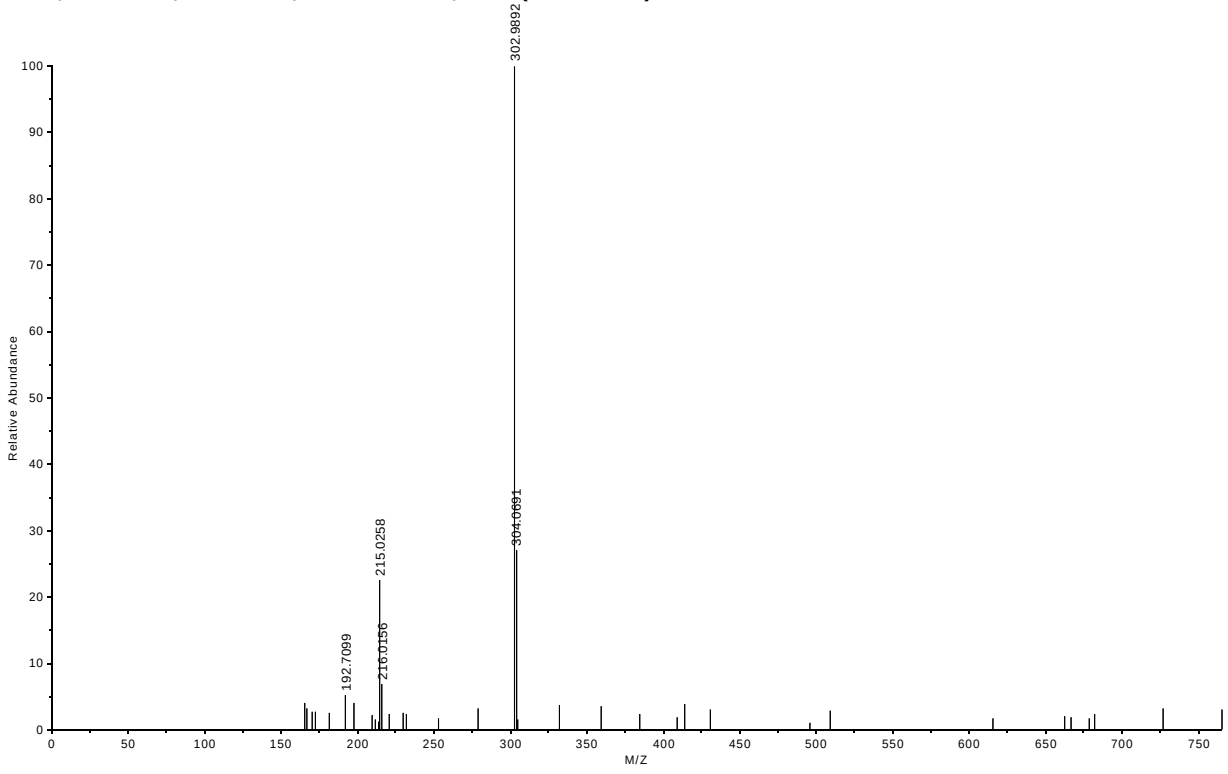


	RT Mins	Height	Height %	Area	Area %
1	2.21	4436131	37.06	7559109	28.84
2	2.48	2753262	23.00	5477222	20.90
3	3.08	11970382	100.00	26211472	100.00
4	3.35	2122933	17.73	4134615	15.77
5	3.72	10737293	89.70	24349240	92.90

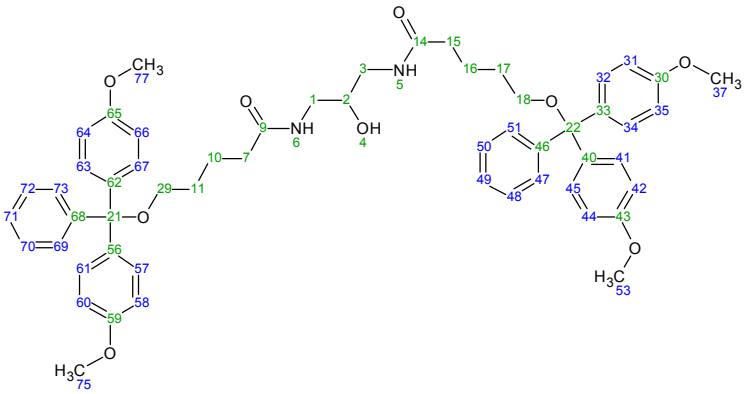
Peak 1, RT 2.213, Scan 830, NL 2.837E06, MS2 (150:1500) ES+



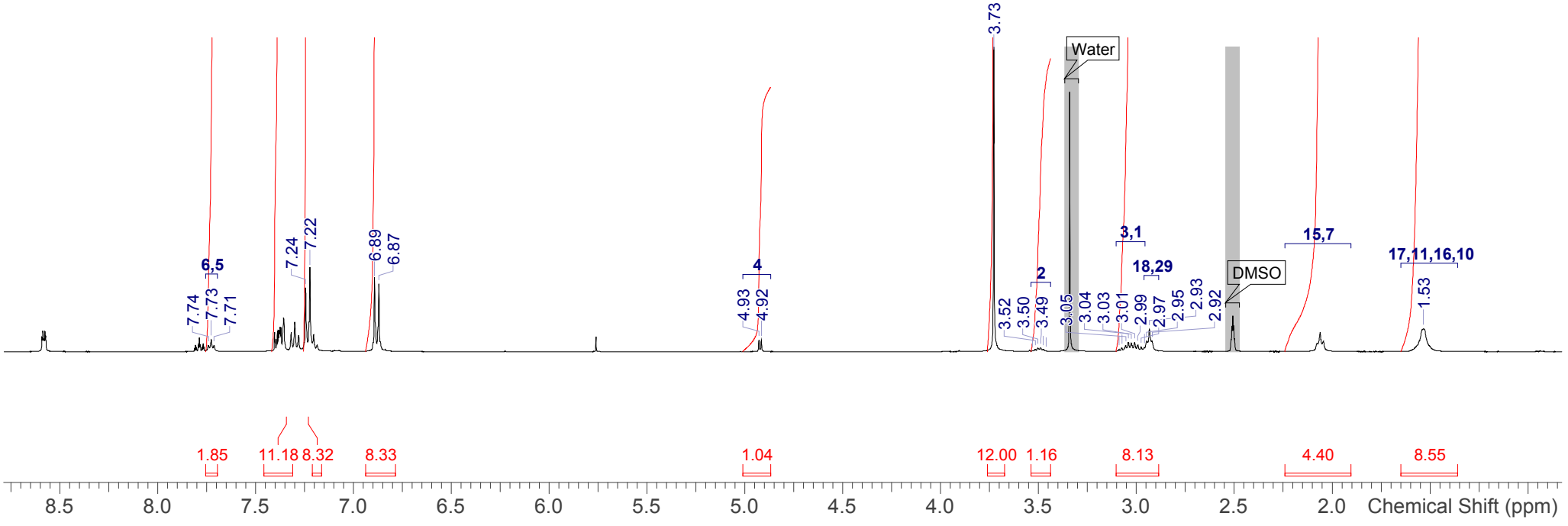
Peak 2, RT 2.480, Scan 930, NL 2.447E06, MS2 (150:1500) ES+



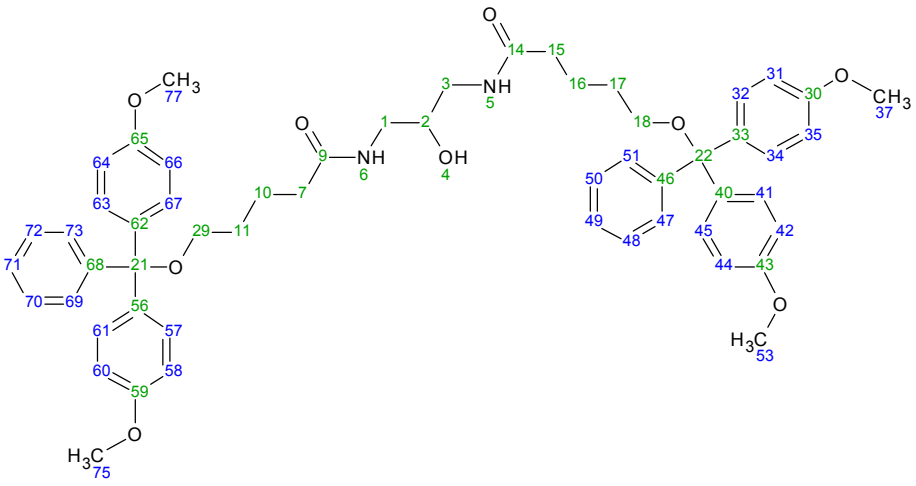
Acquisition Time (sec)	4.0894	***Comment***	mm/7500/3 Bis-DMT-dialcohol monoamidite F15-26
Date	08 Jan 2015 03:47:27		
Date Stamp	08 Jan 2015 03:47:27		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\ja0715mm1 BisDMT alcohol doubler\10\PDATA\1\1r		
Frequency (MHz)	400.1300	***Nucleus***	1H
Number of Transients	16	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	524288	***Pulse Sequence***	zg30
Receiver Gain	58.58	***SW(cyclical) (Hz)***	8012.82
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	2471.0203
Spectrum Type	standard	***Sweep Width (Hz)***	8012.81
Temperature (degree C)	25.003		



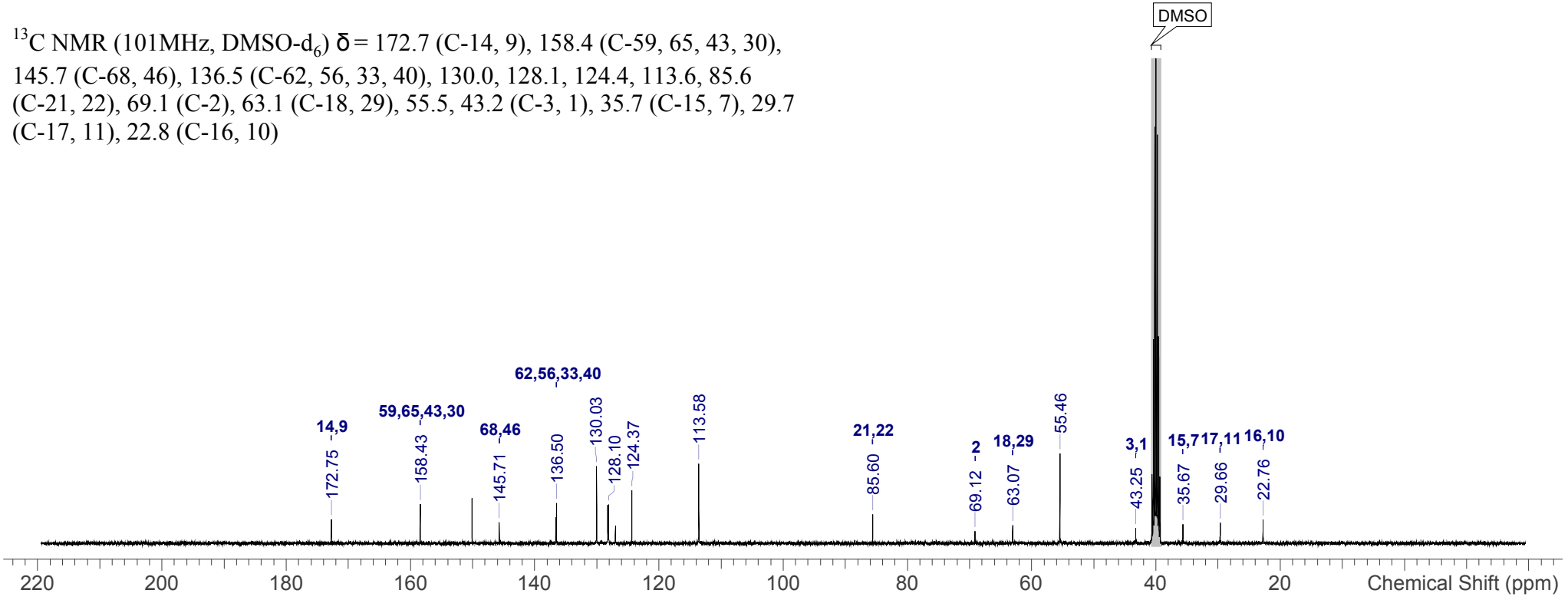
^{1}H NMR (400MHz, DMSO-d_6) δ = 7.73 (2H, t, J = 5.8 Hz, H-6, 5), 7.27 - 7.42 (11H, m, M10), 7.23 (8H, d, J = 8.9 Hz, M09), 6.88 (8H, d, J = 8.8 Hz, M03), 4.92 (1H, d, J = 4.9 Hz, H-4), 3.73 (12H, s, M01), 3.44 - 3.54 (1H, m, H-2), 2.96 - 3.10 (4H, m, H-3, 1), 2.89 - 2.96 (3H, m, H-18, 29), 1.90 - 2.24 (4H, m, H-15, 7), 1.53 (9H, br s, H-17, 11, 16, 10)



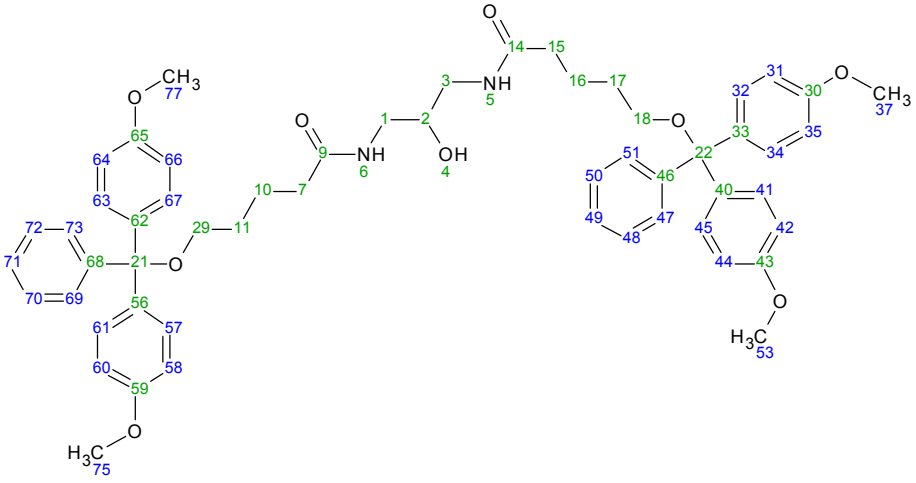
Acquisition Time (sec)	1.3631		
Comment	mm/7500/3 Bis-DMT-dialcohol monoamidite F15-26		
Date	08 Jan 2015 04:17:59		
Date Stamp	08 Jan 2015 04:17:59		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\ja0715mm1 BisDMT alcohol doubler\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	10060.3320
Spectrum Type	standard	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	25.000		



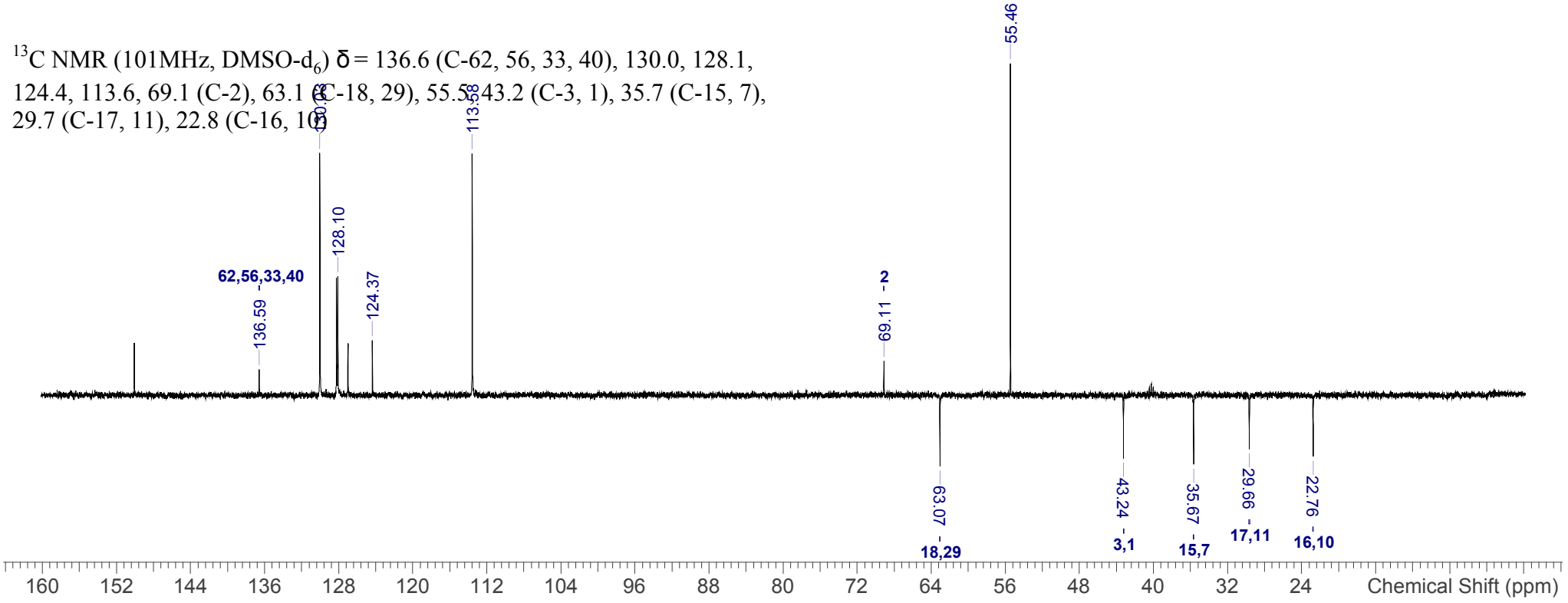
^{13}C NMR (101MHz, DMSO-d_6) δ = 172.7 (C-14, 9), 158.4 (C-59, 65, 43, 30), 145.7 (C-68, 46), 136.5 (C-62, 56, 33, 40), 130.0, 128.1, 124.4, 113.6, 85.6 (C-21, 22), 69.1 (C-2), 63.1 (C-18, 29), 55.5, 43.2 (C-3, 1), 35.7 (C-15, 7), 29.7 (C-17, 11), 22.8 (C-16, 10)



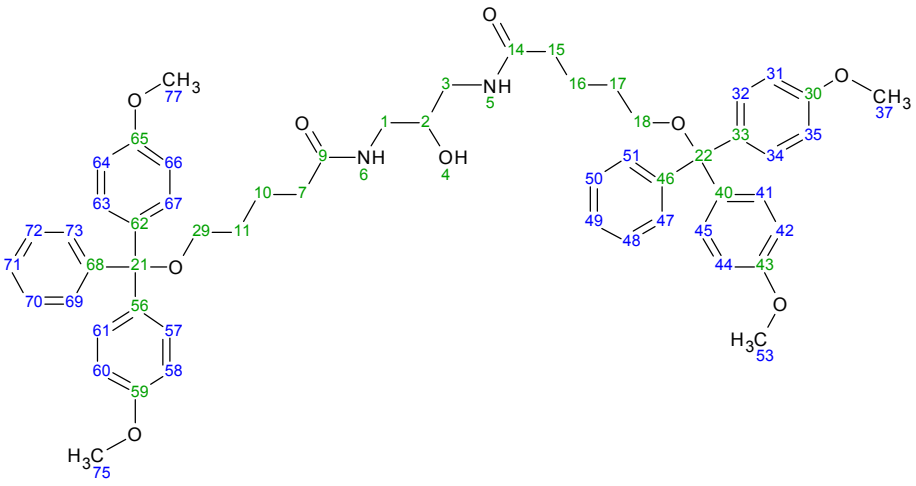
Acquisition Time (sec)	2.0316		
Comment	mm/7500/3 Bis-DMT-dialcohol monoamidite F15-26		
Date	08 Jan 2015 04:36:38		
Date Stamp	08 Jan 2015 04:36:38		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\ja0715mm1 BisDMT alcohol doubler\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	8047.9619
Spectrum Type	DEPT135	***Sweep Width (Hz)***	16128.54
Temperature (degree C)	25.002		



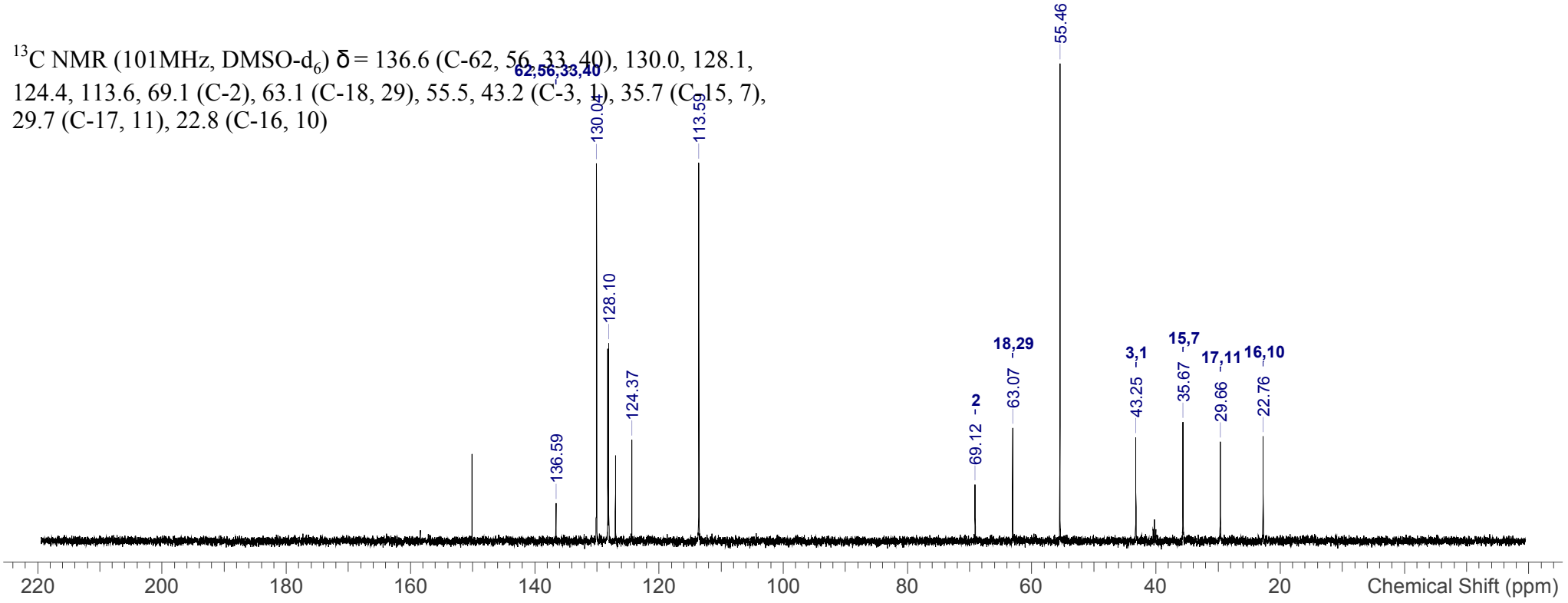
^{13}C NMR (101MHz, DMSO-d$_6$) δ = 136.6 (C-62, 56, 33, 40), 130.0, 128.1, 124.4, 113.6, 69.1 (C-2), 63.1 (C-18, 29), 55.5, 43.2 (C-3, 1), 35.7 (C-15, 7), 29.7 (C-17, 11), 22.8 (C-16, 10)



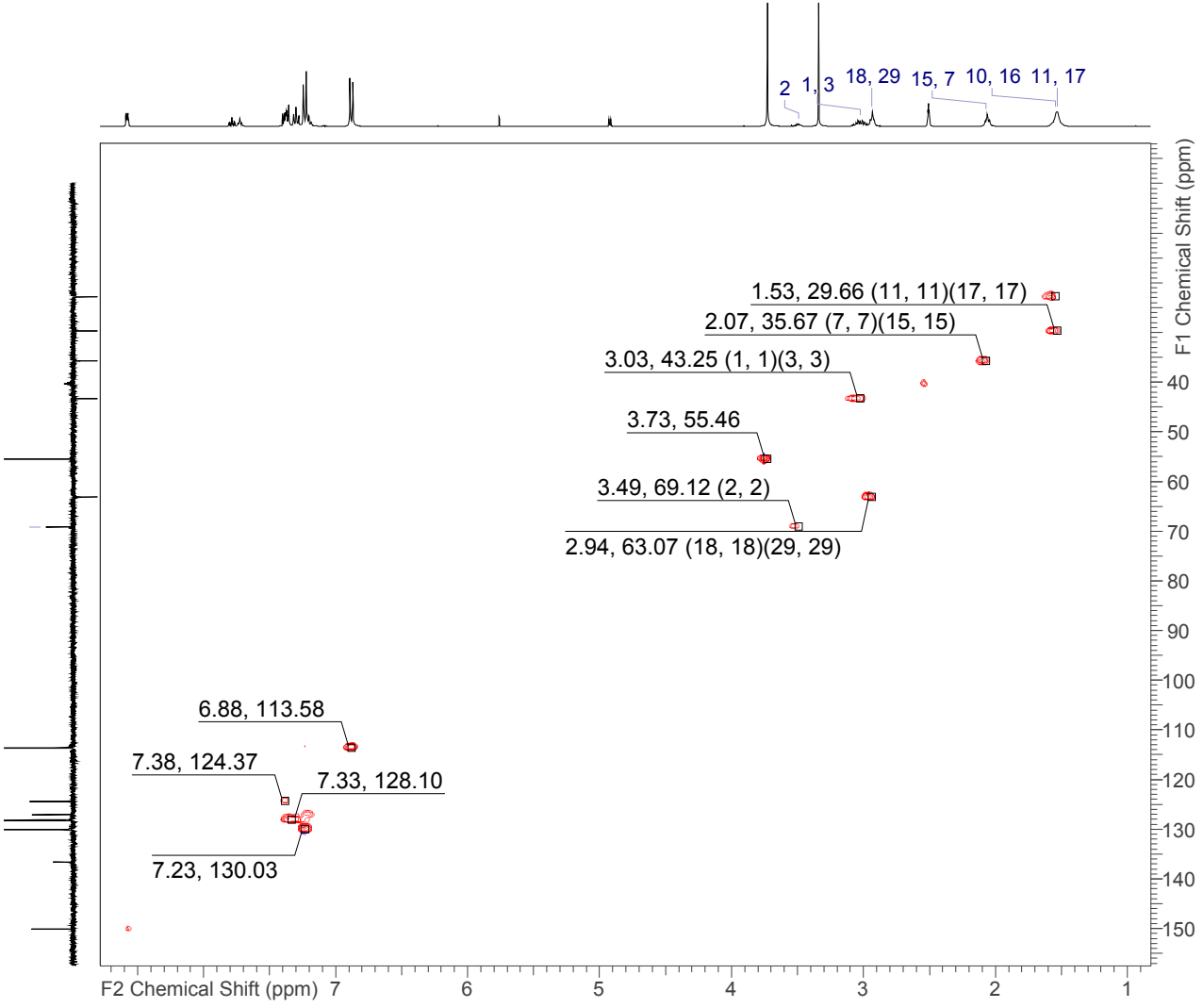
Acquisition Time (sec)	1.3631		
Comment	mm/7500/3 Bis-DMT-dialcohol monoamidite F15-26		
Date	08 Jan 2015 05:30:20		
Date Stamp	08 Jan 2015 05:30:20		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\ja0715mm1 BisDMT alcohol doubler\15\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp45
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	DMSO-d6	***Spectrum Offset (Hz)***	10061.0684
Spectrum Type	DEPT45	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	25.002		

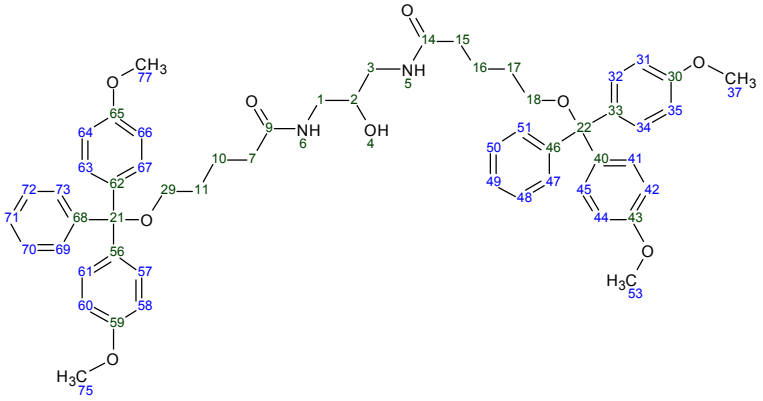


^{13}C NMR (101MHz, DMSO-d_6) δ = 136.6 (C-62, 56, 33, 40), 130.0, 128.1, 124.4, 113.6, 69.1 (C-2), 63.1 (C-18, 29), 55.5, 43.2 (C-3, 1), 35.7 (C-15, 7), 29.7 (C-17, 11), 22.8 (C-16, 10)

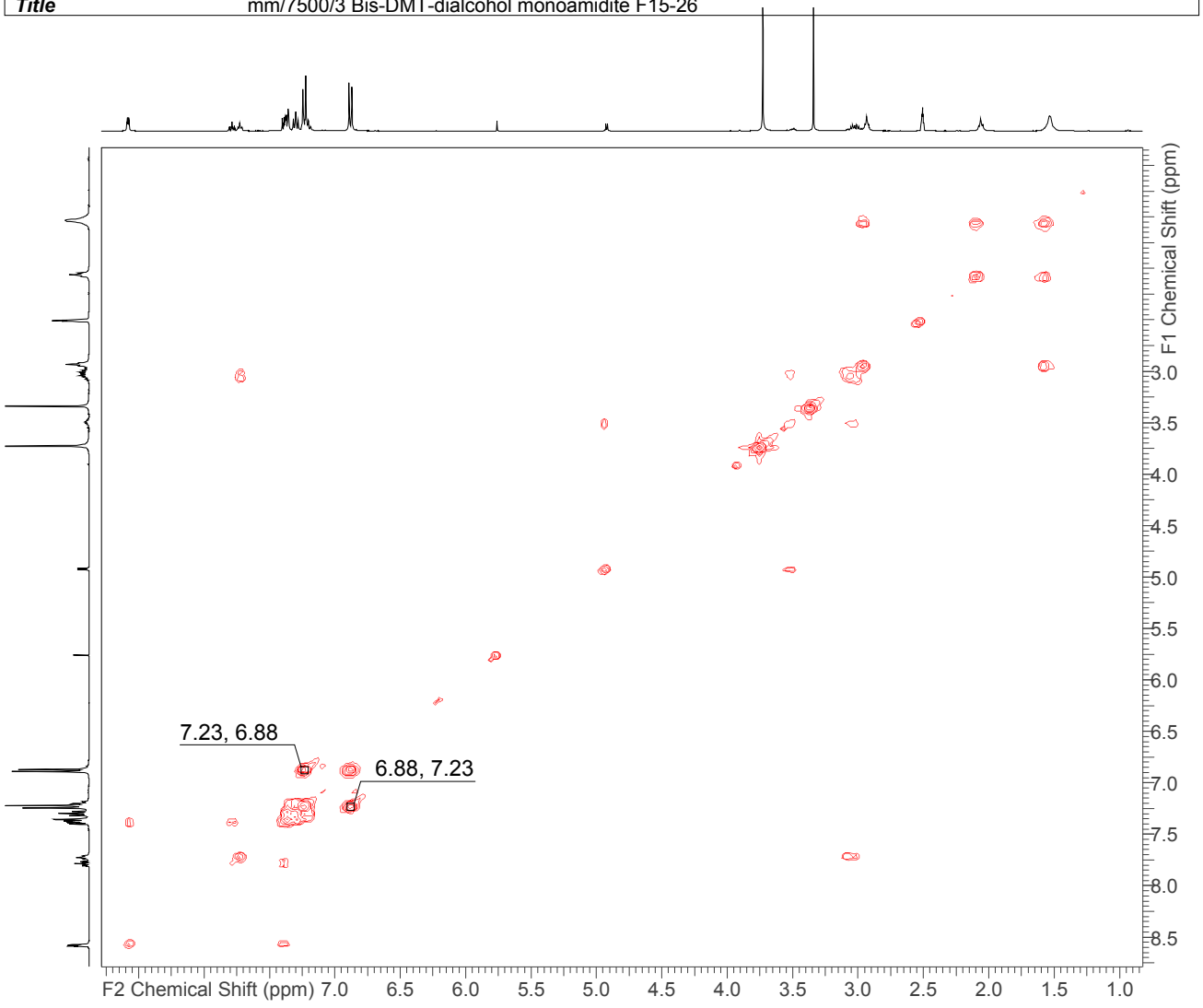


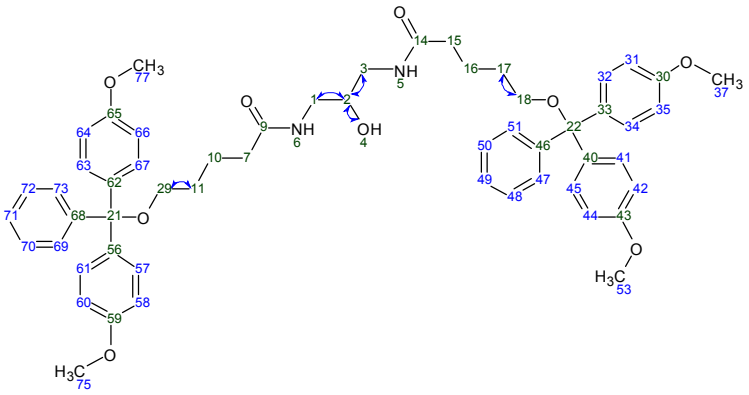
Acquisition Time (sec)	(0.1413, 0.0154)	***Comment***	mm/7500/3 Bis-DMT-dialcohol monoamidite F15-26
Date	08 Jan 2015 05:45:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\ja0715mm1 BisDMT alcohol doubler\16\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)	***Nucleus***	(1H, 13C)
Number of Transients	2	***Origin***	spect
Original Points Count	(512, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	hsqcetgp
Solvent	DMSO-d6	***Spectrum Type***	HSQC
Sweep Width (Hz)	(3619.65, 16650.39)	***Temperature (degree C)***	25.055
Title	mm/7500/3 Bis-DMT-dialcohol monoamidite F15-26		





Acquisition Time (sec)	(0.2826, 0.0707)	***Comment***	mm/7500/3 Bis-DMT-dialcohol monoamidite F15-26
Date	08 Jan 2015 04:53:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\ja0715mm1 BisDMT alcohol doubler\13\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)	***Nucleus***	(1H, 1H)
Number of Transients	2	***Origin***	spect
Original Points Count	(1024, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	cosygpqf
Solvent	DMSO-d6	***Spectrum Type***	COSY
Sweep Width (Hz)	(3619.65, 3619.65)	***Temperature (degree C)***	24.997
Title	mm/7500/3 Bis-DMT-dialcohol monoamidite F15-26		

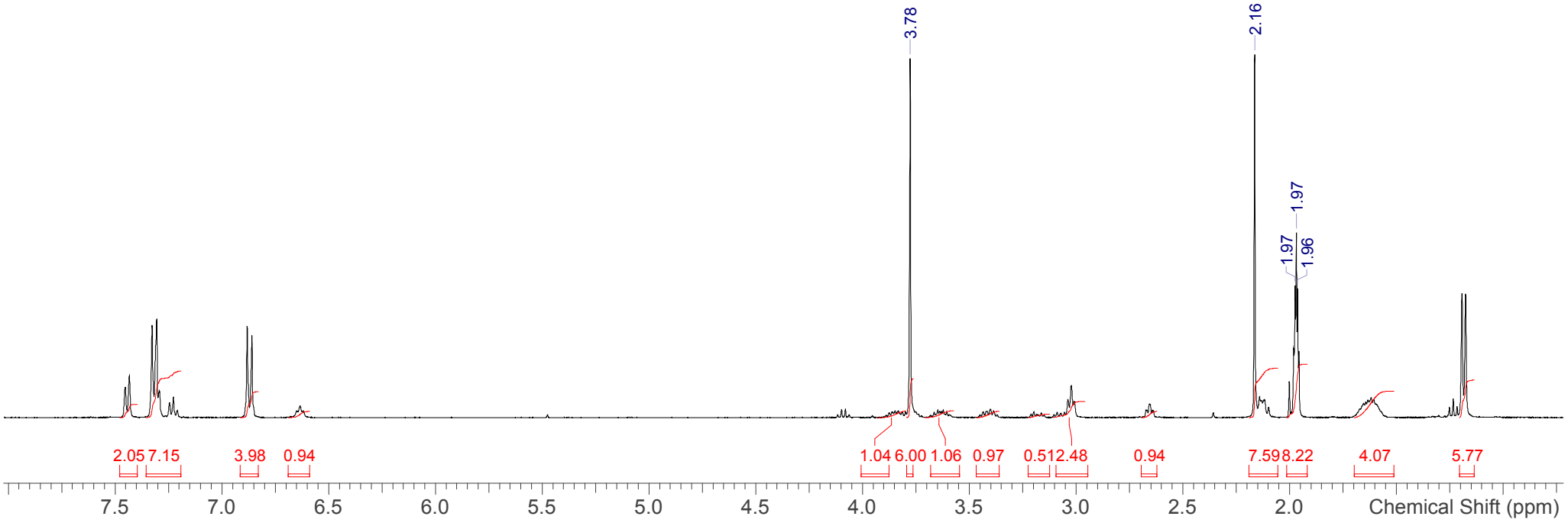




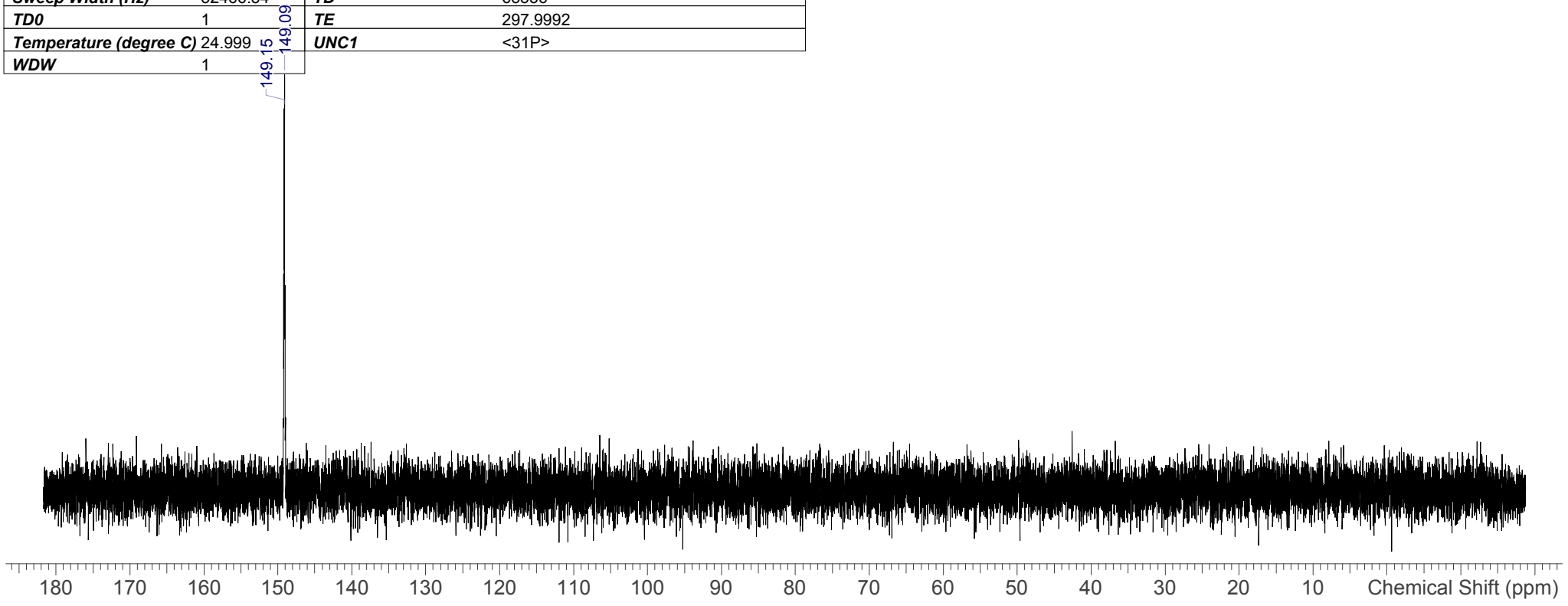
A.26 DMT Doubler amidite

Acquisition Time (sec)	4.0894	***Comment***	Doubler Phosphoramidite		
D	0.0002	***DE***	18.00442	***DS***	2
Date	24 Aug 2015 22:10:18			***Date Stamp***	24 Aug 2015 22:10:18
File Name	\\filestore.soton.ac.uk\users\mm29g10\mydesktop\au2415mm5\12\PDATA\1\1r				
Frequency (MHz)	400.1300	***GB***	0	***INSTRUM***	<spect>
LB	0.1	***NS***	16	***Nucleus***	1H
Number of Transients	16	***Origin***	spect	***Original Points Count***	32768
Owner	nmr	***PC***	1		
PROBHD	<5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218 >			***PULPROG***	<zg30>
Points Count	65536	***Pulse Sequence***	zg30	***Receiver Gain***	168.59
SF	400.13	***SFO1***	400.132470966543		
SI	65536	***SSB***	0	***SW(cyclical) (Hz)***	8012.82
SWH	8012.82051282051			***Solvent***	ACETONITRILE-d3
Spectrum Offset (Hz)	2470.9668	***Spectrum Type***	standard	***Sweep Width (Hz)***	8012.70
TD	65536	***TD0***	1	***TE***	298.0031
Temperature (degree C)	25.003	***UNC1***	<1H>	***WDW***	1

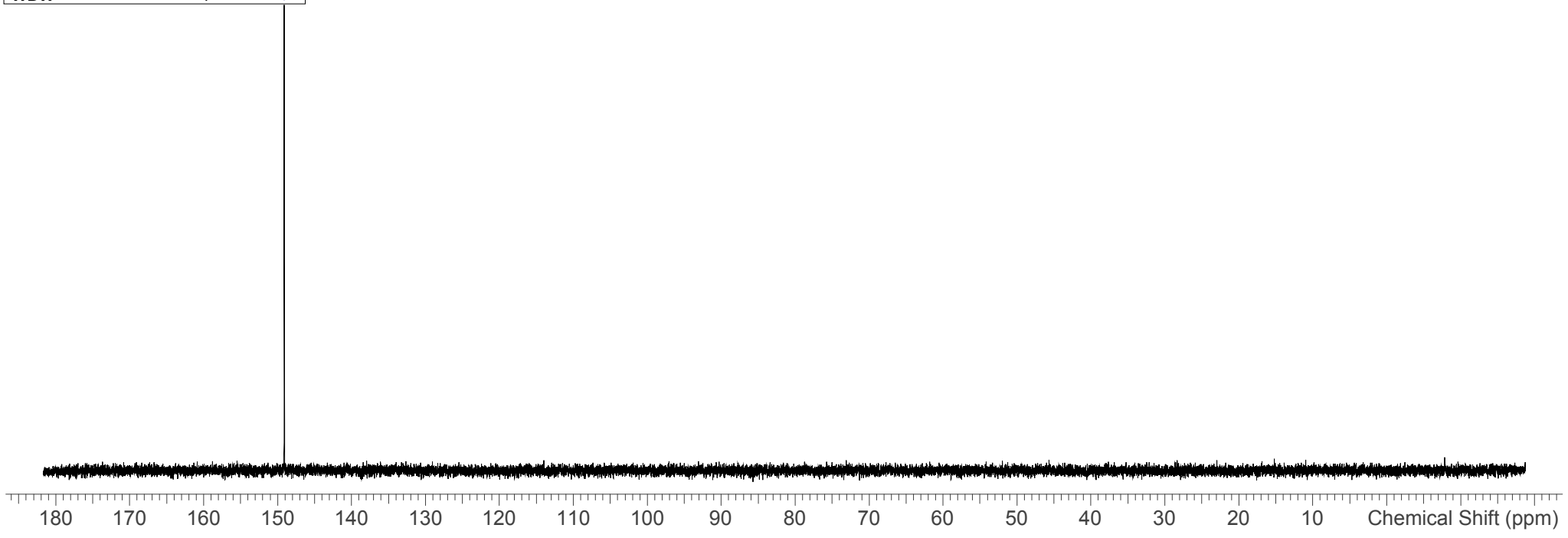
^{1}H NMR (400 MHz, ACETONITRILE-d_3) δ ppm 1.14 - 1.20 (m, 6 H) 1.51 - 1.70 (m, 4 H) 1.97 (t, *J*=2.45 Hz, 8 H) 2.16 (s, 8 H) 2.62 - 2.69 (m, 1 H) 2.96 - 3.11 (m, 2 H) 3.12 - 3.22 (m, 1 H) 3.36 - 3.47 (m, 1 H) 3.57 - 3.71 (m, 1 H) 3.78 (s, 6 H) 3.80 - 3.93 (m, 1 H) 6.59 - 6.69 (m, 1 H) 6.83 - 6.91 (m, 4 H) 7.19 - 7.36 (m, 7 H) 7.40 - 7.48 (m, 2 H)



Acquisition Time (sec)	1.0093	***Comment***	Doubler Phosphoramidite
D	0.0002	***DE***	6.5
DS	2	***Date***	24 Aug 2015 22:03:37
Date Stamp	24 Aug 2015 22:03:37		
File Name	\\filestore.soton.ac.uk\users\mm29g10\mydesktop\au2415mm5\10\PDATA\1\1r		
Frequency (MHz)	161.9754	***GB***	0
INSTRUM	<spect>	***LB***	1
NS	64	***Nucleus***	31P
Number of Transients	64	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
PC	1.4		
PROBHD	<5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218 >		
PULPROG	<zg30>	***Points Count***	32768
Pulse Sequence	zg30	***Receiver Gain***	212.69
SF	161.975351110001		
SFO1	161.98855104744		
SI	32768	***SSB***	0
SW(cyclical) (Hz)	32467.53	***SWH***	32467.5324675325
Solvent	ACETONITRILE-d3		
Spectrum Offset (Hz)	13199.9404	***Spectrum Type***	standard
Sweep Width (Hz)	32466.54	***TD***	65536
TD0	1	***TE***	297.9992
Temperature (degree C)	24.999	***UNC1***	<31P>
WDW	1		



Acquisition Time (sec)	1.0093	***Comment***	Doubler Phosphoramidite
D	0.03	***DE***	6.5
DS	2	***Date***	24 Aug 2015 22:08:02
Date Stamp	24 Aug 2015 22:08:02		
File Name	\\filestore.soton.ac.uk\users\mm29g10\mydesktop\au2415mm5\11\PDATA\1\1r		
Frequency (MHz)	161.9754	***GB***	0
INSTRUM	<spect>	***LB***	1
NS	64	***Nucleus***	31P
Number of Transients	64	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
PC	1.4		
PROBHD	<5 mm PABBO BB/19F-1H/D Z-GRD Z116098/0218 >		
PULPROG	<zgpg30>	***Points Count***	32768
Pulse Sequence	zgpg30	***Receiver Gain***	212.69
SF	161.975351110001		
SFO1	161.98855104744		
SI	32768	***SSB***	0
SW(cyclical) (Hz)	32467.53	***SWH***	32467.5324675325
Solvent	ACETONITRILE-d3		
Spectrum Offset (Hz)	13193.9902	***Spectrum Type***	standard
Sweep Width (Hz)	32466.54	***TD***	65536
TD0	1	***TE***	297.9969
Temperature (degree C)	24.997	***UNC1***	<31P>
WDW	1		

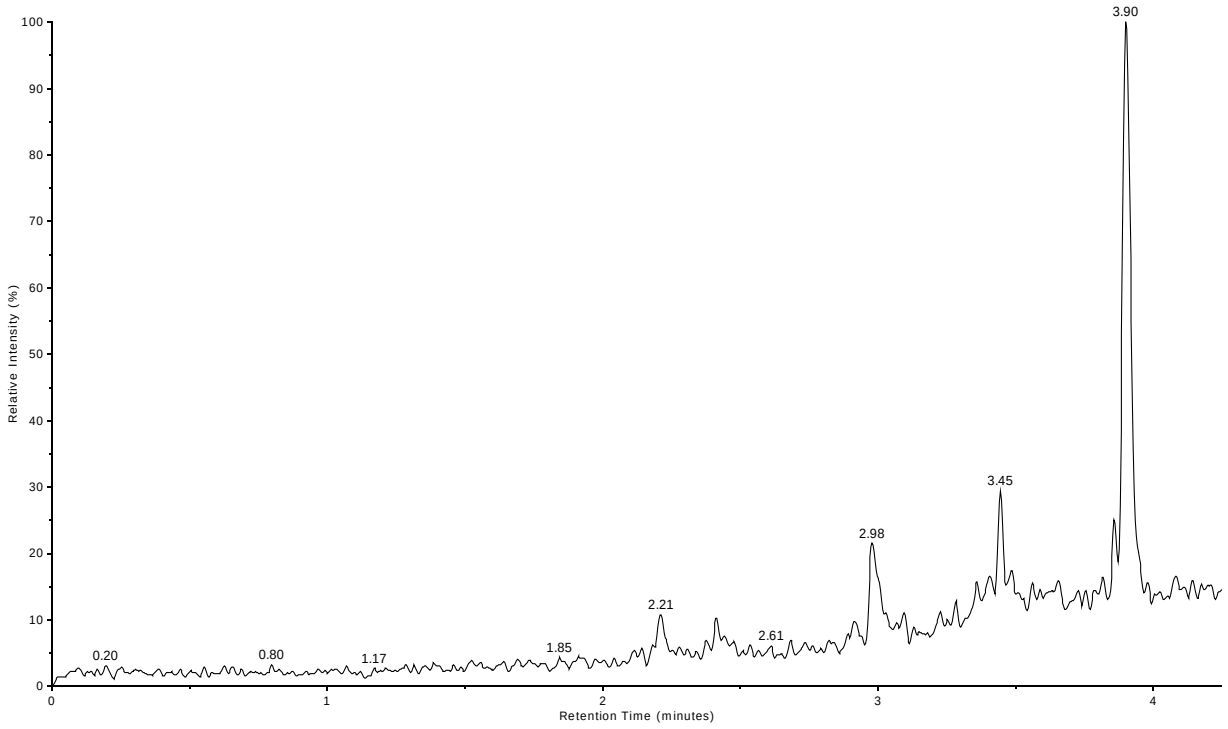


A.27 DMT Doubler Succ

Top 5 Peak Report

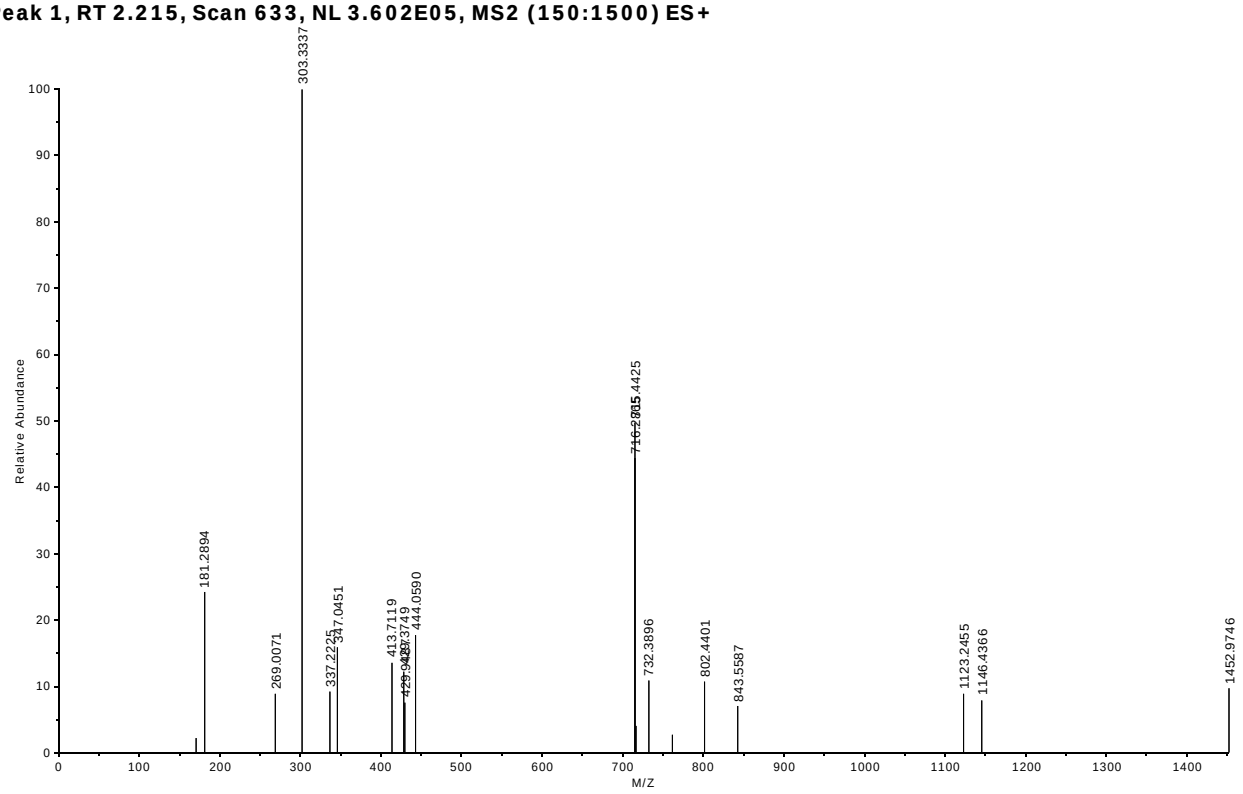
Group: Watts, J
Submitter: Michael Moazami
Project: RP LC C18 < 2 cmpds
Sample ID: mm/7500/41 succinyl doubler dmt
Experiment: BLUE ESIPOS C18 5 min
Filename: mm750041_succinyl_doubler_dmt_Michael_Moa
Instrument: Blue RP UHPLC-MS (B30:1023)
Acquisition Date: 05/05/2015 13:23:15

TIC, NL 1.261E07, MS2 (150:1500) ES+

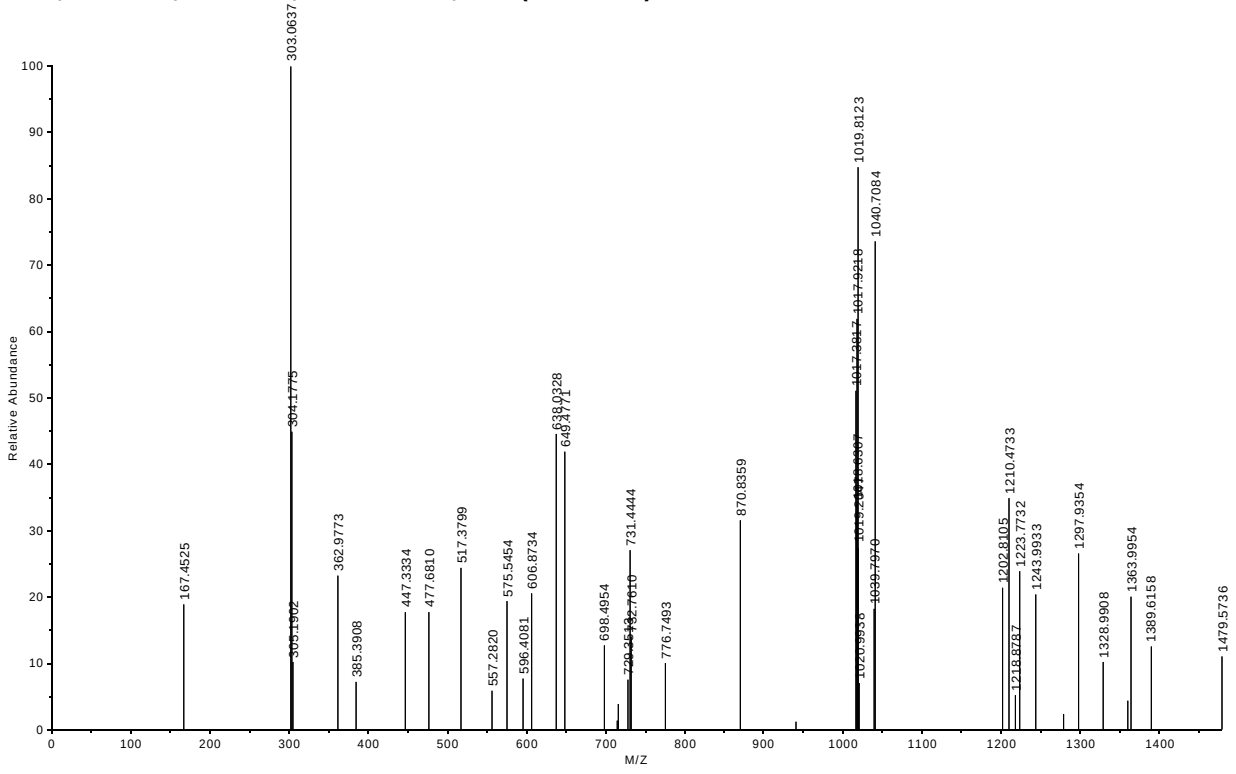


	RT Mins	Height	Height %	Area	Area %
1	2.21	667564	6.33	1127759	4.89
2	2.98	1637793	15.52	4678127	20.28
3	3.45	1868156	17.70	2201009	9.54
4	3.86	1147960	10.88	1689624	7.32
5	3.90	10553170	100.00	23066967	100.00

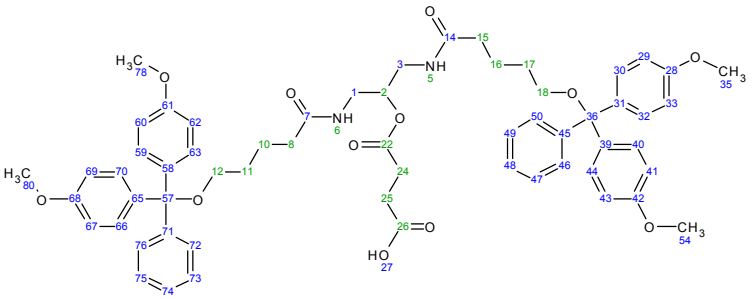
Peak 1, RT 2.215, Scan 633, NL 3.602E05, MS2 (150:1500) ES+



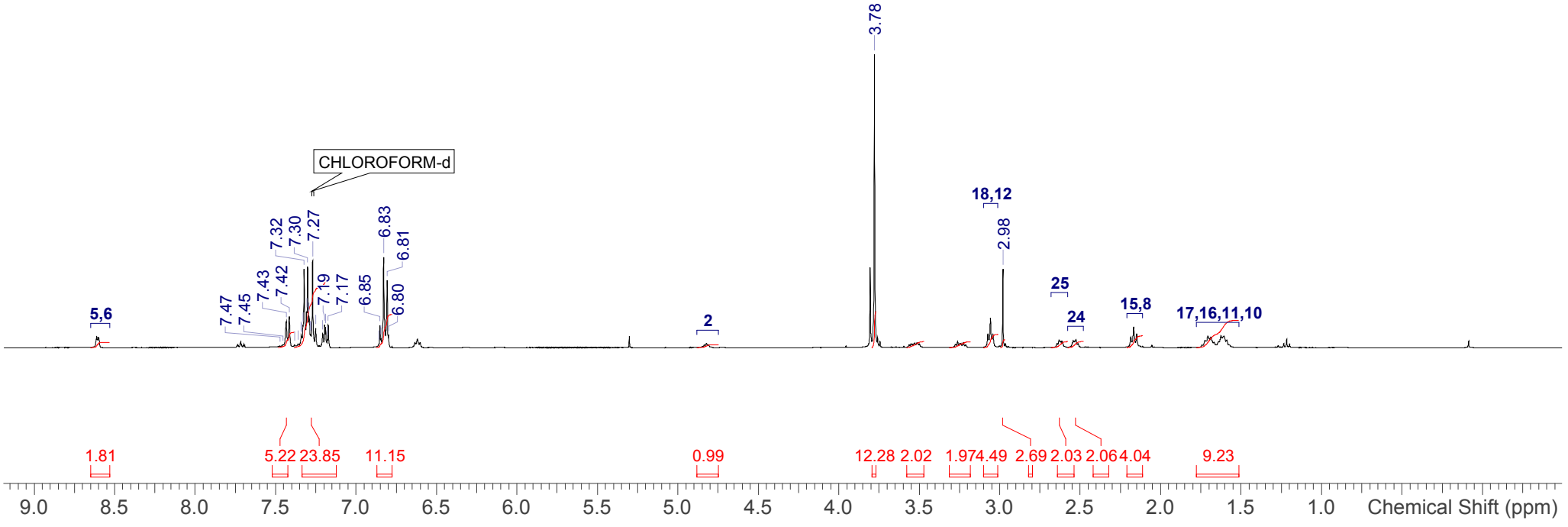
Peak 2, RT 2.981, Scan 852, NL 2.357E05, MS2 (150:1500) ES+



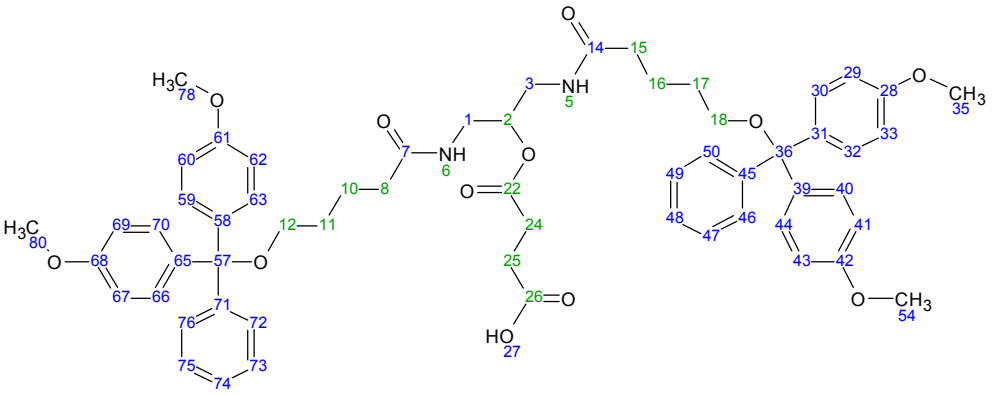
Acquisition Time (sec)	4.0894	***Comment***	mm/7500/41 Bis DMT succinyl doubler modifier
Date	05 May 2015 15:21:29		
Date Stamp	05 May 2015 15:21:29		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\my0515mm1 succinylated DMT DOublert\10\PDATA\1\1r		
Frequency (MHz)	400.1300	***Nucleus***	1H
Number of Transients	16	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	65536	***Pulse Sequence***	zg30
Receiver Gain	58.58	***SW(cyclical) (Hz)***	8012.82
Solvent	CHLOROFORM-d	***Spectrum Offset (Hz)***	2465.5647
Spectrum Type	standard	***Sweep Width (Hz)***	8012.70
Temperature (degree C)	26.913		



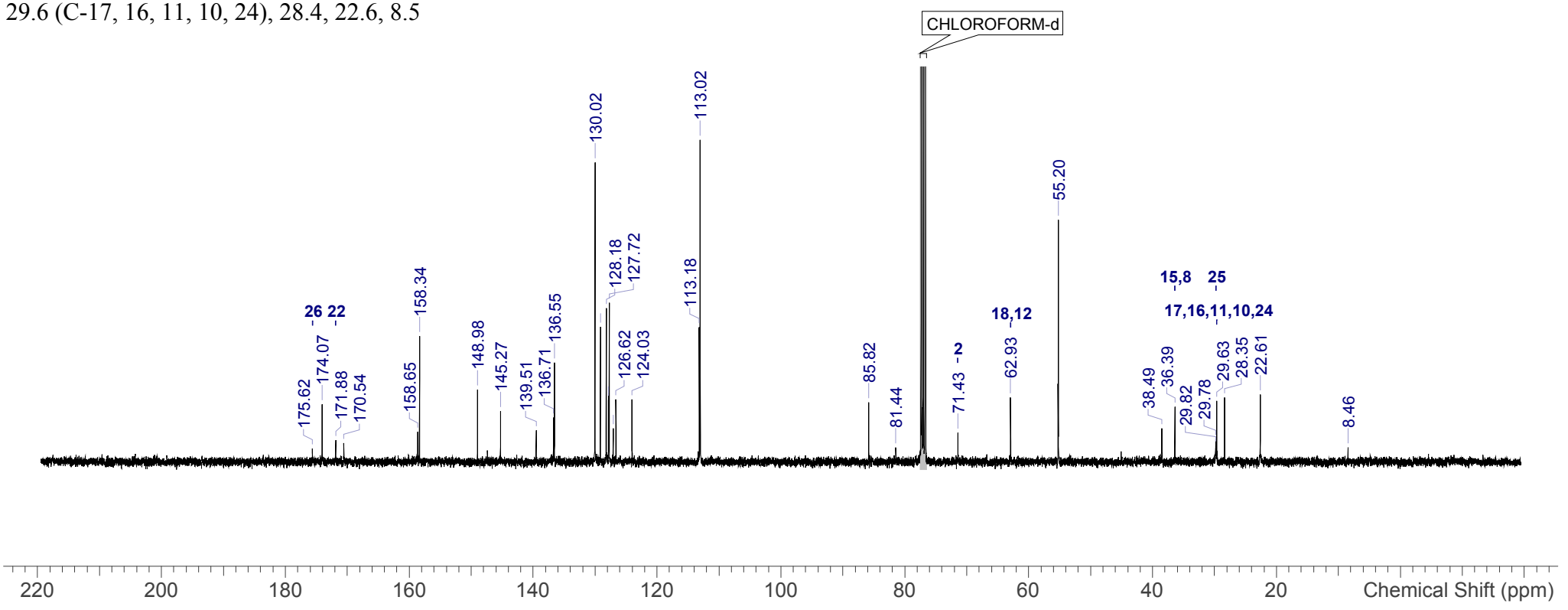
^{1}H NMR (400MHz, CHLOROFORM-d) δ = 8.53 - 8.65 (2H, m, H-5, 6), 7.38 - 7.48 (5H, m, M12), 7.17 - 7.38 (25H, m, M11), 6.78 - 6.87 (11H, m, M10), 4.75 - 4.88 (1H, m, H-2), 3.78 (12H, s, M09), 3.47 - 3.58 (2H, m, M06), 3.18 - 3.31 (2H, m, M05), 3.01 - 3.10 (5H, m, H-18, 12), 2.98 (3H, s, M14), 2.58 - 2.68 (2H, m, H-25), 2.48 - 2.58 (2H, m, H-24), 2.11 - 2.21 (4H, m, H-15, 8), 1.51 - 1.78 (9H, m, H-17, 16, 11, 10)



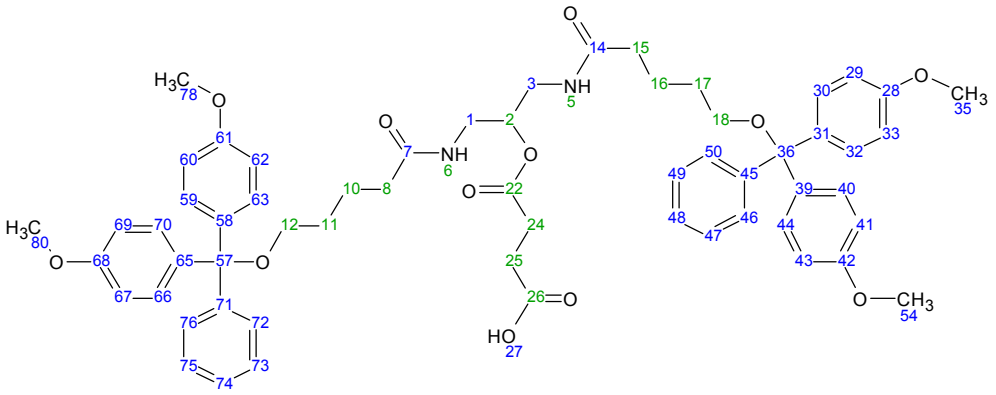
Acquisition Time (sec)	1.3631		
Comment	mm/7500/41 Bis DMT succinyl doubler modifier		
Date	05 May 2015 18:06:18		
Date Stamp	05 May 2015 18:06:18		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\my0515mm1 succinylated DMT DOublert\11\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	512	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d	***Spectrum Offset (Hz)***	10060.3320
Spectrum Type	standard	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	28.319		



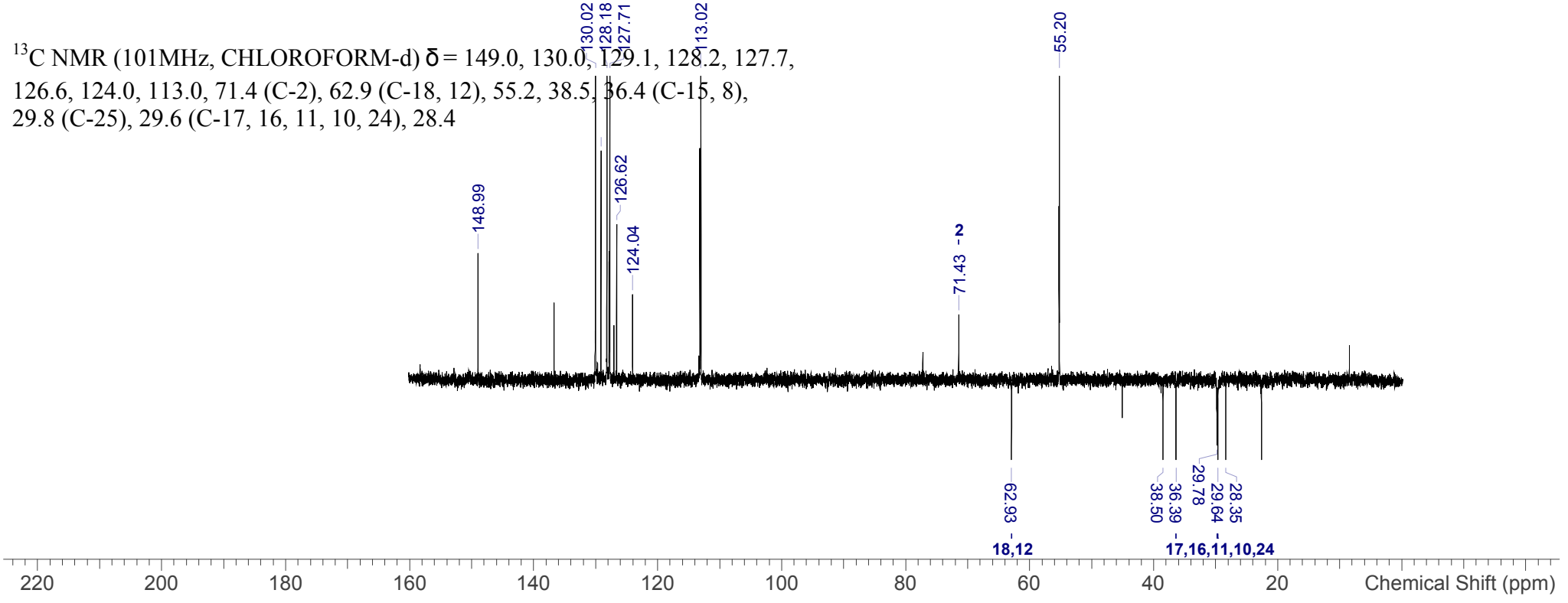
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 175.6 (C-26), 174.1, 171.9 (C-22), 170.5, 158.6, 158.3, 149.0, 145.3, 139.5, 136.7, 136.6, 130.1, 130.0, 129.1, 128.2, 127.8, 127.8, 127.7, 127.1, 126.6, 124.0, 113.2, 113.0, 85.8, 81.4, 71.4 (C-2), 62.9 (C-18, 12), 55.3, 55.2, 38.5, 36.4 (C-15, 8), 29.8, 29.8 (C-25), 29.6 (C-17, 16, 11, 10, 24), 28.4, 22.6, 8.5



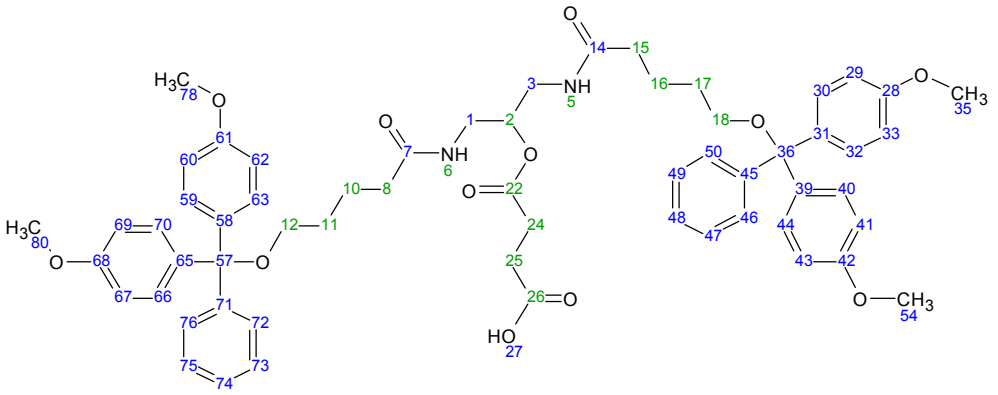
Acquisition Time (sec)	2.0316		
Comment	mm/7500/41 Bis DMT succinyl doubler modifier		
Date	05 May 2015 18:24:55		
Date Stamp	05 May 2015 18:24:55		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\my0515mm1 succinylated DMT DOublert\12\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp135
Receiver Gain	212.69	***SW(cyclical) (Hz)***	16129.03
Solvent	CHLOROFORM-d	***Spectrum Offset (Hz)***	8047.8672
Spectrum Type	DEPT135	***Sweep Width (Hz)***	16128.54
Temperature (degree C)	28.252		



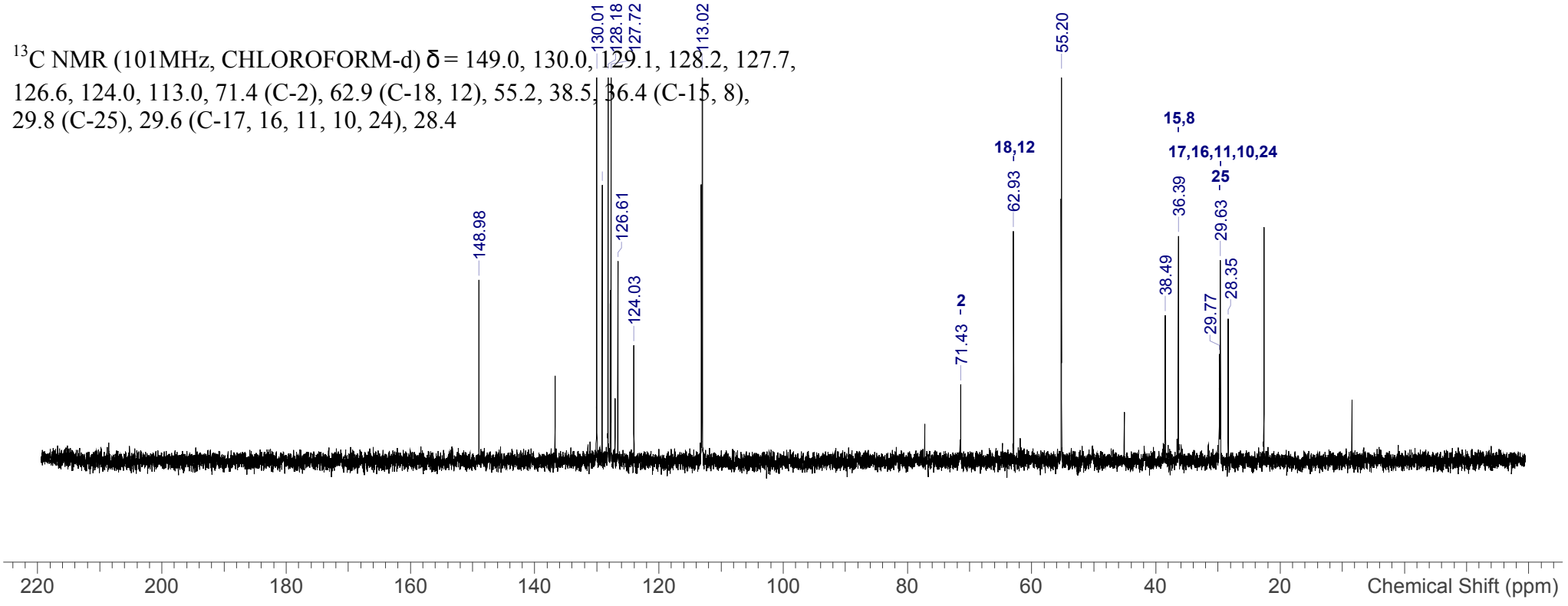
^{13}C NMR (101MHz, CHLOROFORM-d) δ = 149.0, 130.0, 129.1, 128.2, 127.7, 126.6, 124.0, 113.0, 71.4 (C-2), 62.9 (C-18, 12), 55.2, 38.5, 36.4 (C-15, 8), 29.8 (C-25), 29.6 (C-17, 16, 11, 10, 24), 28.4



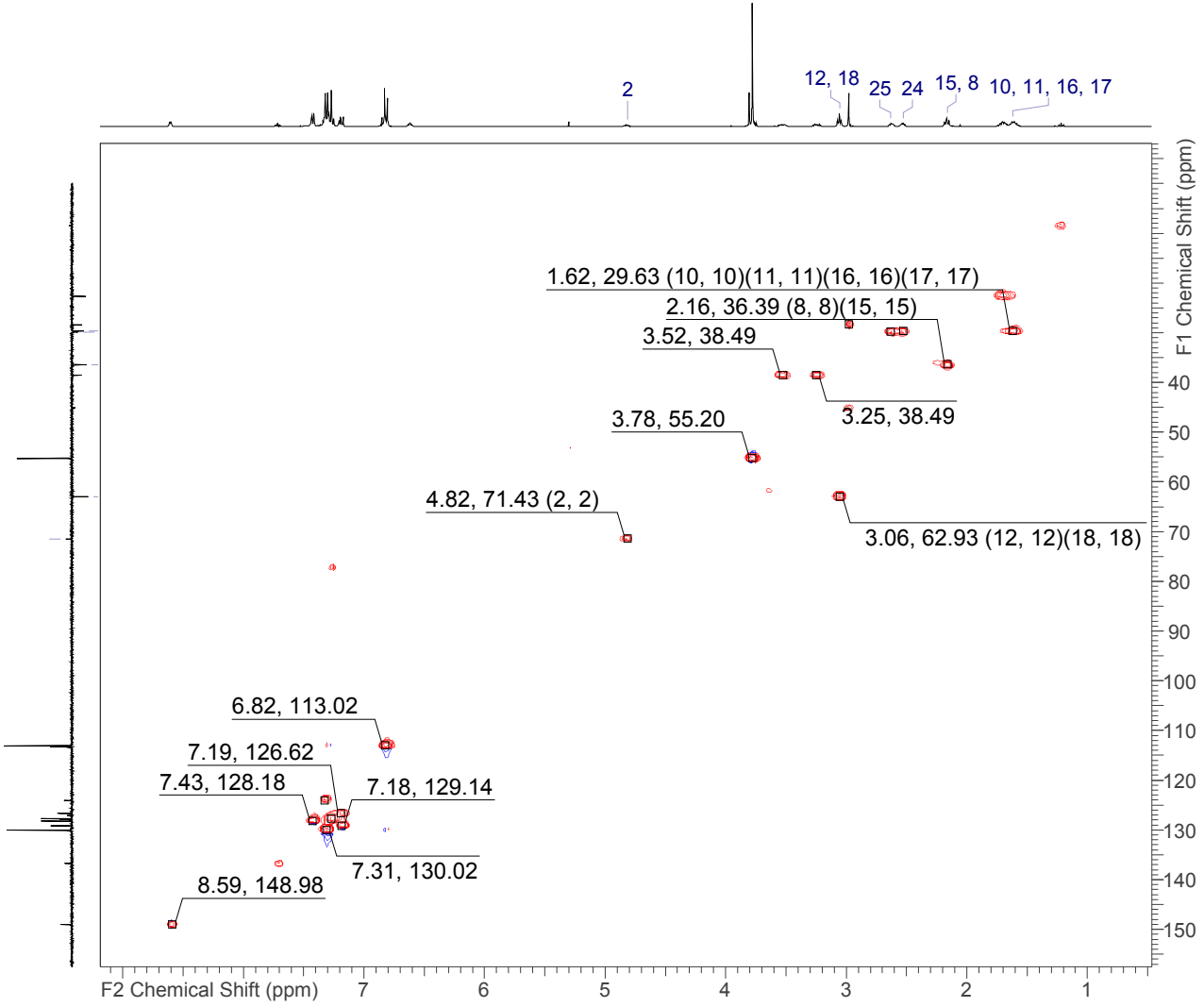
Acquisition Time (sec)	1.3631		
Comment	mm/7500/41 Bis DMT succinyl doubler modifier		
Date	05 May 2015 19:18:34		
Date Stamp	05 May 2015 19:18:34		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\my0515mm1 succinylated DMT DOublert\15\PDATA\1\1r		
Frequency (MHz)	100.6128	***Nucleus***	13C
Number of Transients	256	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	deptsp45
Receiver Gain	212.69	***SW(cyclical) (Hz)***	24038.46
Solvent	CHLOROFORM-d	***Spectrum Offset (Hz)***	10060.3564
Spectrum Type	DEPT45	***Sweep Width (Hz)***	24037.73
Temperature (degree C)	28.259		

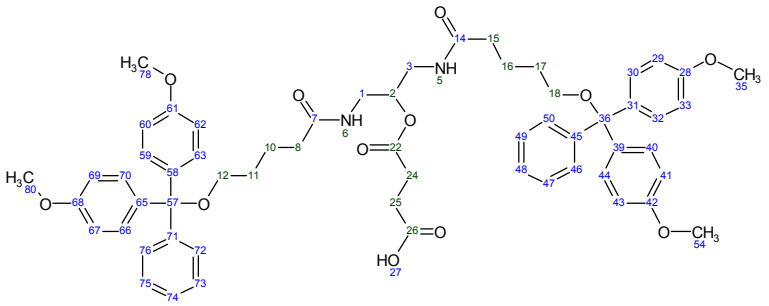


^{13}C NMR (101MHz, CHLOROFORM-d) δ = 149.0, 130.0, 129.1, 128.2, 127.7, 126.6, 124.0, 113.0, 71.4 (C-2), 62.9 (C-18, 12), 55.2, 38.5, 36.4 (C-15, 8), 29.8 (C-25), 29.6 (C-17, 16, 11, 10, 24), 28.4

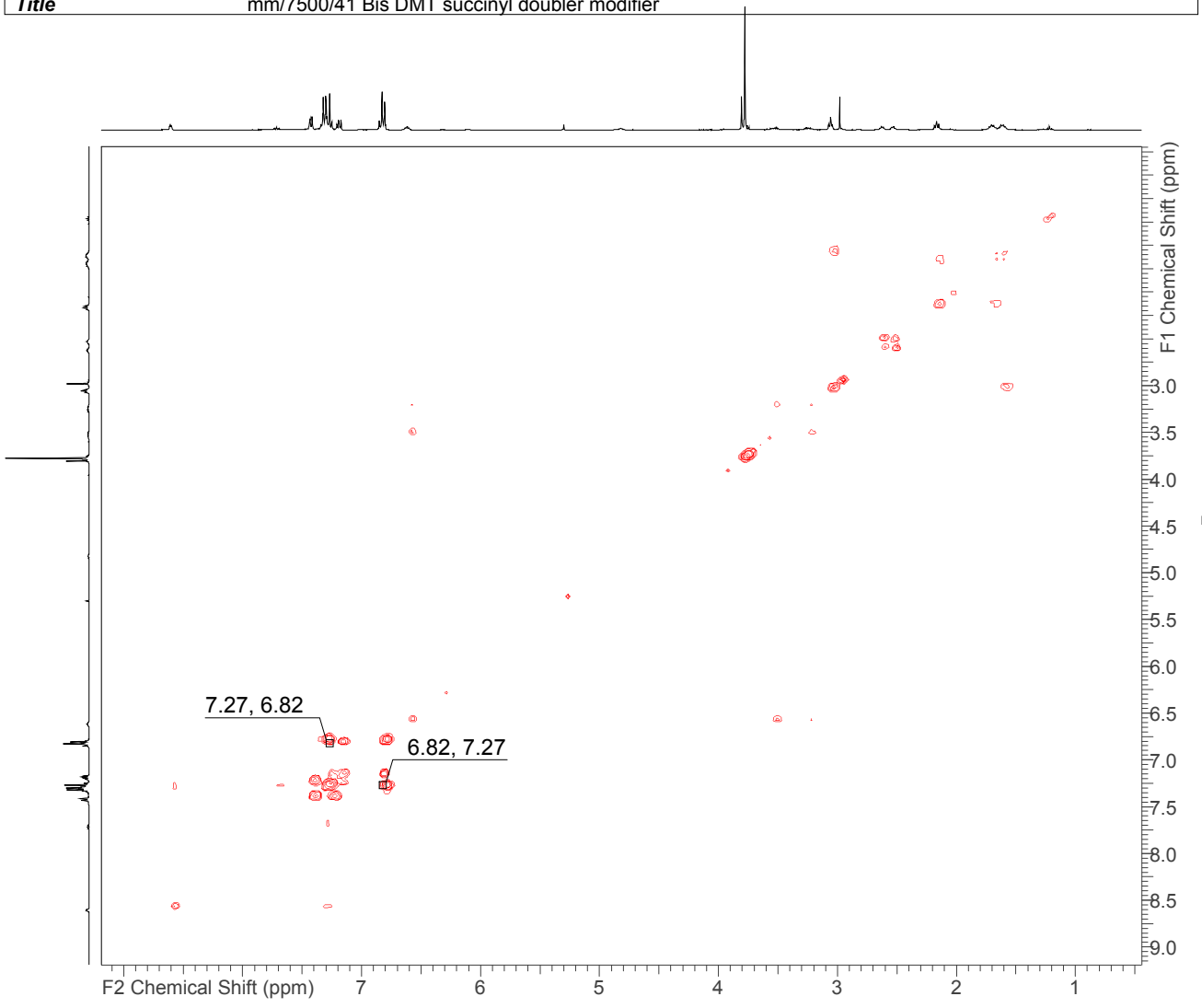


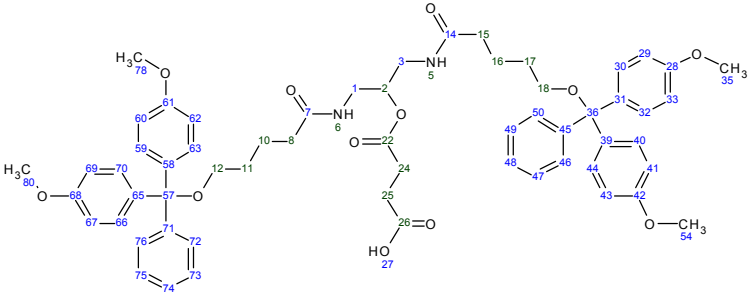
Acquisition Time (sec)	(0.1393, 0.0154)	***Comment***	mm/7500/41 Bis DMT succinyl doubler modifier
Date	05 May 2015 00:00:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\my0515mm1 succinylated DMT DOublert\16\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 100.6128)	***Nucleus***	(1H, 13C)
Number of Transients	2	***Origin***	spect
Original Points Count	(512, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	hsqcetgp
Solvent	CHLOROFORM-d	***Spectrum Type***	HSQC
Sweep Width (Hz)	(3672.88, 16650.39)	***Temperature (degree C)***	28.224
Title	mm/7500/41 Bis DMT succinyl doubler modifier		





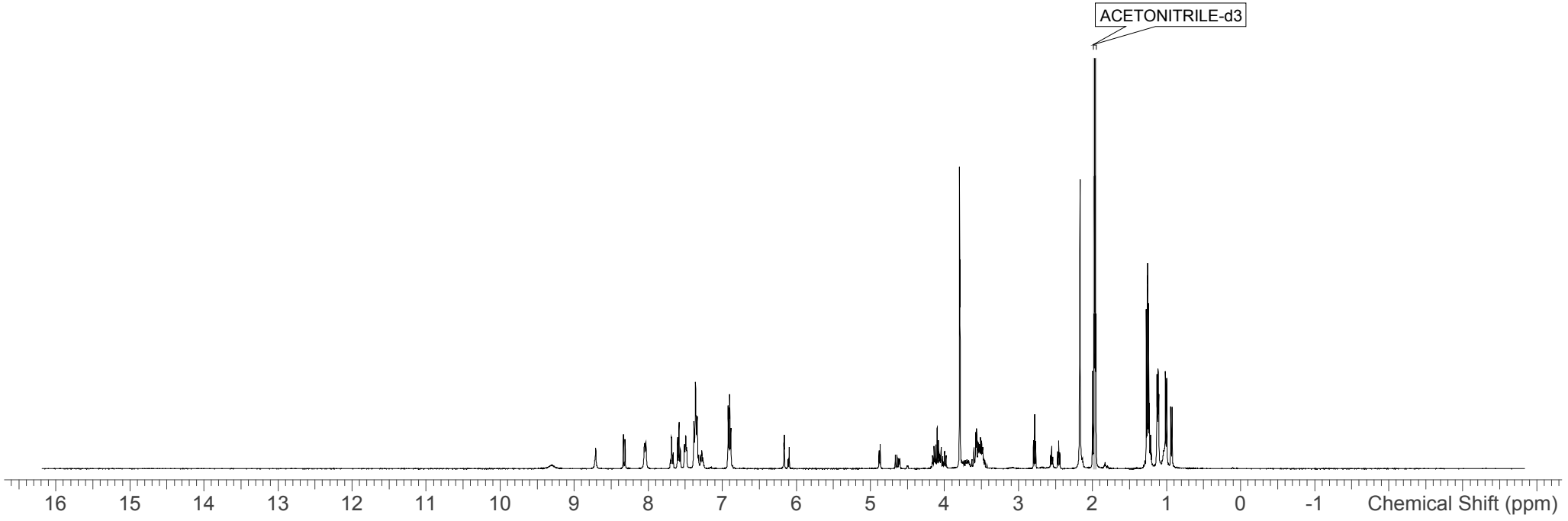
Acquisition Time (sec)	(0.2785, 0.0696)	***Comment***	mm/7500/41 Bis DMT succinyl doubler modifier
Date	05 May 2015 00:00:00		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\my0515mm1 succinylated DMT DOublert\13\PDATA\1\2rr		
Frequency (MHz)	(400.1300, 400.1300)	***Nucleus***	(1H, 1H)
Number of Transients	2	***Origin***	spect
Original Points Count	(1024, 256)	***Owner***	nmr
Points Count	(1024, 1024)	***Pulse Sequence***	cosygpqf
Solvent	CHLOROFORM-d	***Spectrum Type***	COSY
Sweep Width (Hz)	(3672.88, 3672.88)	***Temperature (degree C)***	28.008
Title	mm/7500/41 Bis DMT succinyl doubler modifier		





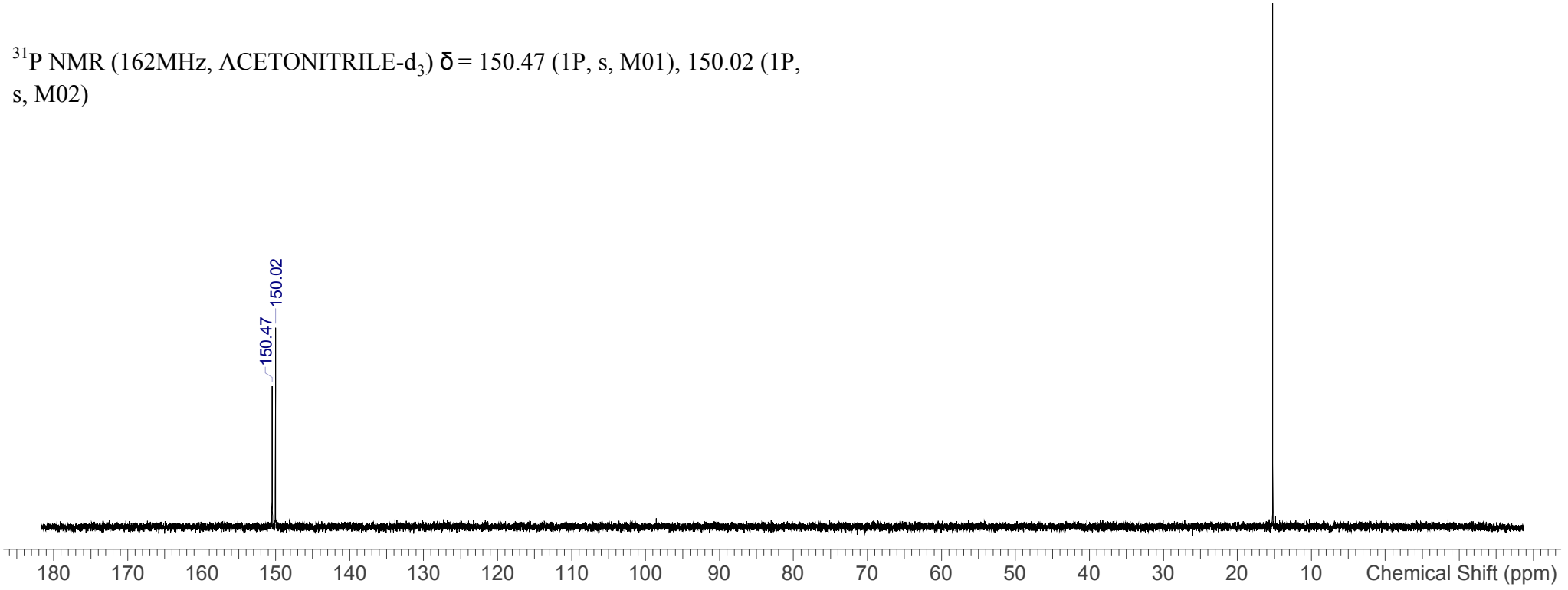
A.28 LNA-A

Acquisition Time (sec)	4.0894	***Comment***	mm/7500/69 LNA-A		
Date	20 Jun 2015 19:37:16				
Date Stamp	20 Jun 2015 19:37:16				
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\7500-69\12\PDATA\1\1r				
Frequency (MHz)	400.1300	***Nucleus***	1H	***Number of Transients***	16
Origin	spect	***Original Points Count***	32768	***Owner***	nmr
Points Count	65536	***Pulse Sequence***	zg30	***Receiver Gain***	100.45
SW(cyclical) (Hz)	8012.82	***Solvent***	ACETONITRILE-d3		
Spectrum Offset (Hz)	2470.9668	***Spectrum Type***	standard	***Sweep Width (Hz)***	8012.70
Temperature (degree C)	25.003				



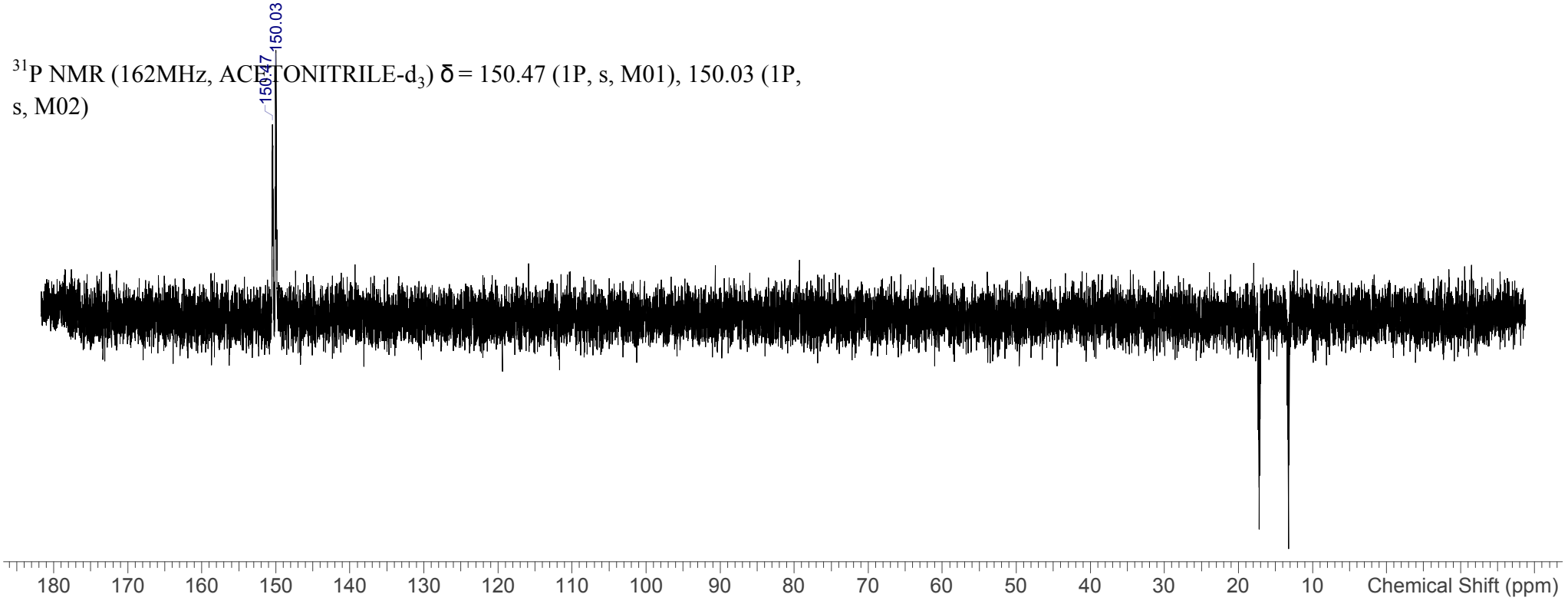
Acquisition Time (sec)	1.0093	***Comment***	mm/7500/69 LNA-A
Date	20 Jun 2015 19:30:52		
Date Stamp	20 Jun 2015 19:30:52		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\7500-69\10\PDATA\1\1r		
Frequency (MHz)	161.9754	***Nucleus***	31P
Number of Transients	64	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	32467.53
Solvent	ACETONITRILE-d3		
Spectrum Offset (Hz)	13199.9404	***Spectrum Type***	standard
Sweep Width (Hz)	32466.54	***Temperature (degree C)***	24.999

^{31}P NMR (162MHz, ACETONITRILE-d_3) δ = 150.47 (1P, s, M01), 150.02 (1P, s, M02)



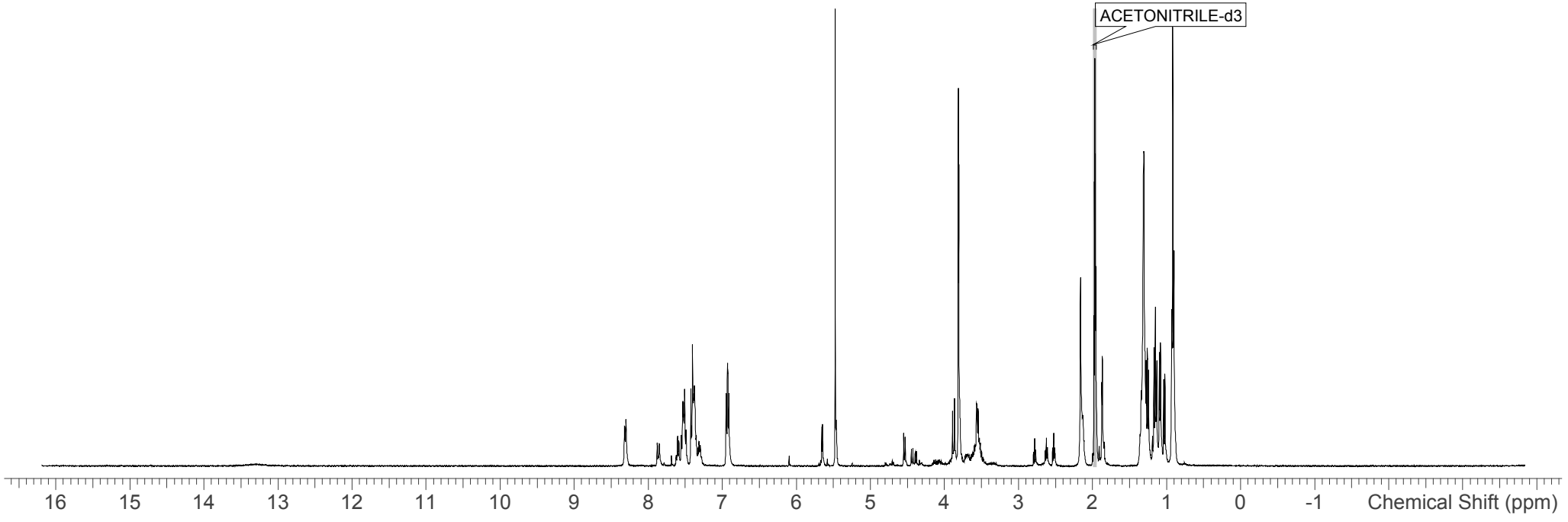
Acquisition Time (sec)	1.0093	***Comment***	mm/7500/69 LNA-A
Date	20 Jun 2015 19:34:58		
Date Stamp	20 Jun 2015 19:34:58		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\7500-69\11\PDATA\1\1r		
Frequency (MHz)	161.9754	***Nucleus***	31P
Number of Transients	64	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	32467.53
Solvent	ACETONITRILE-d3		
Spectrum Offset (Hz)	13196.9453	***Spectrum Type***	standard
Sweep Width (Hz)	32466.54	***Temperature (degree C)***	25.000

^{31}P NMR (162MHz, ACETONITRILE-d_3) δ = 150.47 (1P, s, M01), 150.03 (1P, s, M02)



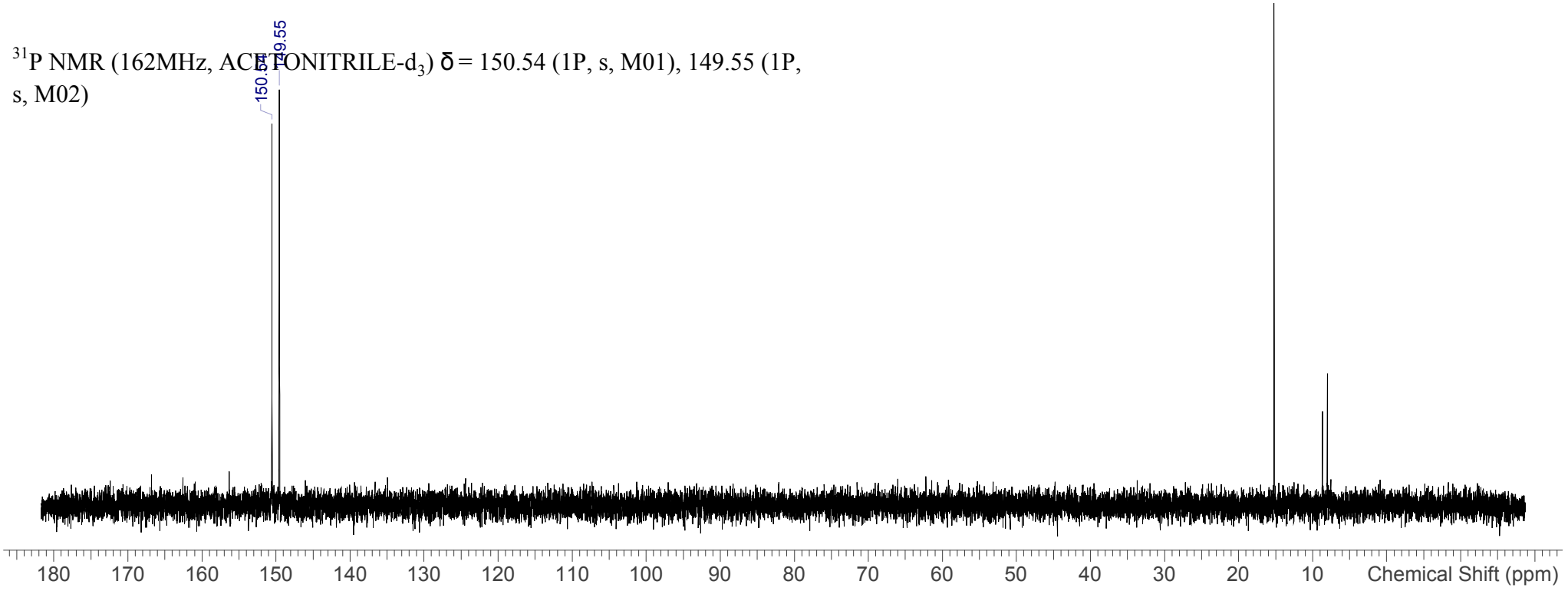
A.29 LNA-C

Acquisition Time (sec)	4.0894	***Comment***	mm/7500/76 LNA C		
Date	07 Jul 2015 05:46:50			***Date Stamp***	07 Jul 2015 05:46:50
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\7500-76\12\PDATA\1\1r				
Frequency (MHz)	400.1300	***Nucleus***	1H	***Number of Transients***	16
Origin	AVII400	***Original Points Count***	32768	***Owner***	nmr
Points Count	65536	***Pulse Sequence***	zg30	***Receiver Gain***	193.87
SW(cyclical) (Hz)	8012.82	***Solvent***	ACETONITRILE-d3		
Spectrum Offset (Hz)	2470.9668	***Spectrum Type***	standard	***Sweep Width (Hz)***	8012.70
Temperature (degree C)	25.160				



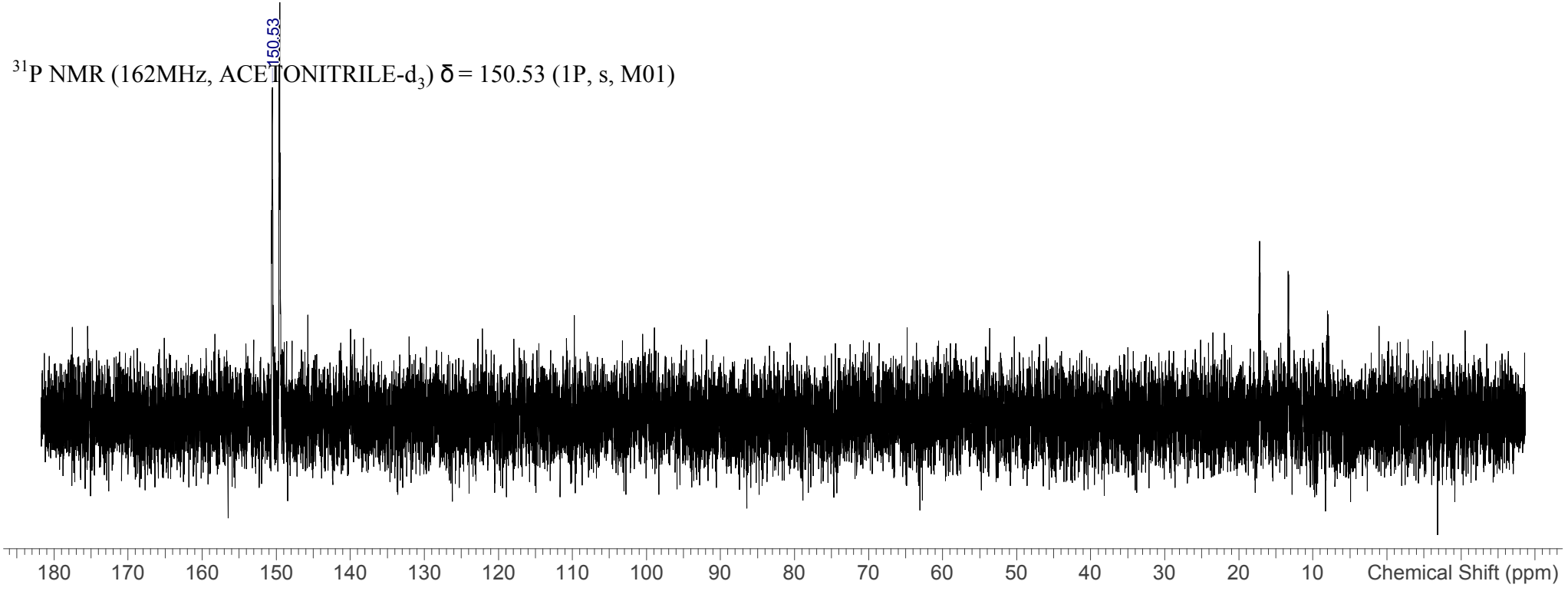
Acquisition Time (sec)	1.0093	***Comment***	mm/7500/76 LNA C
Date	07 Jul 2015 05:39:03		
Date Stamp	07 Jul 2015 05:39:03		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\7500-76\10\PDATA\1\1r		
Frequency (MHz)	161.9754	***Nucleus***	31P
Number of Transients	64	***Origin***	AVII400
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	1620.00	***SW(cyclical) (Hz)***	32467.53
Solvent	ACETONITRILE-d3		
Spectrum Offset (Hz)	13200.3633	***Spectrum Type***	standard
Sweep Width (Hz)	32466.54	***Temperature (degree C)***	25.160

^{31}P NMR (162MHz, ACETONITRILE-d_3) δ = 150.54 (1P, s, M01), 149.55 (1P, s, M02)



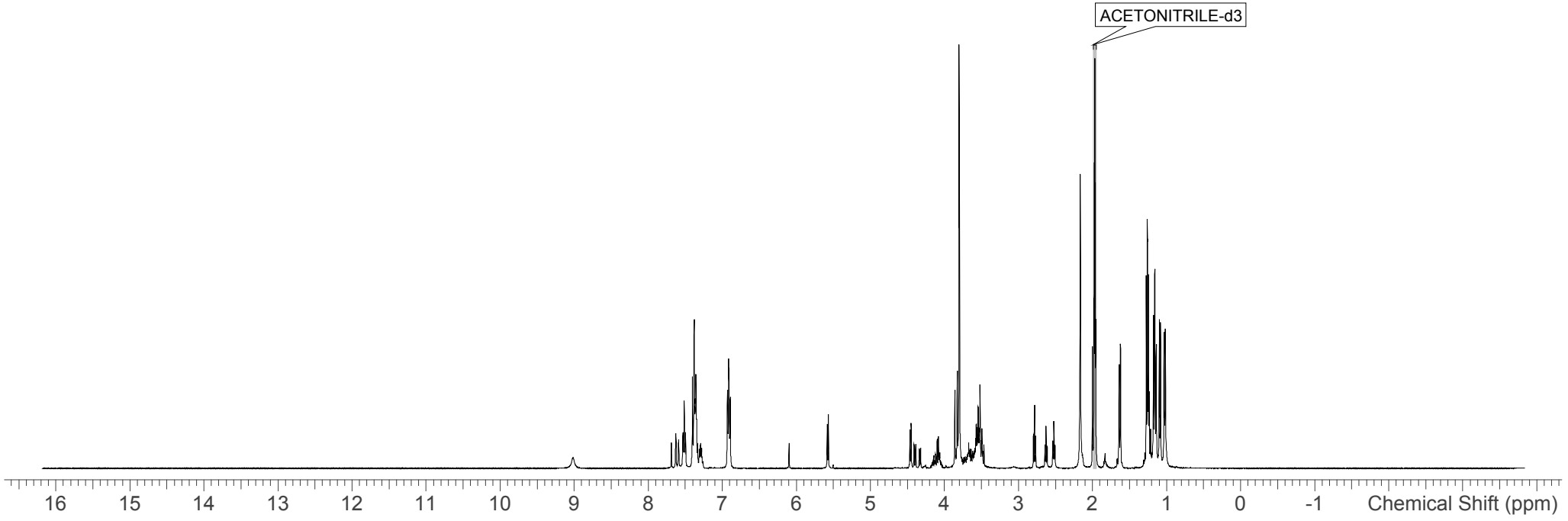
Acquisition Time (sec)	1.0093	***Comment***	mm/7500/76 LNA C
Date	07 Jul 2015 05:43:49		
Date Stamp	07 Jul 2015 05:43:49		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\7500-76\11\PDATA\1\1r		
Frequency (MHz)	161.9754	***Nucleus***	31P
Number of Transients	64	***Origin***	AVII400
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zg30
Receiver Gain	1150.00	***SW(cyclical) (Hz)***	32467.53
Solvent	ACETONITRILE-d3		
Spectrum Offset (Hz)	13205.3164	***Spectrum Type***	standard
Sweep Width (Hz)	32466.54	***Temperature (degree C)***	25.060

^{31}P NMR (162MHz, ACETONITRILE-d_3) δ = 150.53 (1P, s, M01)



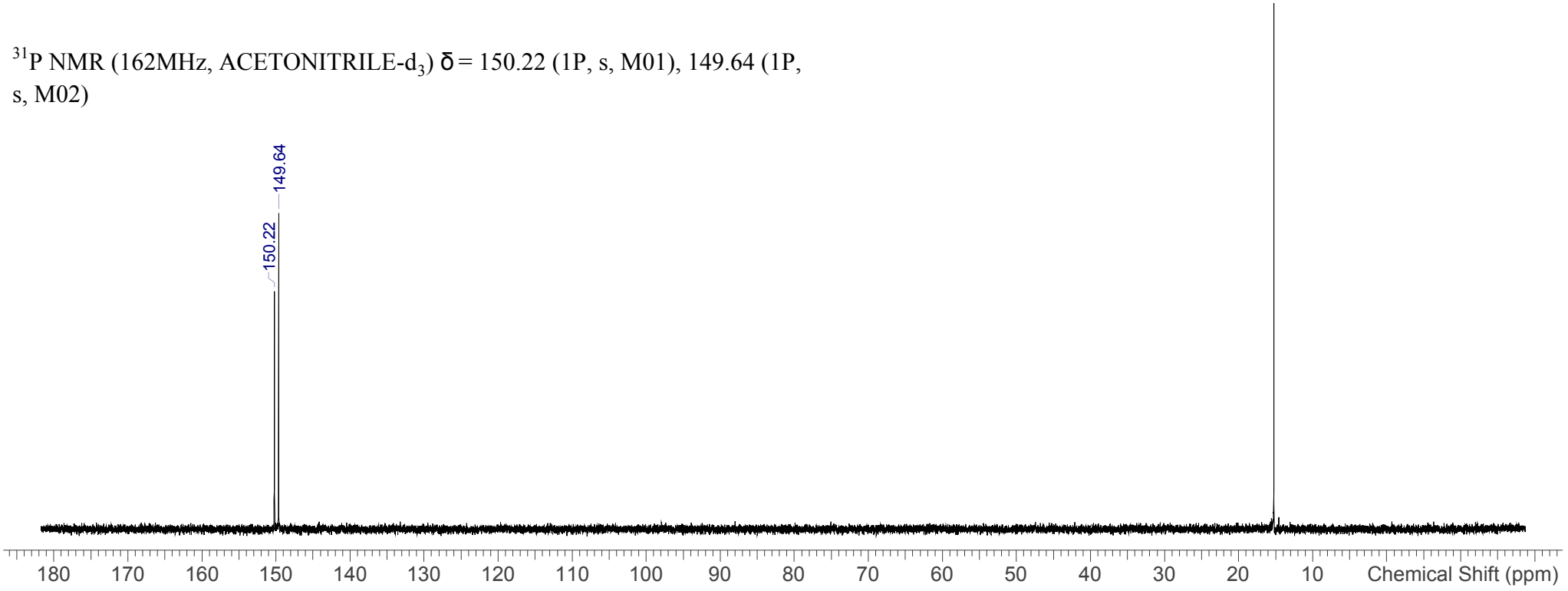
A.30 LNA-T

Acquisition Time (sec)	4.0894	***Comment***	mm/7500/70 LNA-T		
Date	20 Jun 2015 19:51:33				
Date Stamp	20 Jun 2015 19:51:33				
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\7500-70\12\PDATA\1\1r				
Frequency (MHz)	400.1300	***Nucleus***	1H	***Number of Transients***	16
Origin	spect	***Original Points Count***	32768	***Owner***	nmr
Points Count	65536	***Pulse Sequence***	zg30	***Receiver Gain***	89.37
SW(cyclical) (Hz)	8012.82	***Solvent***	ACETONITRILE-d3		
Spectrum Offset (Hz)	2470.9668	***Spectrum Type***	standard	***Sweep Width (Hz)***	8012.70
Temperature (degree C)	24.999				



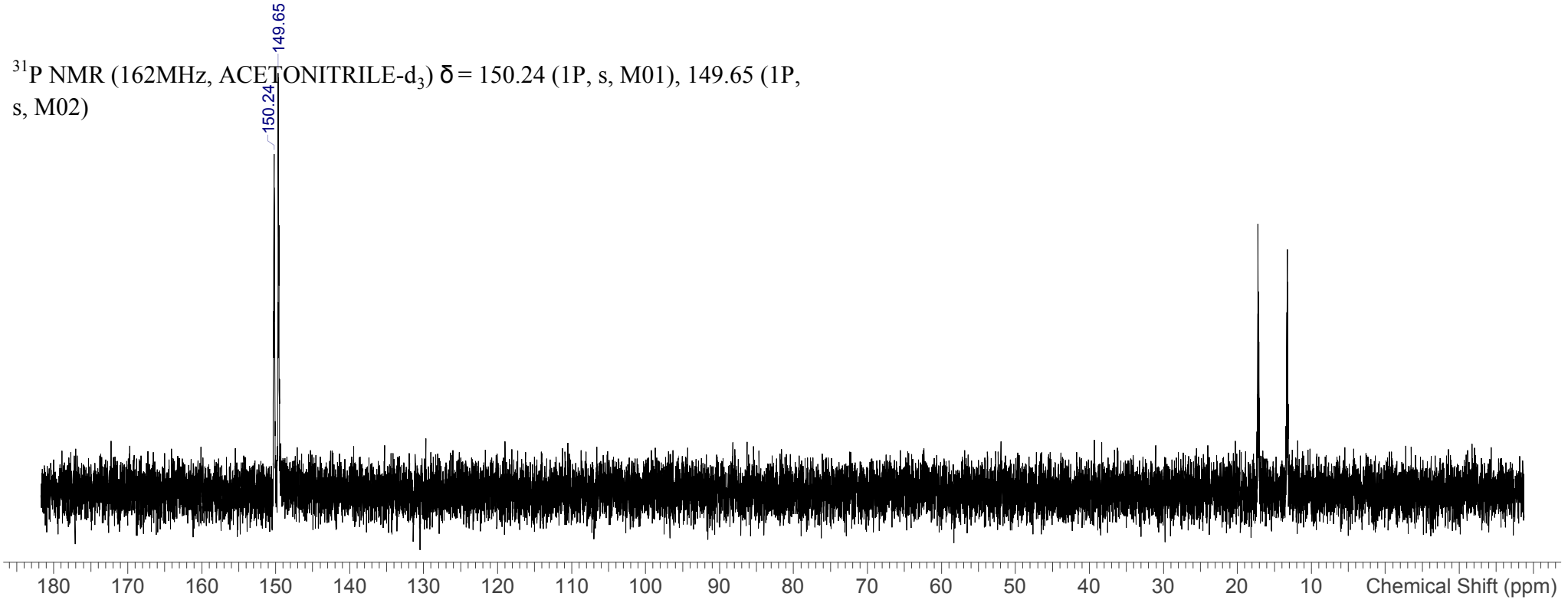
Acquisition Time (sec)	1.0093	***Comment***	mm/7500/70 LNA-T
Date	20 Jun 2015 19:45:06		
Date Stamp	20 Jun 2015 19:45:06		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\7500-70\10\PDATA\1\1r		
Frequency (MHz)	161.9754	***Nucleus***	31P
Number of Transients	64	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	32467.53
Solvent	ACETONITRILE-d3		
Spectrum Offset (Hz)	13199.9404	***Spectrum Type***	standard
Sweep Width (Hz)	32466.54	***Temperature (degree C)***	24.998

^{31}P NMR (162MHz, ACETONITRILE-d_3) δ = 150.22 (1P, s, M01), 149.64 (1P, s, M02)



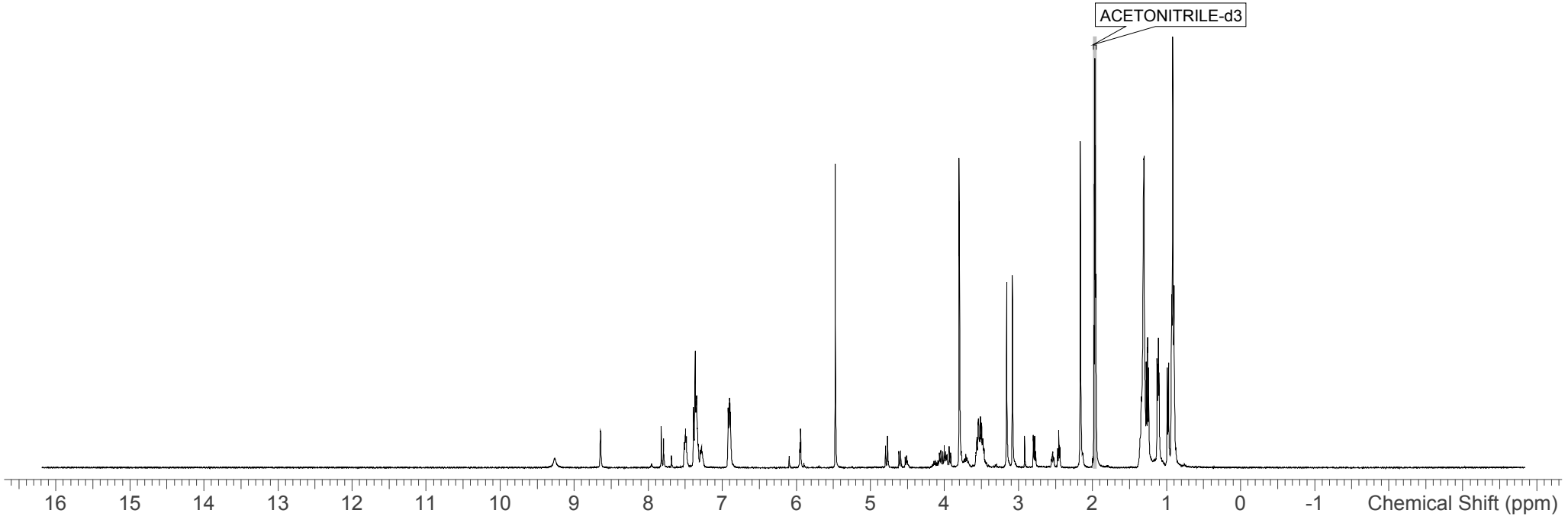
Acquisition Time (sec)	1.0093	***Comment***	mm/7500/70 LNA-T
Date	20 Jun 2015 19:49:13		
Date Stamp	20 Jun 2015 19:49:13		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\7500-70\11\PDATA\1\1r		
Frequency (MHz)	161.9754	***Nucleus***	31P
Number of Transients	64	***Origin***	spect
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zg30
Receiver Gain	212.69	***SW(cyclical) (Hz)***	32467.53
Solvent	ACETONITRILE-d3		
Spectrum Offset (Hz)	13199.9404	***Spectrum Type***	standard
Sweep Width (Hz)	32466.54	***Temperature (degree C)***	24.999

^{31}P NMR (162MHz, ACETONITRILE-d_3) δ = 150.24 (1P, s, M01), 149.65 (1P, s, M02)



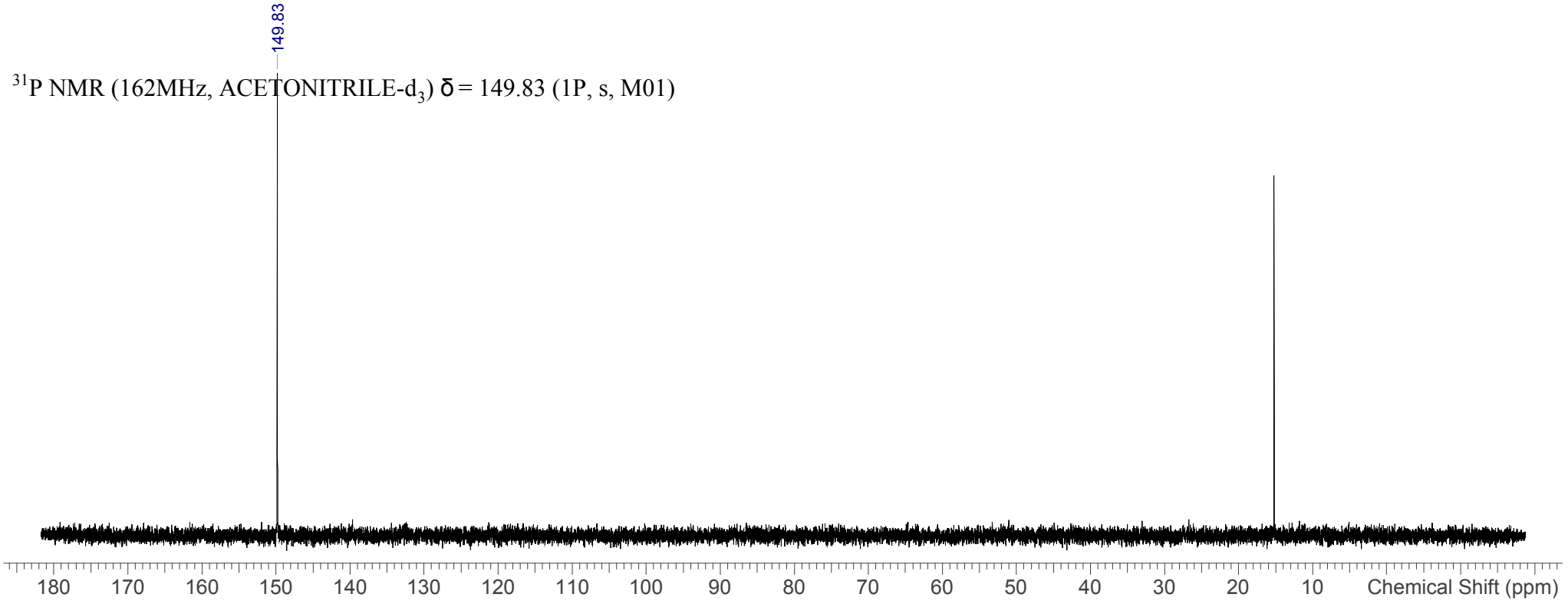
A.31 LNA-G

Acquisition Time (sec)	4.0894	***Comment***	mm/7500/77 LNA G		
Date	07 Jul 2015 06:08:06			***Date Stamp***	07 Jul 2015 06:08:06
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\7500-77\12\PDATA\1\1r				
Frequency (MHz)	400.1300	***Nucleus***	1H	***Number of Transients***	16
Origin	AVII400	***Original Points Count***	32768	***Owner***	nmr
Points Count	65536	***Pulse Sequence***	zg30	***Receiver Gain***	193.87
SW(cyclical) (Hz)	8012.82	***Solvent***	ACETONITRILE-d3		
Spectrum Offset (Hz)	2470.9668	***Spectrum Type***	standard	***Sweep Width (Hz)***	8012.70
Temperature (degree C)	25.160				



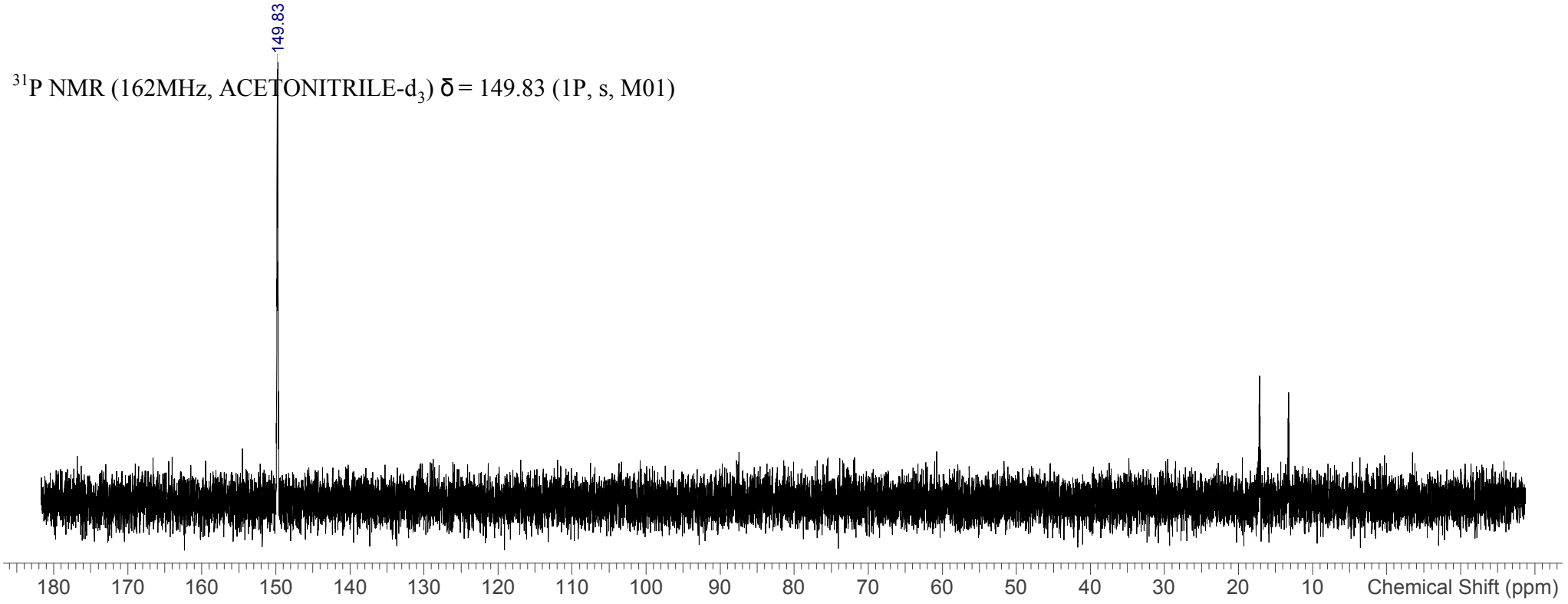
Acquisition Time (sec)	1.0093	***Comment***	mm/7500/77 LNA G
Date	07 Jul 2015 06:00:31		
Date Stamp	07 Jul 2015 06:00:31		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\7500-77\10\PDATA\1\1r		
Frequency (MHz)	161.9754	***Nucleus***	31P
Number of Transients	64	***Origin***	AVII400
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zgpg30
Receiver Gain	1620.00	***SW(cyclical) (Hz)***	32467.53
Solvent	ACETONITRILE-d3		
Spectrum Offset (Hz)	13200.3633	***Spectrum Type***	standard
Sweep Width (Hz)	32466.54	***Temperature (degree C)***	25.160

^{31}P NMR (162MHz, ACETONITRILE-d_3) δ = 149.83 (1P, s, M01)



Acquisition Time (sec)	1.0093	***Comment***	mm/7500/77 LNA G
Date	07 Jul 2015 06:05:09		
Date Stamp	07 Jul 2015 06:05:09		
File Name	\\soton.ac.uk\ude\personalfiles\users\mm29g10\mydesktop\7500-77\11\PDATA\1\1r		
Frequency (MHz)	161.9754	***Nucleus***	31P
Number of Transients	64	***Origin***	AVII400
Original Points Count	32768	***Owner***	nmr
Points Count	32768	***Pulse Sequence***	zg30
Receiver Gain	1150.00	***SW(cyclical) (Hz)***	32467.53
Solvent	ACETONITRILE-d3		
Spectrum Offset (Hz)	13198.4238	***Spectrum Type***	standard
Sweep Width (Hz)	32466.54	***Temperature (degree C)***	25.060

^{31}P NMR (162MHz, ACETONITRILE-d_3) δ = 149.83 (1P, s, M01)



Appendix B

Oligonucleotide LCMS

Name	Sequence	Predicted Mass	Found Mass
ASO 82- Disulphide	+A*+A*+C* A*C*G* T*C*T* A*T*A* +C*+G*+C-$C_6H_{12}SSC_6H_{12}OH$	5328.57	5327.48
ASO 82- Disulphide (Reduced)	+A*+A*+C* A*C*G* T*C*T* A*T*A* +C*+G*+C-$C_6H_{12}SH$	5195.57	5195.50
ASO 132- 1 PS	A*AC ACG TCT ATA CGC-Biotin	5044.29	5043.99
ASO 139- Fully PS	A*A*C* A*C*G* T*C*T* A*T*A* C*G*C*-Biotin	5269.16	5268.67
DNA Dimer	(ACT GAT TGA CTG C)$_2$ Shchepinov doubler	8313.51	8312.54
DNA Tetramer	(ACT GAT TGA CTG C)$_4$ (Shchepinov doubler)$_2$	17041.9	17041.9
LNA Dimer	(+A*+G*+C*A*T*C*C*A*A*A*T*G*T*+G*+A*G*)$_2$ doubler	11065.04	11065.09
LNA Tetramer	(+A*+G*+C*A*T*C*C*A*A*A*T*G*T*+G*+A*G*)$_4$(Doubler)$_2$	22576.49	22541.60*
MOE Monomer	&G*&G*&G*&T*&C* A*G*C* T*G*C* C*A*A* T* &G*&C*&T*&A*&G	7274.15	7273.15
MOE Dimer	(&G*&G*&G*&T*&C* A*G*C* T*G*C* C*A*A* T* &G*&C*&T*&A*&G TEG)$_2$ Doubler	15340	15340*
MOE Tetramer	(&G*&G*&G*&T*&C* A*G*C* T*G*C* C*A*A* T* &G*&C*&T*&A*&G TEG)$_4$ (Doubler-TEG)$_2$	31,472.15	31,464*
MOE Octamer	(&G*&G*&G*&T*&C* A*G*C* T*G*C* C*A*A* T* &G*&C*&T*&A*&G TEG)$_8$ (Doubler-TEG)$_4$	67,811.46	NA
ASO 23	+C*+C*+C* G*G*C* C*C*C* T*A*G* +C*+G*+C	4938	4937.56
ASO 24	+G*+G*+C* C*C*C* T*A*G* C*G*C* +G*+C*+G	4976	4975.49
ASO 25	+C*+C*+C* T*A*G* C*G*C* G*C*G* +A*+C*+T	4963	4962.65
ASO 30	&G*&C*&C*&C*&C* T*A*G* C*G*C* G*C*G* +A*+C*+T	5913,94	5913.66
ASO 31	&C*&C*&C* &G*&G*C* C*C*C* T*A*G* C*G*C* &G*&C*&G* &A*&C	7141.08	7140.92
ASO 32	&G*&C* &C*&C*&C* T*A*G* C*G*C* G*C*&G* &A*&C*&T*&C	6519,58	6518.86
ASO 66	+C*+C*+C* T*A*G* $^{5-Me}$C*G*$^{5-Me}$C* G*$^{5-Me}$C*G* +A*+C*+T	5008	5004.55
ASO 67	+C*+C*$^{5-Me}$C* G*G*$^{5-Me}$C* $^{5-Me}$C*C*$^{5-Me}$C*$^{5-Me}$C* T*A*G* $^{5-Me}$C*G*$^{5-Me}$C* G*$^{5-Me}$C*G*+A*+C	6596.44	6595.74
ASO 68	+G*+C*$^{5-Me}$C*$^{5-Me}$C*$^{5-Me}$C* T*A*G* $^{5-Me}$C*G*$^{5-Me}$C* G*$^{5-Me}$C*G*A*$^{5-Me}$C*+T*+C	5932.88	5832.65
ASO 69	&C*&C*&C* &G*&G*$^{5-Me}$C* $^{5-Me}$C*C*$^{5-Me}$C*$^{5-Me}$C* T*A*G* $^{5-Me}$C*G*$^{5-Me}$C* &G*&C*&G* &A*&C	7225	7224.46
ASO 70	&G*&C* &C*&C*&C* T*A*G* $^{5-Me}$C*G*$^{5-Me}$C* G*$^{5-Me}$C*&G* &A*&C*&T*&C	6561.64	6561.13
ASO 71	&C*&C*&T*&A*&T*A*G*G*A*T*A*T*$^{5-Me}$C*$^{5-Me}$C*&A*&G*&G*&A*&A	6906,88	6909.05
ASO 70- Skip	&G*&C &C*&C&C* TA*G $^{5-Me}$C*G^{5-Me}C* G^{5-Me}C*&G &A*&C&T*&C	6465.64	6458.20
ASO 70- Wings 1	&G*&C &C&C&C T*A*G* $^{5-Me}$C*G*$^{5-Me}$C* G*$^{5-Me}$C&G &A&C&T*&C	6443.64	6432.21
ASO 70- Wings 2	&G*&C &C&C&C* T*A*G* $^{5-Me}$C*G*$^{5-Me}$C* G*$^{5-Me}$C*&G &A&C&T*&C	6465.64	6465.16

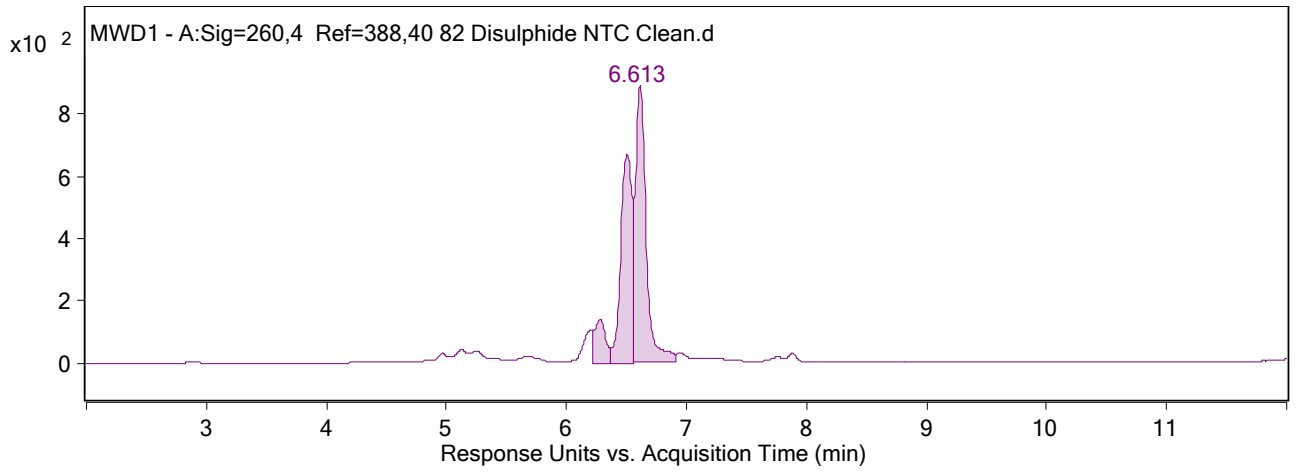
Table B.1: List of synthesised oligonucleotides with calculated and found masses. Asterisk-marked masses are calculated by manual deconvolution of obtained spectra.

B.1 ASO 82- Disulphide

Qualitative Analysis Report

Data File	82 Disulphide NTC Clean.d	**Sample Name**	82 Disulphide NTC
Sample Type	Sample	**Position**	P2-D7
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column.m	**Acquired Time**	2/19/2017 6:56:14 PM
IRM Calibration Status	Success	**DA Method**	MS_Check_Oligos.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

User Chromatograms



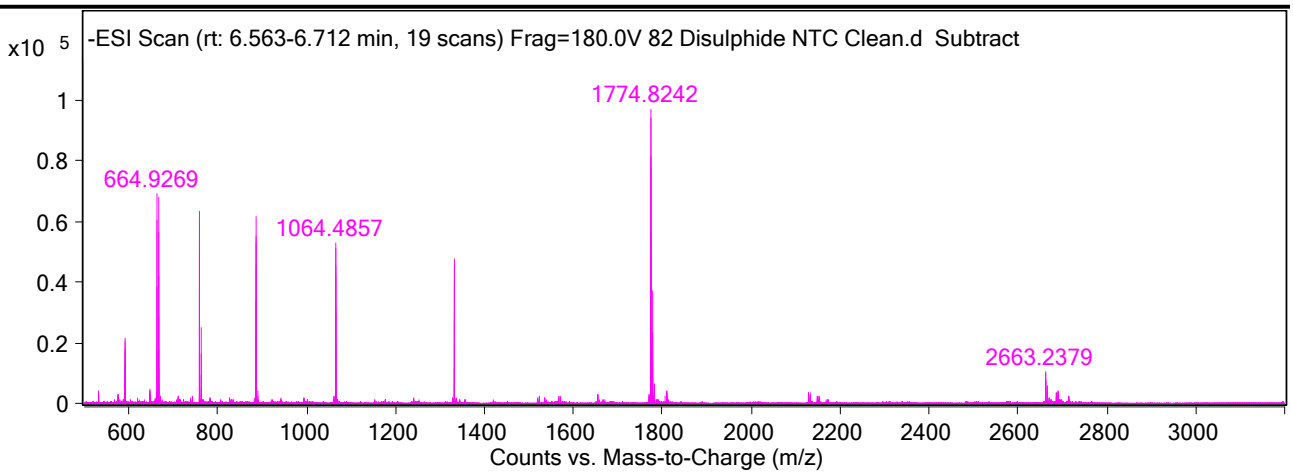
Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	6.22	6.28	6.367	140.56	889.64	16.15
2	6.367	6.5	6.56	668.32	4461.58	81
3	6.56	6.613	6.907	891.12	5508.39	100

User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (3) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

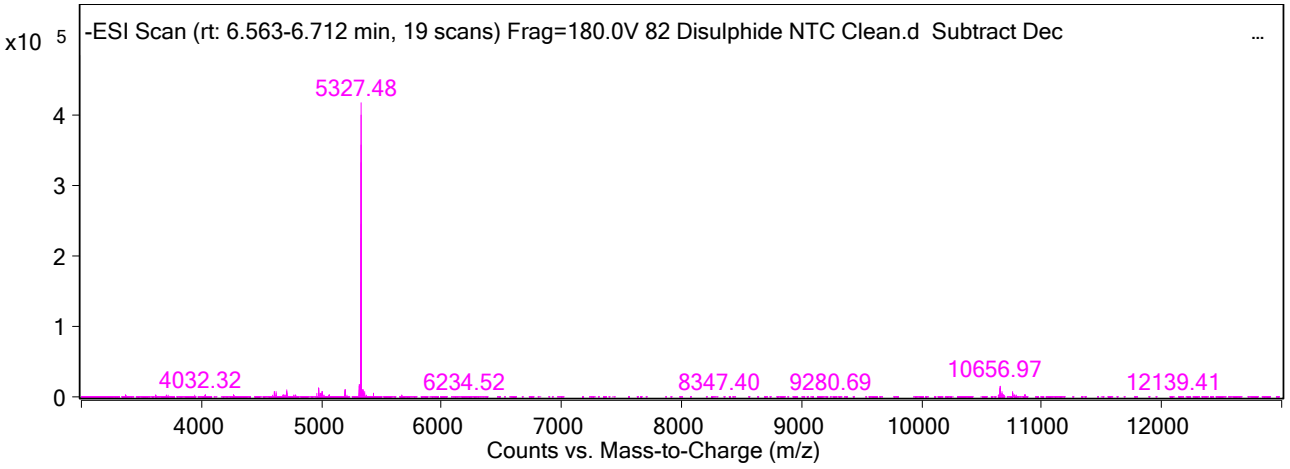
Qualitative Analysis Report



Peak List

m/z	z	Abund
664.8021	8	60417.34
664.9269	8	69001.52
665.0514	8	68070.2
760.0602	7	63434.62
760.203	7	60845.21
886.9042	6	61698.86
1774.4904	3	81411.27
1774.8242	3	96923.33
1775.1575	3	93712.56
1775.4904	3	77963.13

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI

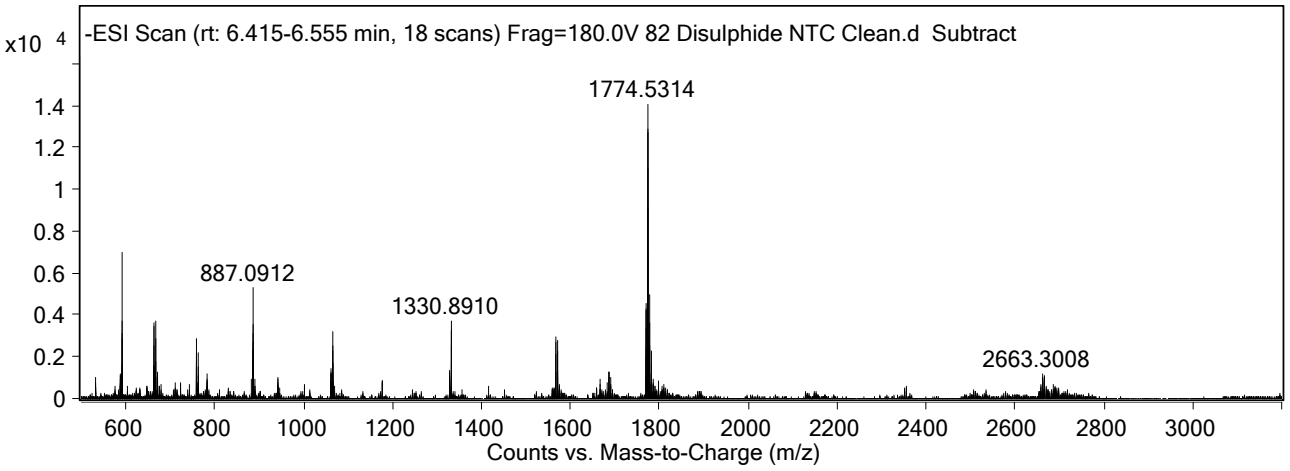


Peak List

m/z	Abund
5324.47	98254.24
5325.47	225301.81
5326.48	357042.63
5327.48	417077.63

5328.47	399168.69
5329.47	330853.03
5330.47	244794.22
5331.47	162430.95
5332.47	102584.38
5333.47	61269.73

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (2) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

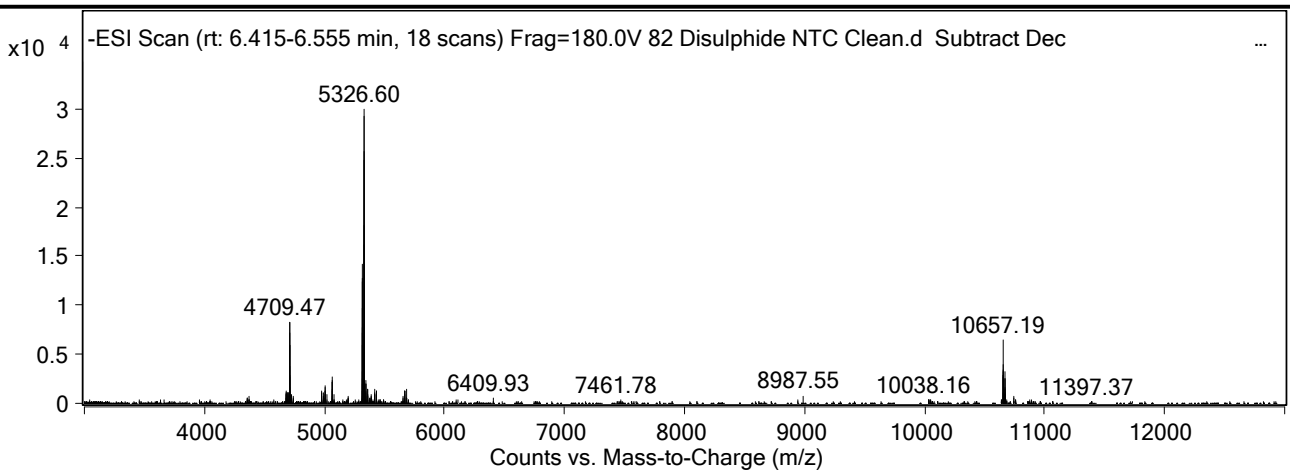


Peak List

m/z	z	Abund
591.3875	9	6993.79
887.0912	12	5333.4
1769.5328	3	4570.76
1774.1982	3	6891.25
1774.5314	3	14060.98
1774.8635	3	12853.34
1775.1966	3	12686.66
1775.5311	3	11015.55
1775.8632	3	9073.02
1776.1974	3	4958.31

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI

Qualitative Analysis Report



Peak List

m/z	**Abund**
5310.61	12827.48
5311.61	14162.83
5312.61	12375.48
5325.6	16154.1
5326.6	29993.48
5327.6	29196.41
5328.6	25679.49
5329.6	24759.14
5330.6	18159.71
5331.58	14293.8

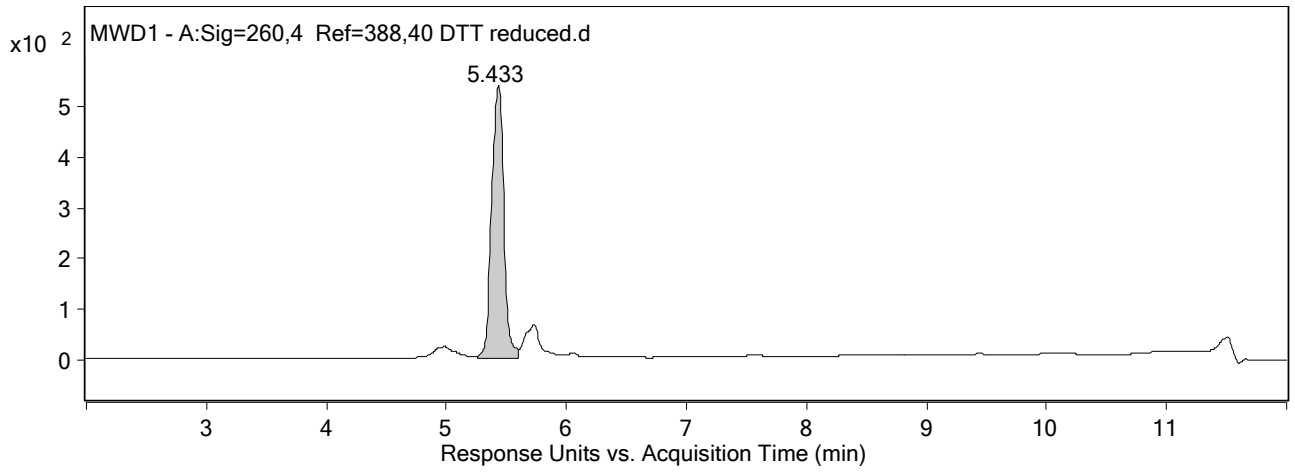
--- End Of Report ---

B.2 ASO 82- Disulphide (Reduced)

Qualitative Analysis Report

Data File	DTT reduced.d	**Sample Name**	Reduced Disulphide Oligo
Sample Type	Sample	**Position**	P2-D1
Instrument Name	Instrument 1	**User Name**	
Acq Method	NEGATIVE MODE A1B1 PORT 1-2.m	**Acquired Time**	3/2/2017 3:12:13 PM
IRM Calibration Status	Success	**DA Method**	MS_Check_Oligos.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

User Chromatograms



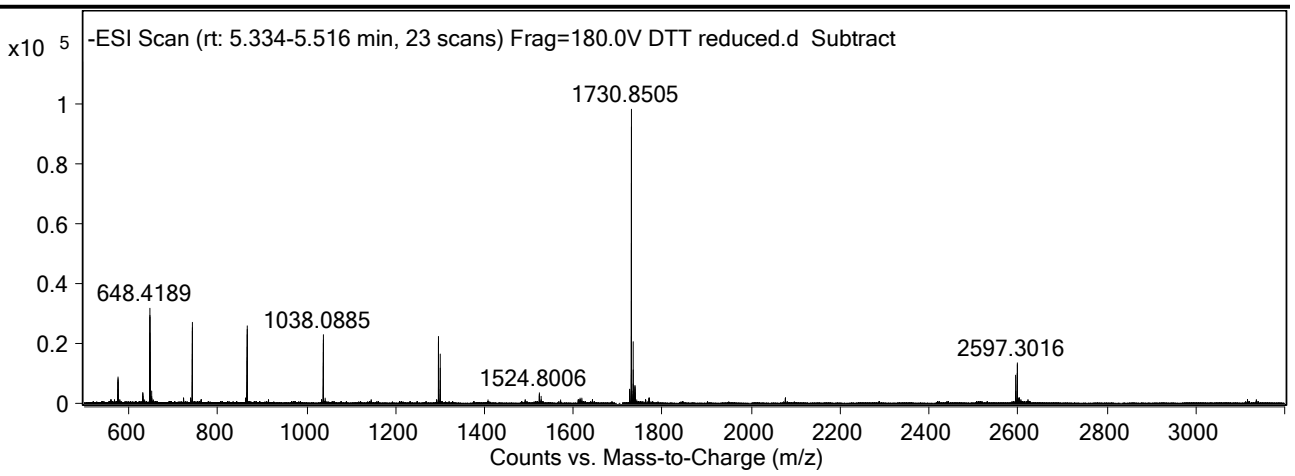
Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	5.253	5.433	5.6	539.36	3799.01	100

User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

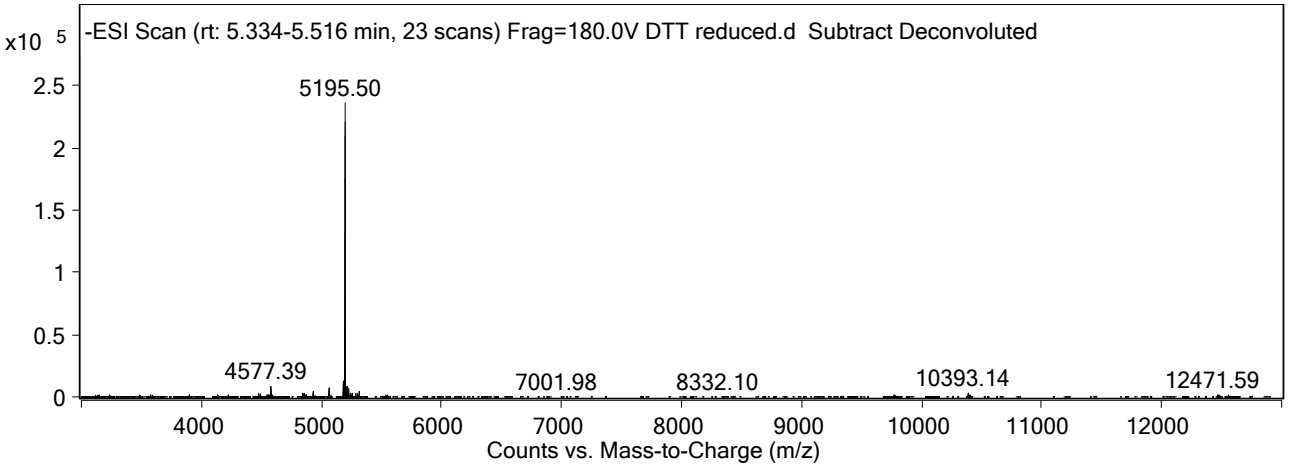
Qualitative Analysis Report



Peak List

m/z	z	Abund
648.2941	8	28580.85
648.4189	8	31503.2
648.5438	8	29347.97
1730.1838	3	53722.39
1730.5167	3	84697.78
1730.8505	3	98250.29
1731.1834	3	92323.01
1731.5165	3	73285.11
1731.8496	3	51295.3
1732.1828	3	34176.82

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund
5192.49	61162.55
5193.5	133168.08
5194.5	208796.88
5195.5	236380.75

5196.5	221288.67
5197.5	176066.91
5198.5	125961.87
5199.5	83240.47
5200.49	51963.69
5201.5	28774.34

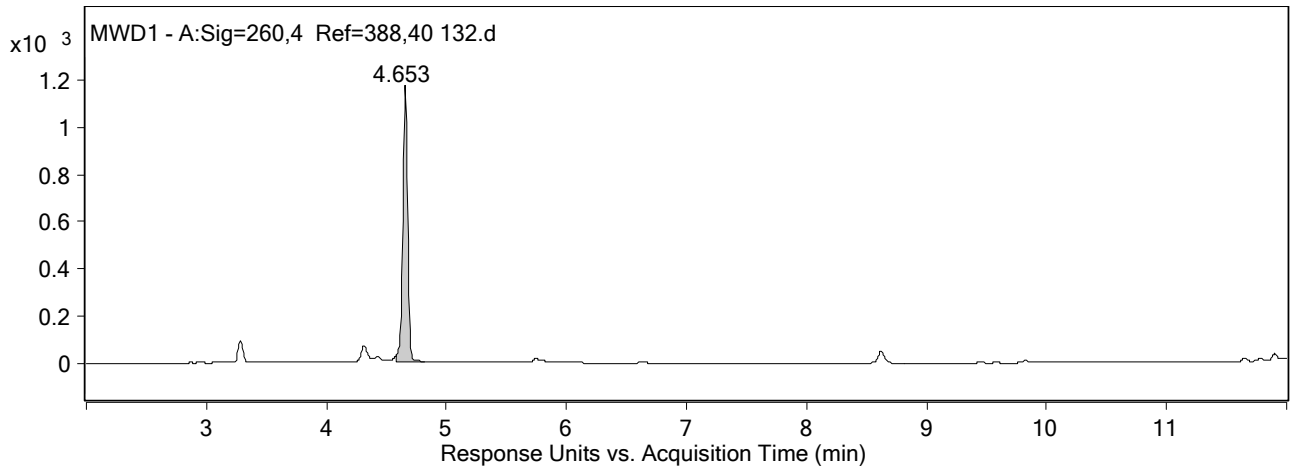
--- End Of Report ---

B.3 ASO 132- 1 PS

Qualitative Analysis Report

Data File	132.d	**Sample Name**	132
Sample Type	Sample	**Position**	P2-B1
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column.m	**Acquired Time**	8/12/2017 3:51:31 PM
IRM Calibration Status	Success	**DA Method**	MS_Check_Oligos.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

User Chromatograms



Integration Peak List

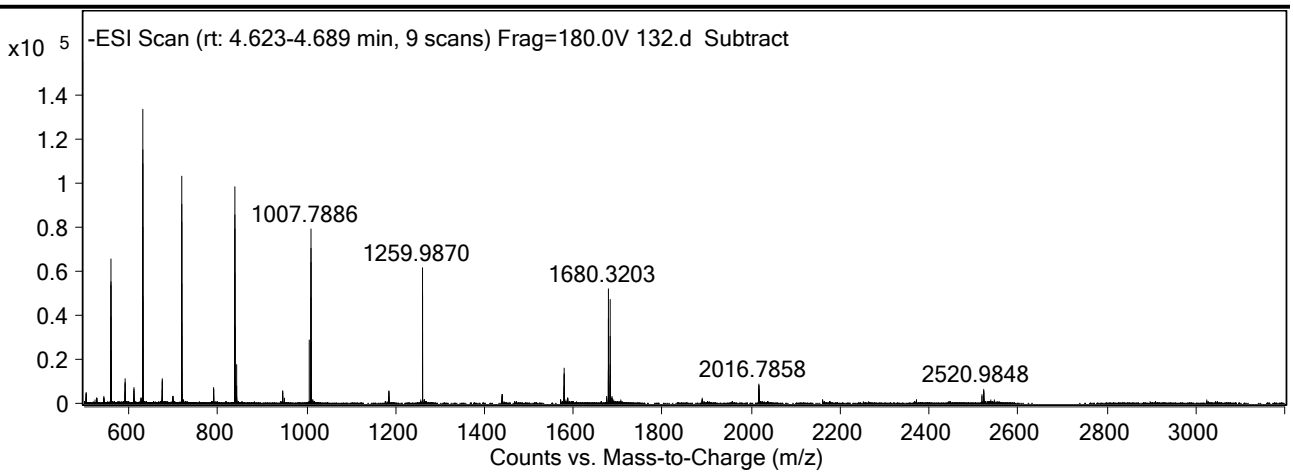
Peak	Start	RT	End	Height	Area	Area %
1	4.58	4.653	4.82	1176.51	3490.71	100

User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

Agilent Technologies

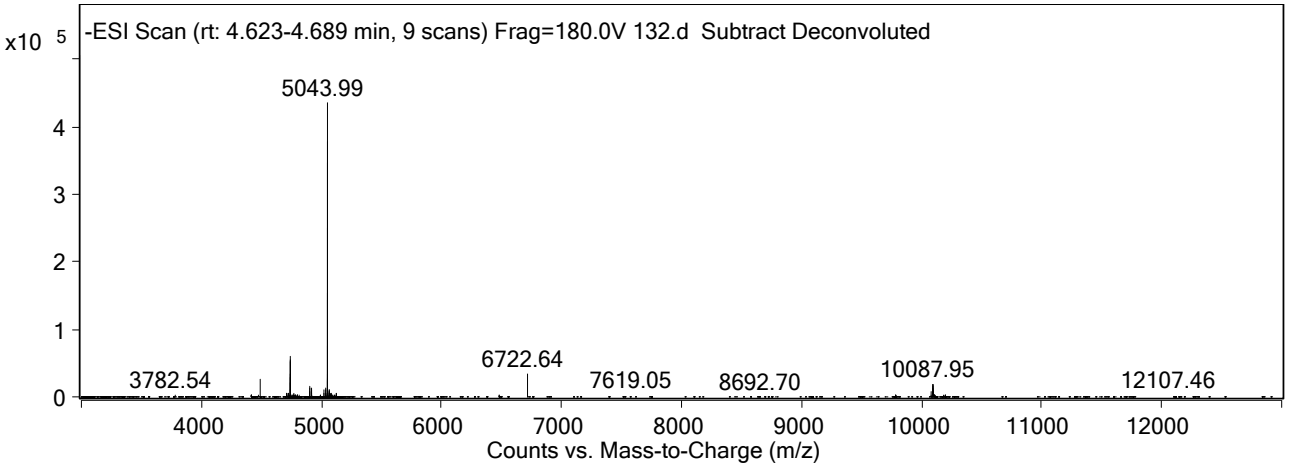
Qualitative Analysis Report



Peak List

m/z	z	Abund
629.3676	8	108762.72
629.4925	8	133740.25
629.6177	8	115008.18
629.7429	8	79878.04
719.4201	7	82183.66
719.5633	7	103214.34
719.7065	7	90075.94
839.4904	6	79448.09
839.6572	6	98747.43
839.8238	6	85748.68

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund
1681	132729.67
1681.33	164745.03
1681.66	140858.44
1682	98402.72

5041.99	153654.72
5042.99	347911.5
5043.99	435906.69
5044.99	377863.31
5045.99	249289.5
5046.99	142530.17

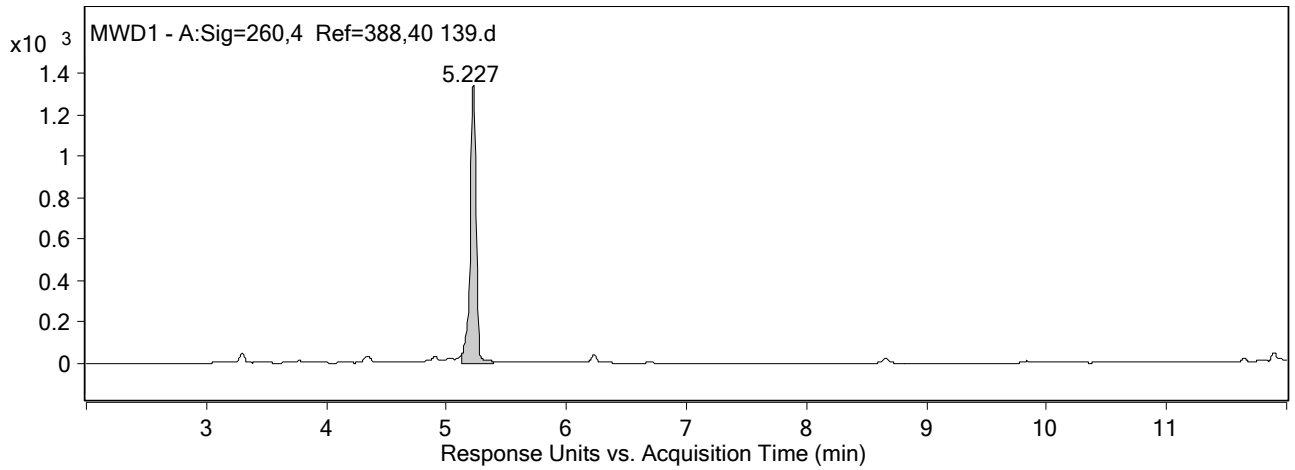
--- End Of Report ---

B.4 ASO 139- Fully PS

Qualitative Analysis Report

Data File	139.d	**Sample Name**	139
Sample Type	Sample	**Position**	P2-B8
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column.m	**Acquired Time**	8/12/2017 5:43:25 PM
IRM Calibration Status	Success	**DA Method**	MS_Check_Oligos.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

User Chromatograms



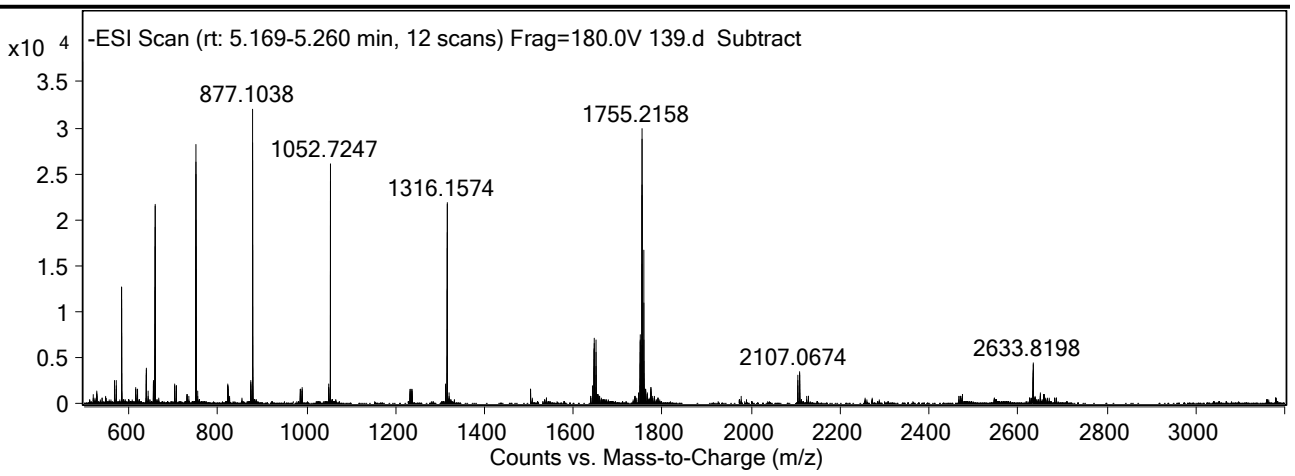
Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	5.12	5.227	5.393	1342.79	4625.82	100

User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

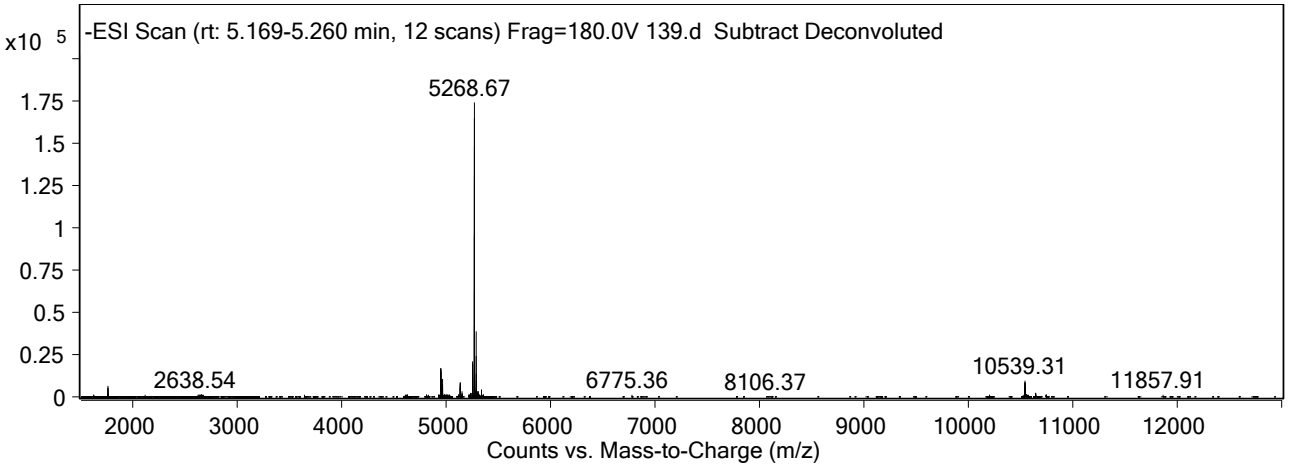
Qualitative Analysis Report



Peak List

m/z	z	Abund
751.5176	7	24901.99
751.6608	7	28145.83
751.8036	7	26311.75
876.9377	6	26454.77
877.1038	6	32050.95
877.2709	6	28312.86
1052.7247	5	26048.02
1052.9247	5	24087.27
1755.2158	3	29952.21
1755.5499	3	28683.97

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund
5265.67	41994.96
5266.67	97173.98
5267.67	147680.81
5268.67	173863.38

5269.67	164569.64
5270.67	130492.6
5271.67	95012.44
5272.66	62808.96
5273.66	38245.47
5274.65	23629.71

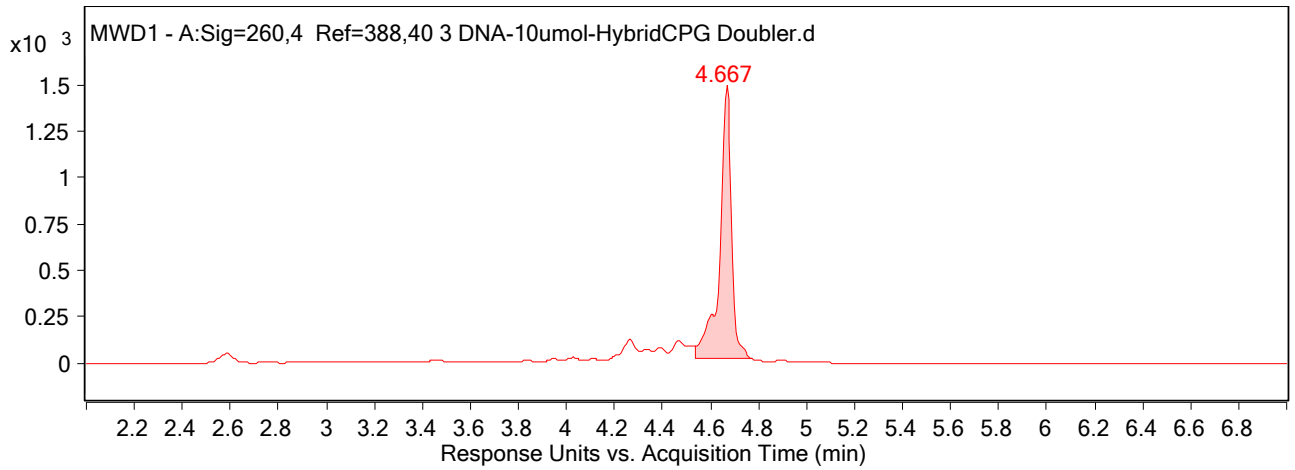
--- End Of Report ---

B.5 DNA Dimer

Qualitative Analysis Report

Data File	3 DNA-10umol-HybridCPG Doubler.d	**Sample Name**	3 DNA-10umol-HybridCPG Doubler
Sample Type	Sample	**Position**	P2-D2
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column.m	**Acquired Time**	8/10/2016 5:49:51 PM
IRM Calibration Status	Success	**DA Method**	MS_Check_Oligos.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

User Chromatograms



Integration Peak List

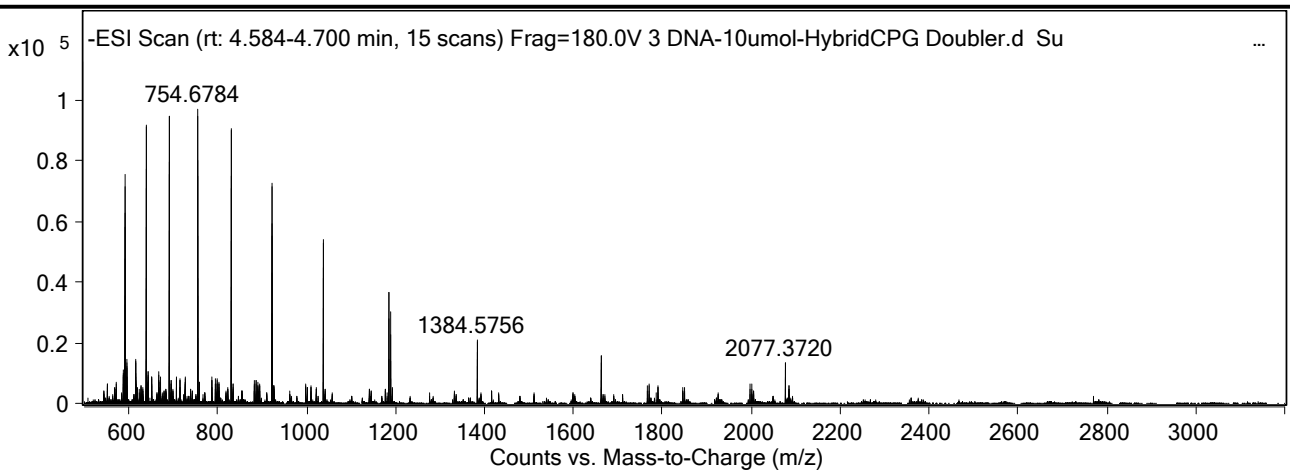
Peak	Start	RT	End	Height	Area	Area %
1	4.54	4.667	4.771	1479.26	5207.05	100

User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

Agilent Technologies

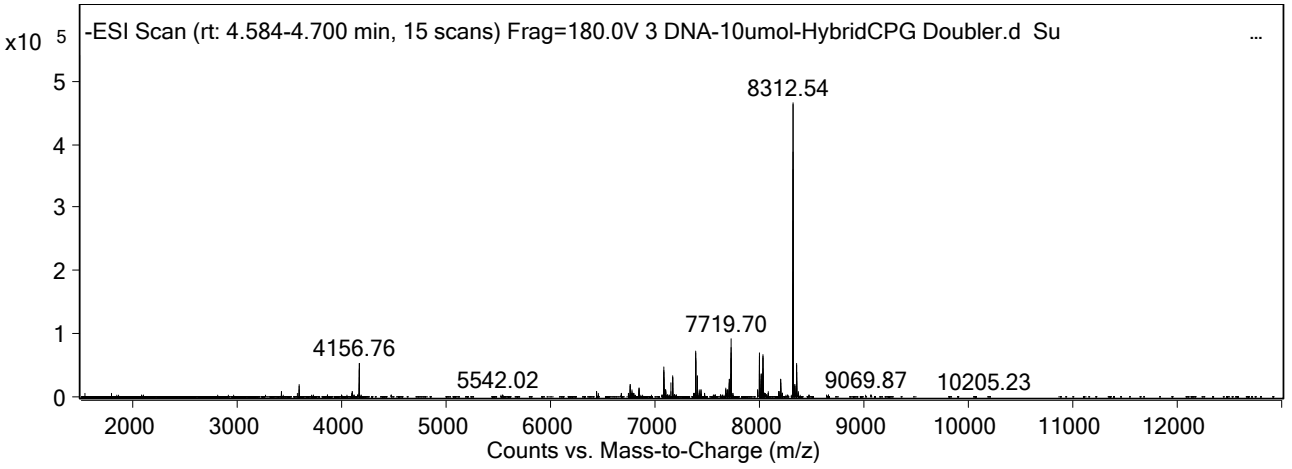
Qualitative Analysis Report



Peak List

m/z	z	Abund
638.4204	13	91822.16
638.4963	13	91220.66
691.7049	12	94875.09
691.7877	12	94607.78
691.8705	12	79799.26
754.6784	11	96978.46
754.7688	11	94347.67
754.8597	11	80190.74
830.2461	10	90444.48
830.3464	10	89672.6

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund
7719.7	92116.19
7720.65	78732.23
8310.54	198557.02
8311.54	359535.59

8312.54	466513.38
8313.53	462969.44
8314.53	389016.22
8315.52	278618.34
8316.52	178245.17
8317.51	108414.63

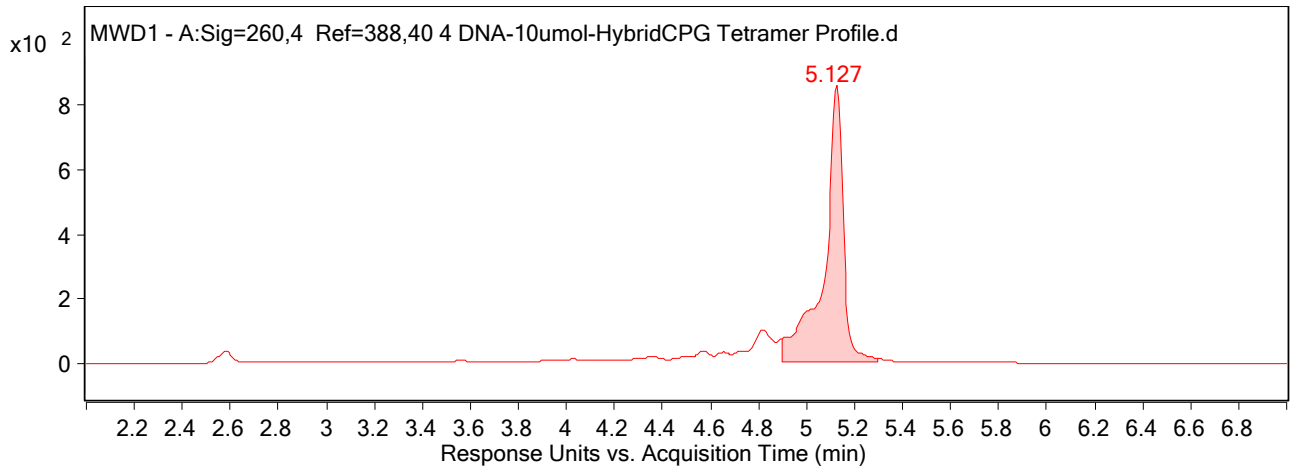
--- End Of Report ---

B.6 DNA Tetramer

Qualitative Analysis Report

Data File	4 DNA-10umol-HybridCPG Tetramer Profile.d	**Sample Name**	4 DNA-10umol-HybridCPG Tetramer Profile
Sample Type	Sample	**Position**	P2-D3
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column PROFILE.m	**Acquired Time**	8/10/2016 6:47:21 PM
IRM Calibration Status	Success	**DA Method**	MS_Check_Oligos.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

User Chromatograms



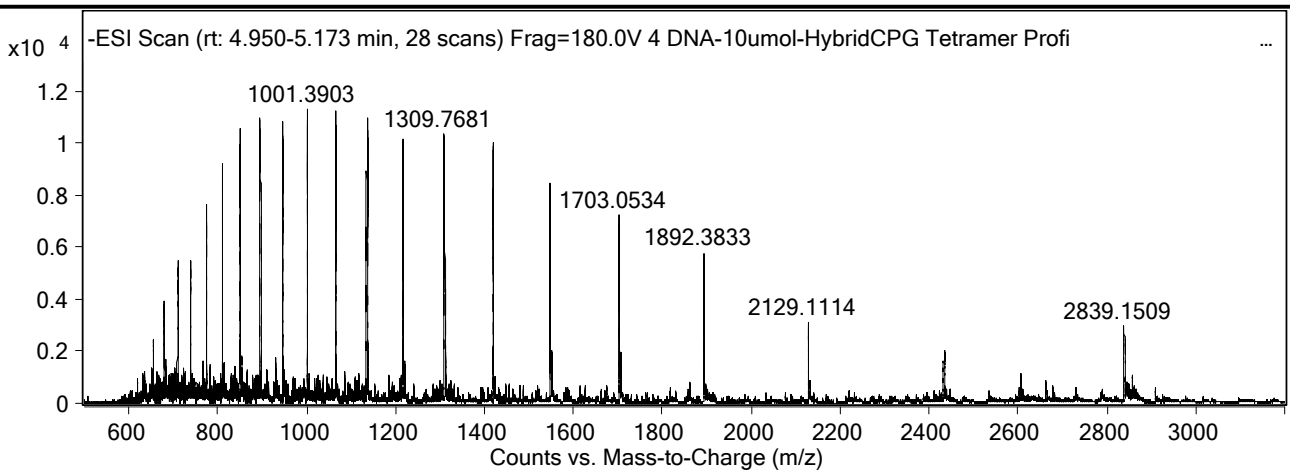
Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	4.9	5.127	5.293	855.29	4742.61	100

User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

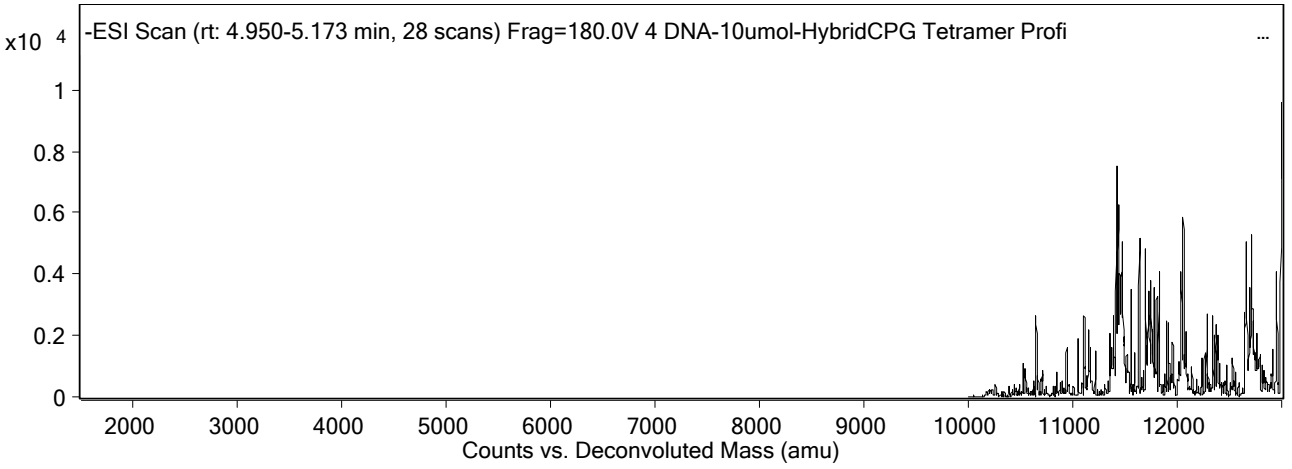
Qualitative Analysis Report



Peak List

m/z	z	Abund
851.0343		10666.44
895.8832	3	10948.68
945.7114	3	10864.63
1001.3903		11357.55
1064.028	2	11350.29
1134.9934	4	11002.42
1135.1303		10313.49
1216.1758	4	10230.79
1309.7681	7	10438.66
1309.8419		10400.21

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund	Score (DB)
14541.81	28091.82	8
16699.78	25579.82	6
16707.84	25290.72	7
16715.72	27569.51	7

Agilent Technologies

17014.4	256921.82	8
17042.59	88131.16	8
17078.12	29357.33	4
17128.96	28266.87	1
17171.9	25822.1	5
18227.74	28004.14	3

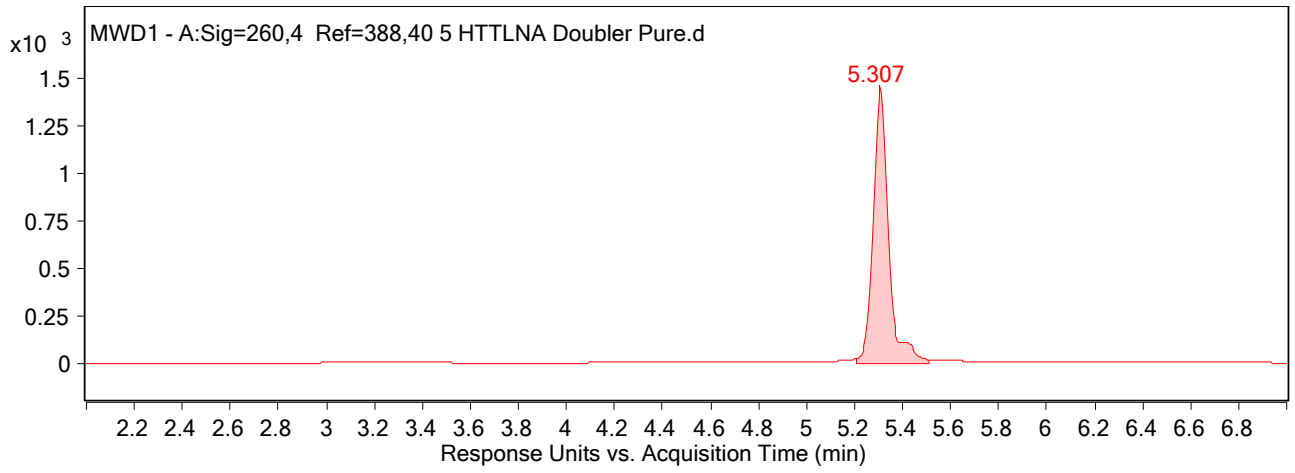
--- End Of Report ---

B.7 LNA Dimer

Qualitative Analysis Report

Data File	5 HTTLNA Doubler Pure.d	**Sample Name**	5 HTTLNA Doubler Pure
Sample Type	Sample	**Position**	P2-B5
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column.m	**Acquired Time**	8/13/2016 6:30:49 PM
IRM Calibration Status	Success	**DA Method**	MS_Check_Oligos.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

User Chromatograms



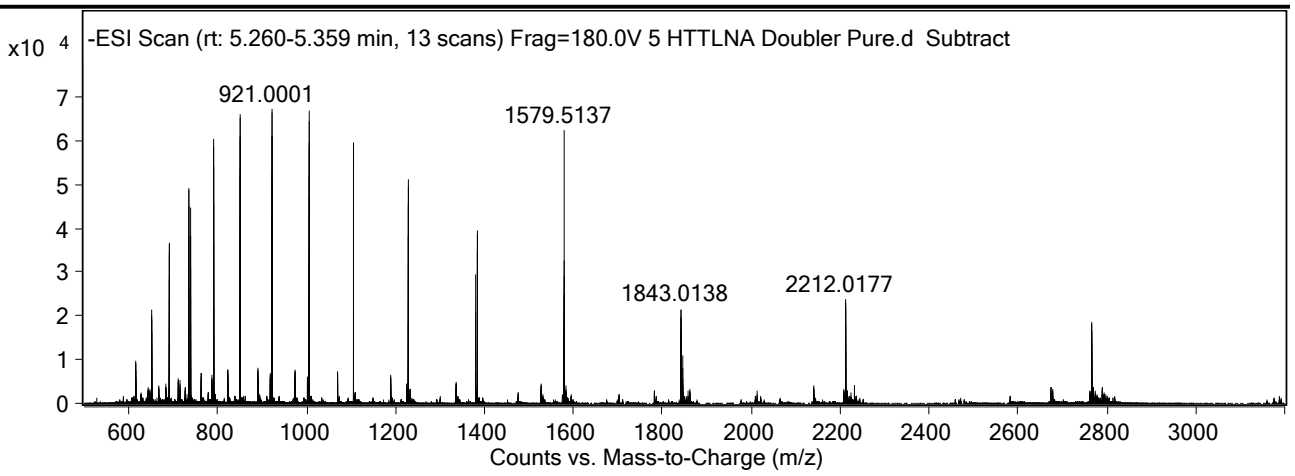
Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	5.207	5.307	5.513	1458.44	6537.44	100

User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

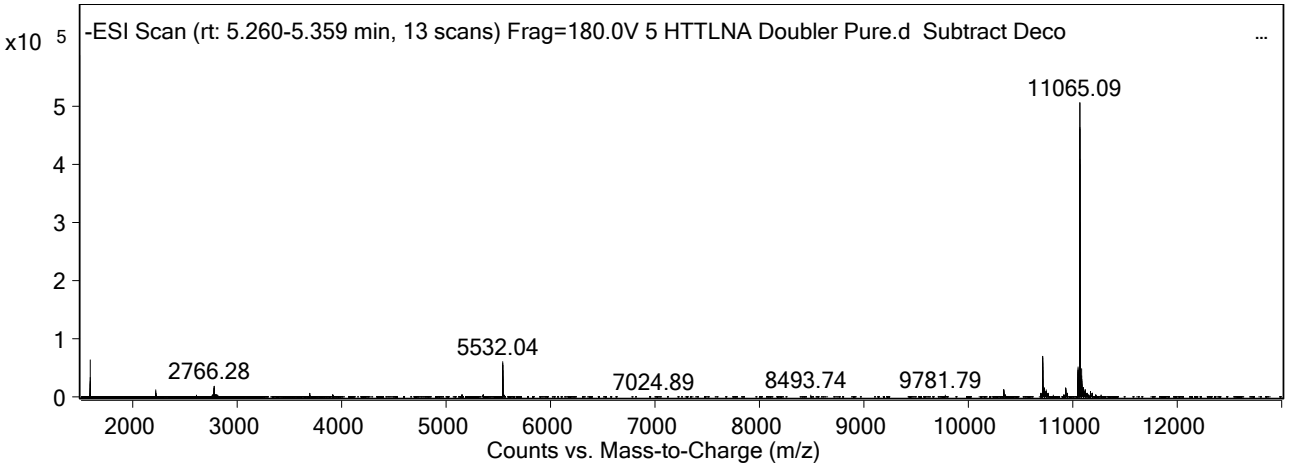
Qualitative Analysis Report



Peak List

m/z	z	Abund
849.9994	13	61131.09
850.0765	13	65237.6
850.1528	13	66210.19
920.9165	12	61280.46
921.0001	12	67295.28
921.0831	12	66318.11
921.1664	12	60819.53
1004.8179	11	64357.3
1004.9086	11	66803.1
1579.5137	5	62462.71

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund
11060.1	190237.02
11061.1	299822.53
11062.09	409152.22
11063.09	463418.31

11064.09	507449.81
11065.09	507668.13
11066.08	462953.28
11067.08	399677.5
11068.08	322960.41
11069.08	250216.48

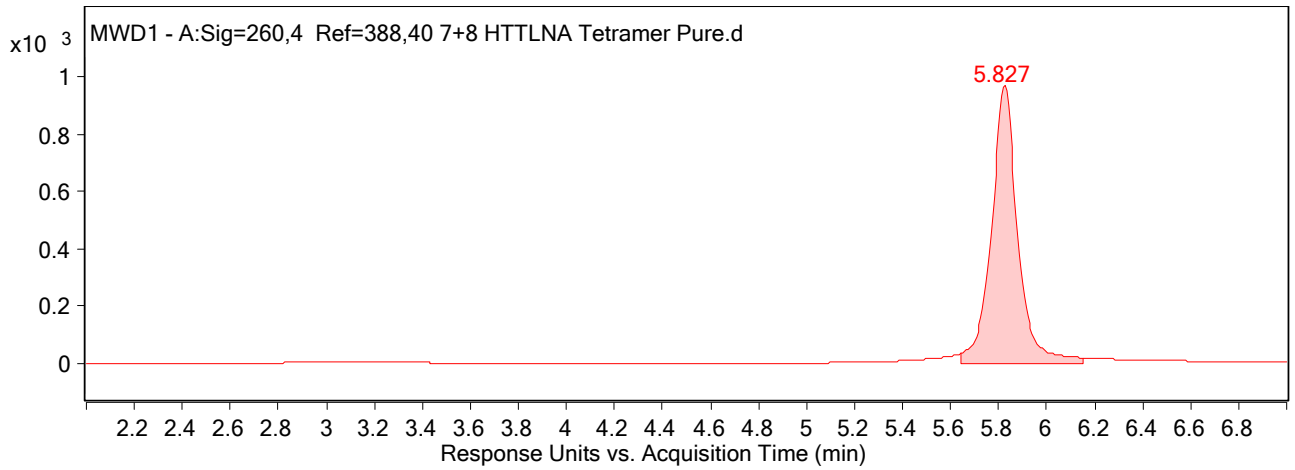
--- End Of Report ---

B.8 LNA Tetramer

Qualitative Analysis Report

Data File	7+8 HTTLNA Tetramer Pure.d	**Sample Name**	7+8 HTTLNA Tetramer Pure
Sample Type	Sample	**Position**	P2-B7
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column PROFILE.m	**Acquired Time**	8/13/2016 7:03:10 PM
IRM Calibration Status	Success	**DA Method**	MS_Check_Oligos.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

User Chromatograms



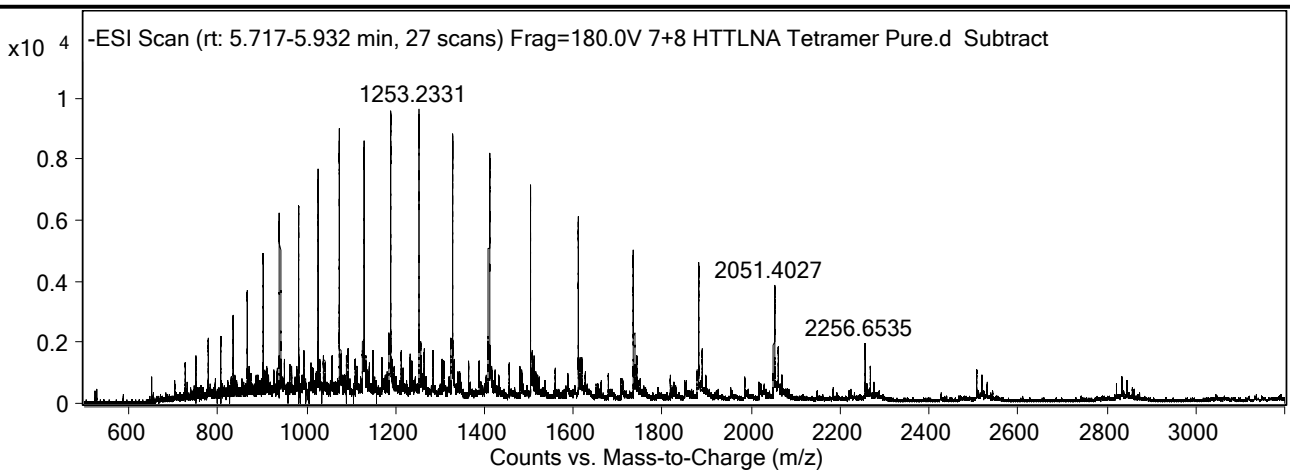
Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	5.64	5.827	6.153	971.94	7427.87	100

User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

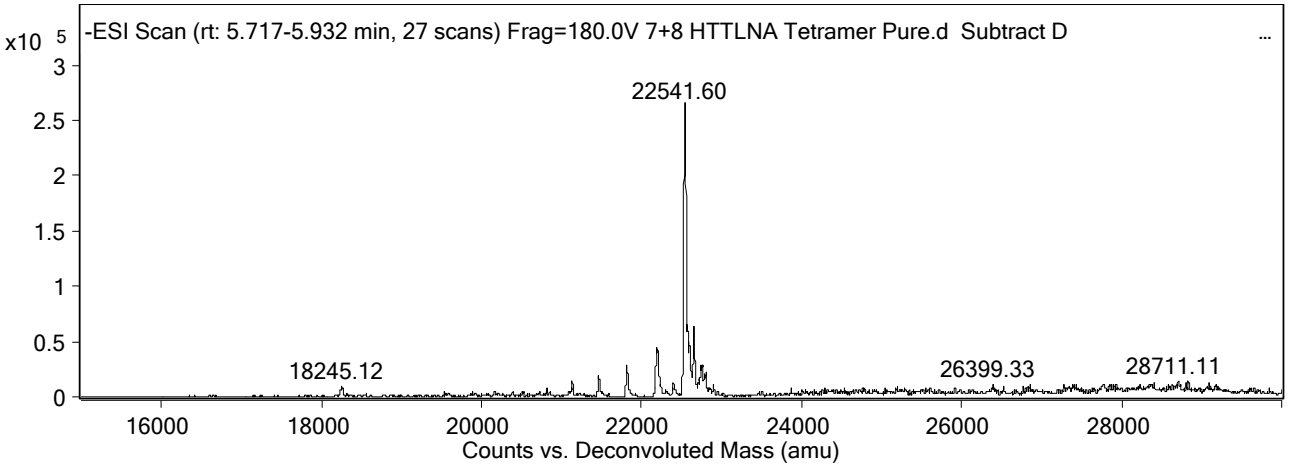
Qualitative Analysis Report



Peak List

m/z	z	Abund
400.985		8265.64
980.5654	2	6473.68
1025.1876		7703.35
1074.0641	2	8984.32
1127.8015	3	8589.06
1187.2323	2	9629.85
1253.2331	2	9643.58
1327.0177	2	8832.71
1410.0166		8207.5
1504.0603		7129.52

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund	Score (DB)
21816.89	26745.34	8
22188.57	42811.82	8
22196.92	43591.81	8
22541.6	262565.1	9

Agilent Technologies

22572.73	63642.18	7
22596.51	48626.76	6
22653.64	64404.78	6
22743.47	28507.61	5
22757.25	28324.7	5
22798.72	22155.23	4

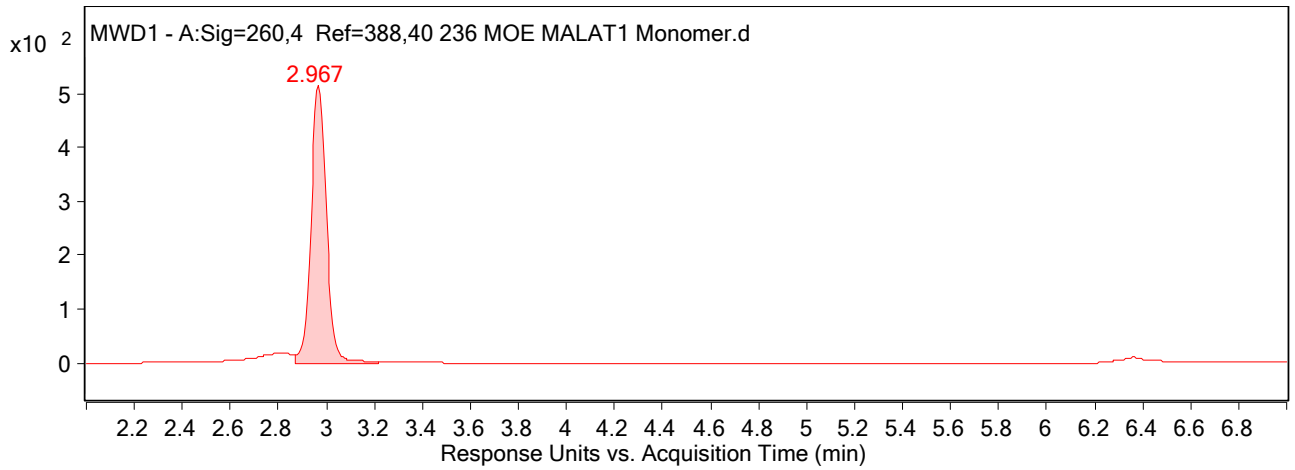
--- End Of Report ---

B.9 MOE Monomer

Qualitative Analysis Report

Data File	236 MOE MALAT1 Monomer.d	**Sample Name**	236 MALAT1 MOE Monomer
Sample Type	Sample	**Position**	P2-B1
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column.m	**Acquired Time**	9/11/2017 3:38:56 PM
IRM Calibration Status	Success	**DA Method**	MS_Check_Oligos.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

User Chromatograms



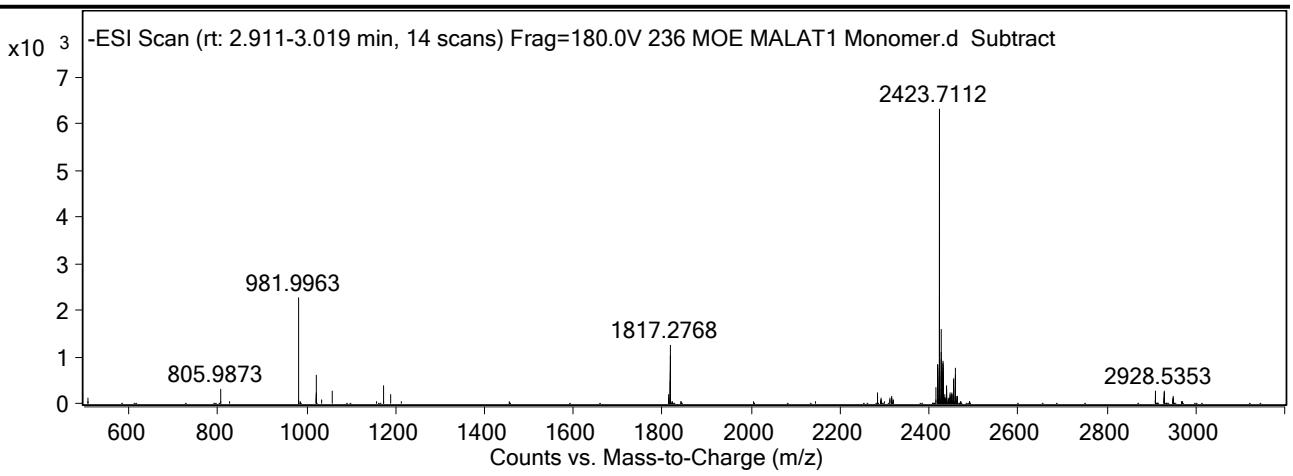
Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	2.873	2.967	3.213	515.41	2297.44	100

User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

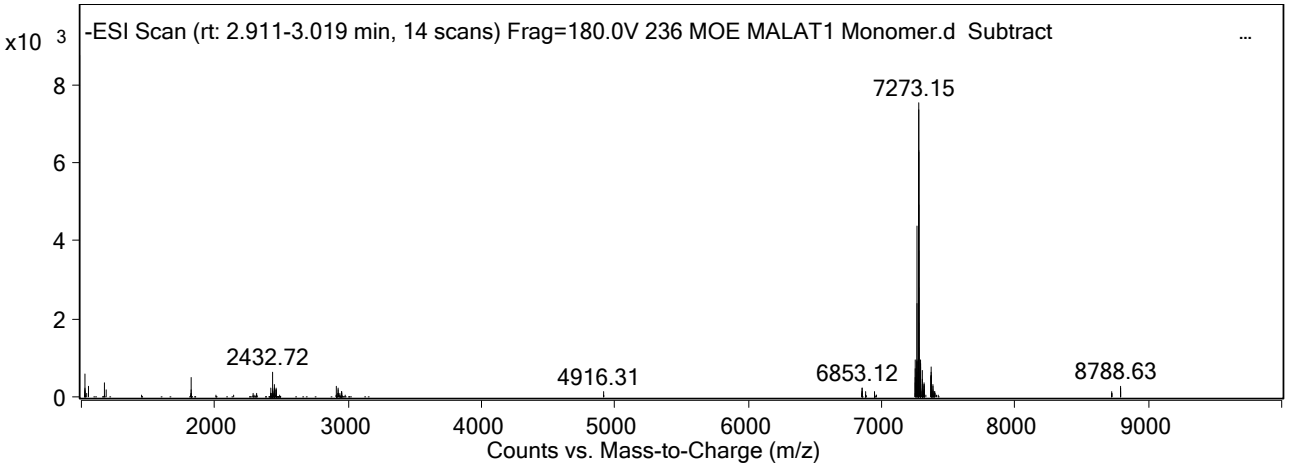
Qualitative Analysis Report



Peak List

m/z	z	Abund
981.9963		2273.21
2422.3787	3	2014.44
2422.7085	3	3759.63
2423.0418	3	5431.5
2423.3756	3	6311.19
2423.7112	3	6326.44
2424.0408	3	5665.96
2424.3735	3	4462.66
2424.7026	3	3261.88
2425.0426	3	2336.95

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund
983	2273.21
7270.15	2388.99
7271.14	4384.27
7272.15	6281.2

Agilent Technologies

7273.15	7548.3
7274.15	7353.25
7275.14	6335.38
7276.13	4927.79
7277.12	3739.45
7278.14	2796.07

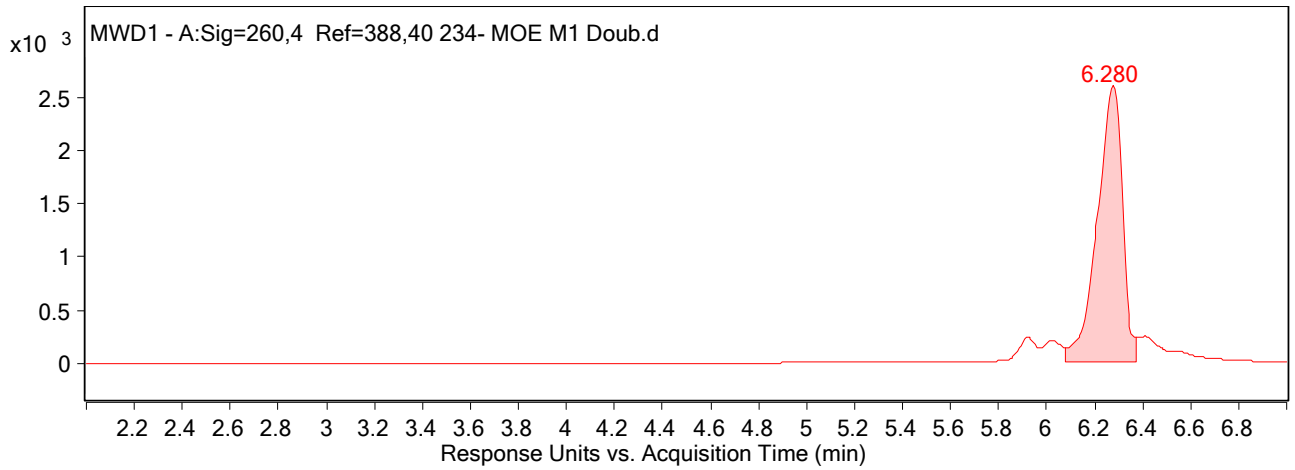
--- End Of Report ---

B.10 MOE Dimer

Qualitative Analysis Report

Data File	234- MOE M1 Doub.d	**Sample Name**	234- MOE M1 Doub
Sample Type	Sample	**Position**	P2-B1
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column PROFILE.m	**Acquired Time**	8/31/2017 10:58:55 AM
IRM Calibration Status	Success	**DA Method**	MS_Check_Oligos.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

User Chromatograms



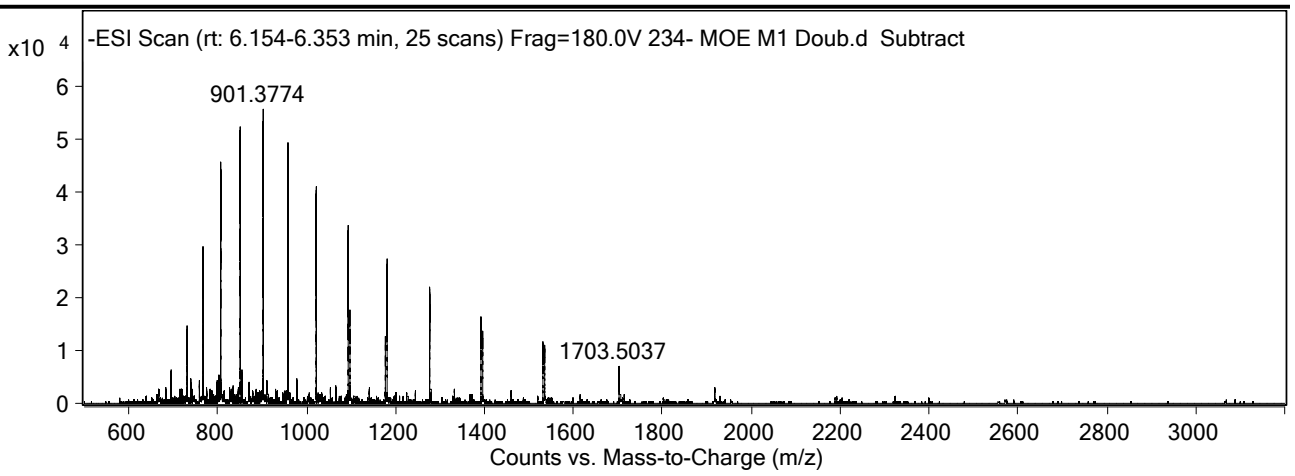
Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	0.327	0.4	0.567	774.6	2304.7	11.98
2	1.167	1.24	1.407	402.57	1253.8	6.52
3	6.08	6.28	6.373	2621.87	19230.82	100

User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (3) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

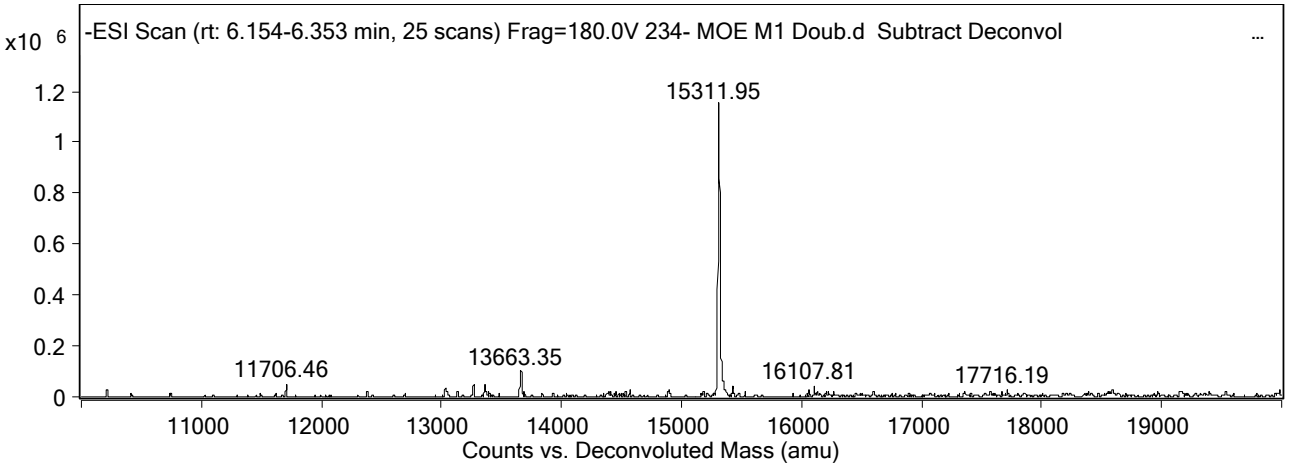
Qualitative Analysis Report



Peak List

m/z	z	Abund
806.3885		45598.08
851.2456	5	52347.7
851.4398	5	37932.37
901.3774	5	56230.46
901.586	5	41633.48
957.7509	5	49242.39
957.9356	5	42449.96
957.9991	5	36019.46
1021.6642	4	41642.74
1021.8633	4	36229.33

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund	Score (DB)
11706.46	46061.44	0
13036.92	33010.52	10
13268.32	49663.21	6
13364.55	50186.45	7

13663.35	100261.8	6
15311.95	1140701.64	6
15427.02	39521.43	8
16064.43	29393.54	6
16107.81	39069.29	8
17716.19	29784.61	0

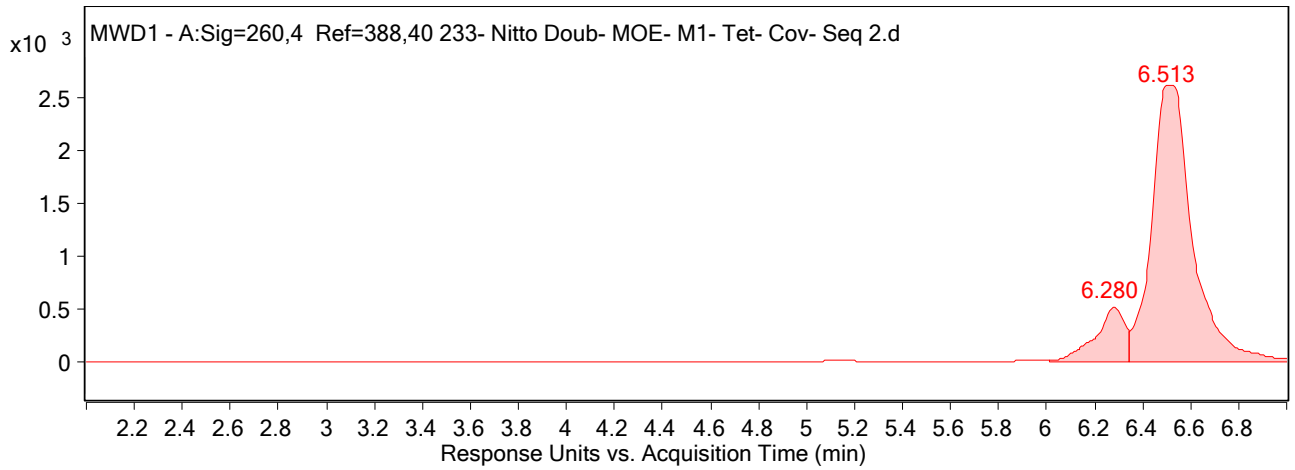
--- End Of Report ---

B.11 MOE Tetramer

Qualitative Analysis Report

Data File	233- Nitto Doub- MOE- M1- Tet- Cov- Seq 2.d	**Sample Name**	233- MOE M1 Tet Seq 2
Sample Type	Sample	**Position**	P2-B2
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column PROFILE.m	**Acquired Time**	8/29/2017 11:26:41 AM
IRM Calibration Status	Success	**DA Method**	MS_Check_Oligos.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

User Chromatograms



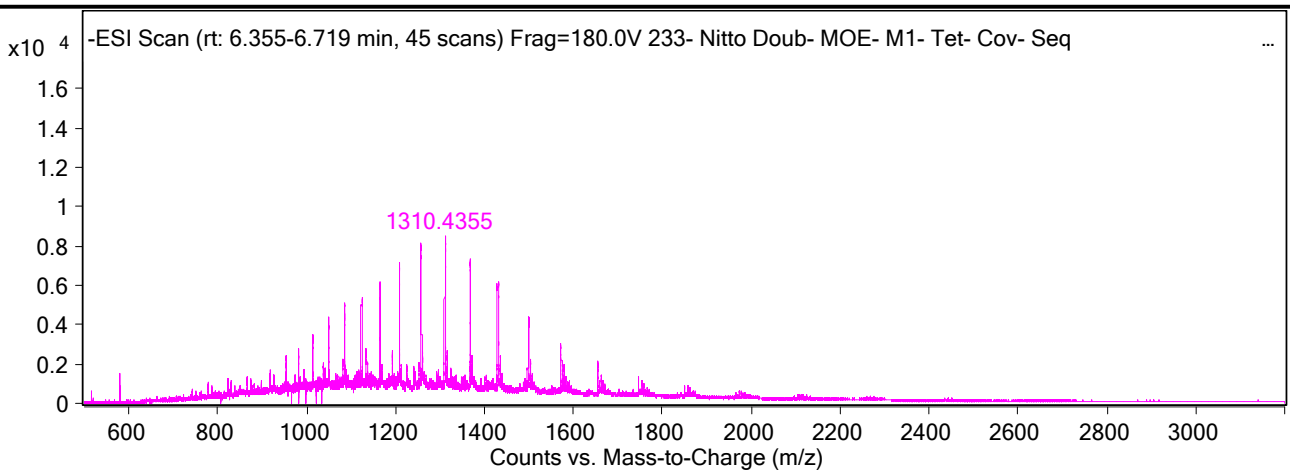
Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	0.34	0.413	0.58	588.46	1752.17	5.57
2	1.167	1.24	1.447	525.72	1756.86	5.59
3	6.013	6.28	6.347	530.88	4627.19	14.71
4	6.347	6.513	7.04	2627.19	31448.67	100

User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (4) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

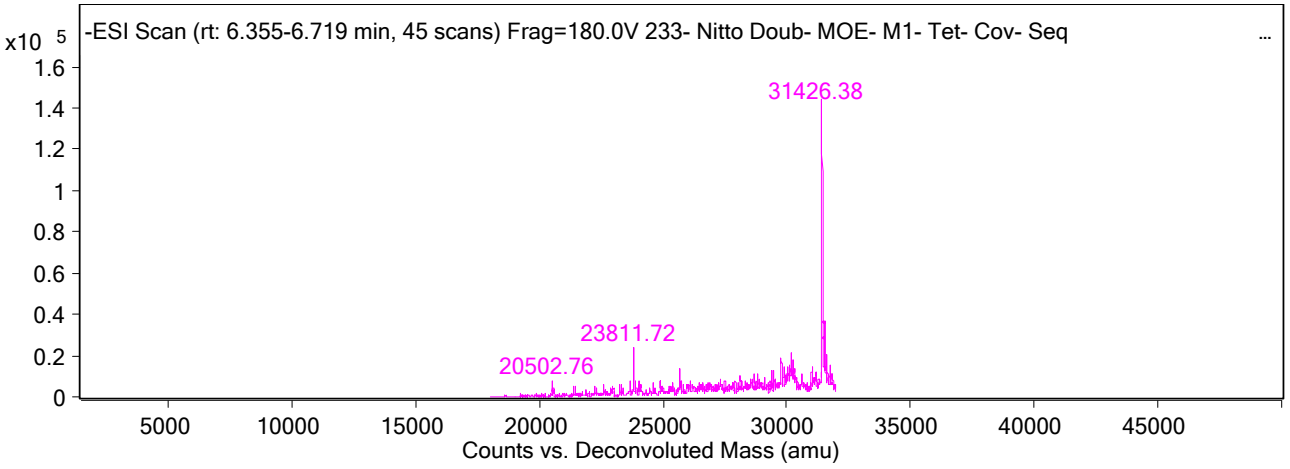
Qualitative Analysis Report



Peak List

m/z	z	Abund
1048.138		4390.63
1084.3109	2	5135.16
1123.0755		5370.18
1164.7016	3	6197.1
1209.5536	1	7137.86
1257.9813		8118.89
1310.4355	1	8527
1367.4519		7313.42
1429.6465		6220.52
1497.7793		4434.39

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund	Score (DB)
23811.72	23827.14	7
29763.98	16610.62	4
29779.59	17973.12	2
30124.42	15445.27	5

30168.91	20955.2	5
30253.5	18057.61	5
31426.38	141154.19	8
31535.06	36223.33	7
31639.54	20749.53	5
31744.94	15447.08	3

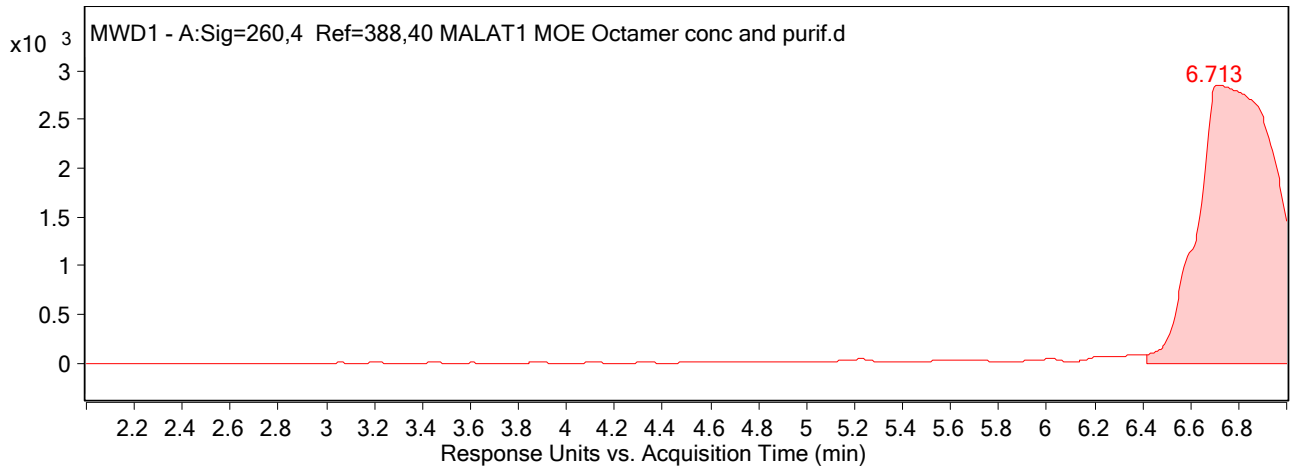
--- End Of Report ---

B.12 MOE Octamer

Qualitative Analysis Report

Data File	MALAT1 MOE Octamer conc and purif.d	**Sample Name**	MALAT1 MOE Octamer conc and purif
Sample Type	Sample	**Position**	p2b3
Instrument Name	Instrument 1	**User Name**	agilent-HP\agilent
Acq Method	Agilent AdvanceBio Column PROFILE.m	**Acquired Time**	9/1/2017 1:46:11 PM
IRM Calibration Status	Success	**DA Method**	MS_Check_Oligos.m
Comment	MALAT1 MOE Octamer conc and purif		
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

User Chromatograms



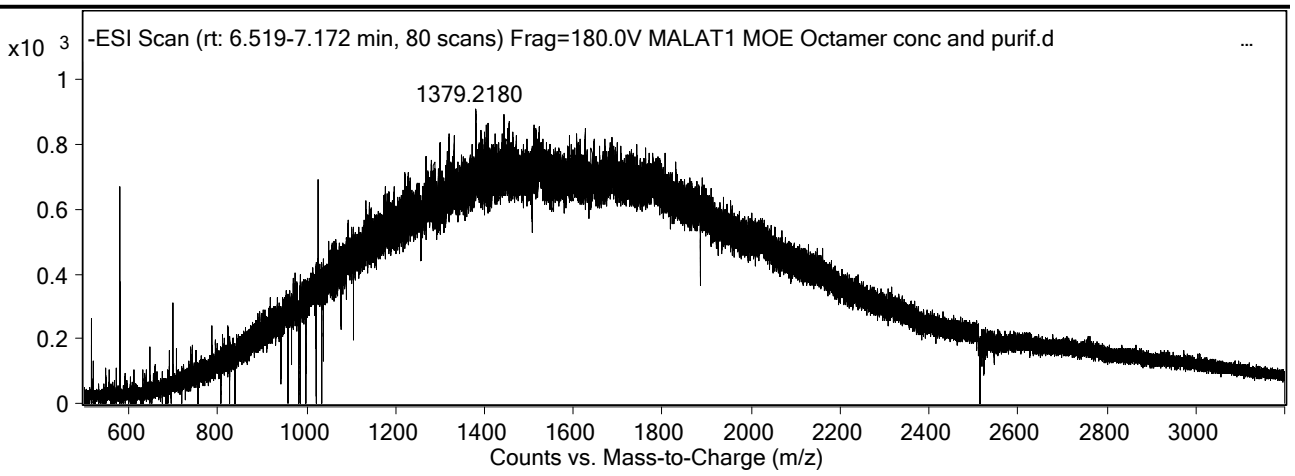
Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	1.167	1.24	1.487	824.69	3468.69	4.56
2	6.42	6.713	8.48	2852.79	76037.85	100

User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (2) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

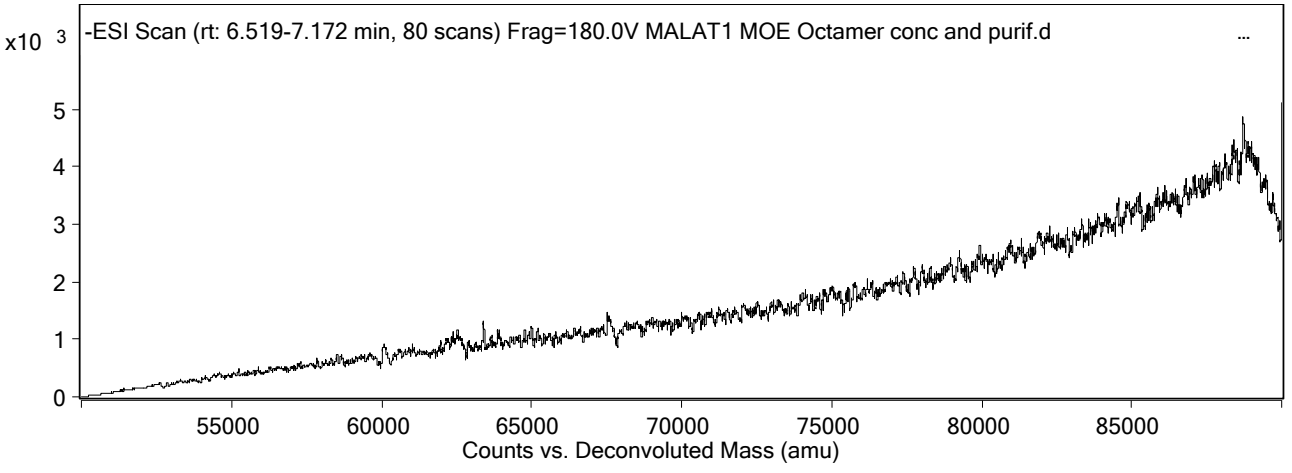
Qualitative Analysis Report



Peak List

m/z	z	Abund
1379.218		914.02
1379.496	1	855.43
1403.6777		862.14
1409.4292		871.55
1442.0412		895.25
1454.2394	1	874.77
1511.3944		862.23
1522.6775	1	859.2
1523.2437	3	858.16
1625.5521		851.59

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund	Score (DB)
88354.79	4372.39	10
88407.06	4482.45	10
88721	4857.39	10
88761.55	4520.77	10

88785.67	4458.92	10
88875.75	4428.69	10
88955.39	4366.8	10
88974.11	4349.47	10
88995.76	4449.65	10
89993.7	4644.06	8

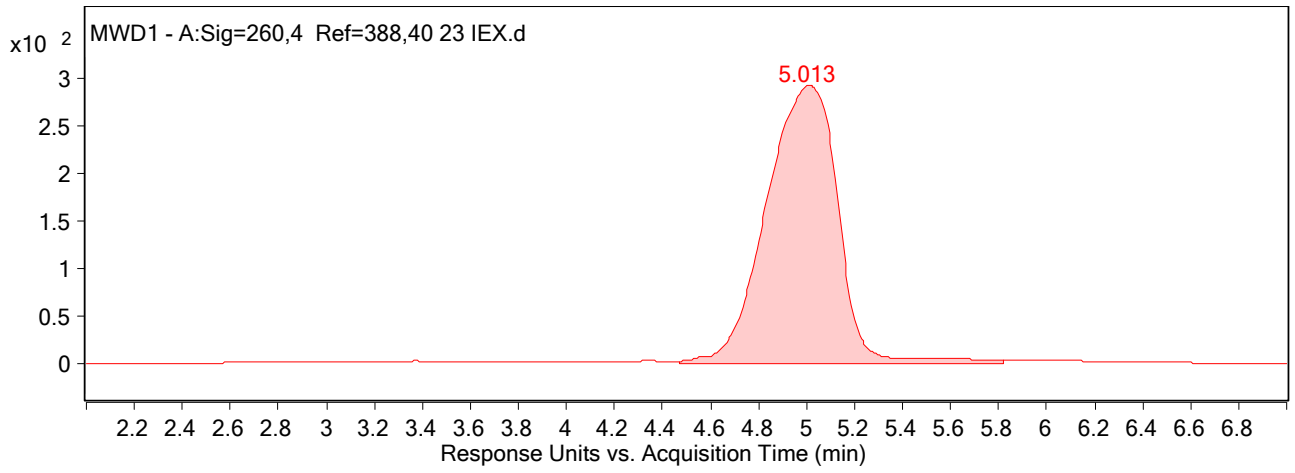
--- End Of Report ---

B.13 ASO 23

Qualitative Analysis Report

Data File	23 IEX.d	**Sample Name**	23 iex
Sample Type	Sample	**Position**	P2-F4
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column.m	**Acquired Time**	9/11/2016 3:27:55 PM
IRM Calibration Status	Success	**DA Method**	NEGATIVE MODE A1B1 PORT 1-2.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

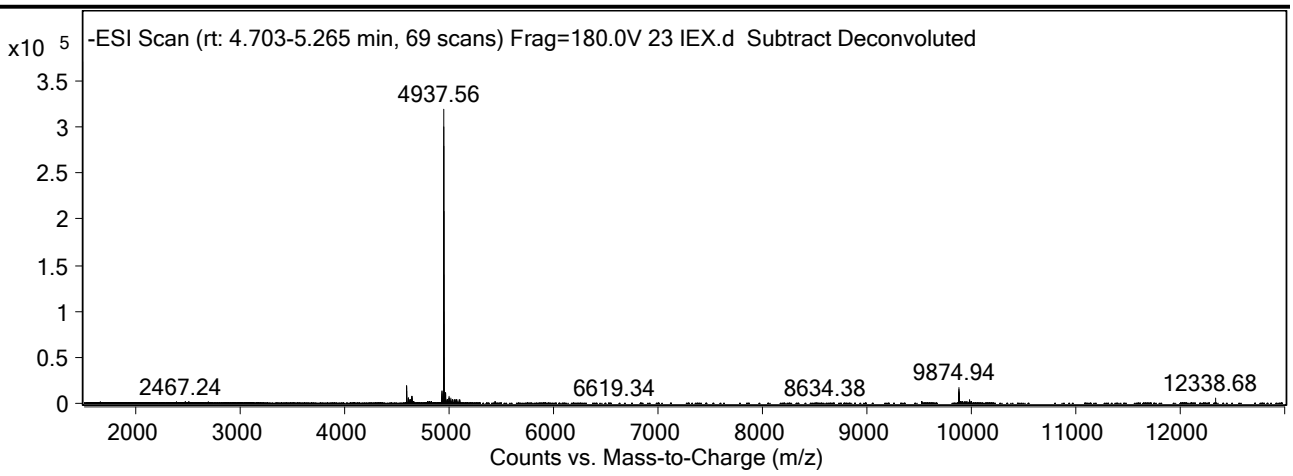
User Chromatograms



User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI

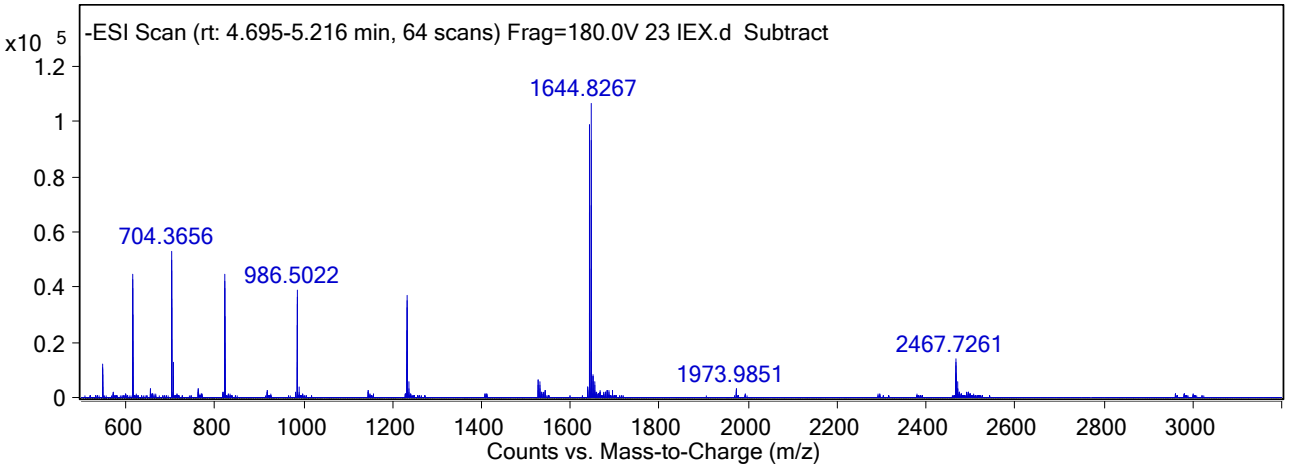
Qualitative Analysis Report



Peak List

m/z	Abund
4934.56	100360.95
4935.56	208951.89
4936.56	300360.25
4937.56	319829.31
4938.56	279325.16
4939.56	208864.09
4940.56	139838.09
4941.55	85919.63
4942.55	50497.02
4943.55	27993.74

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI



Peak List

m/z	z	Abund
704.2227	7	50029.7
704.3656	7	53237.71

704.5083	7	45713.44
821.9226	6	44957.66
1644.1601	3	66745.66
1644.4936	3	99051.18
1644.8267	3	106576.27
1645.1597	3	92973.95
1645.4929	3	69825.66
1645.8256	3	46600.67

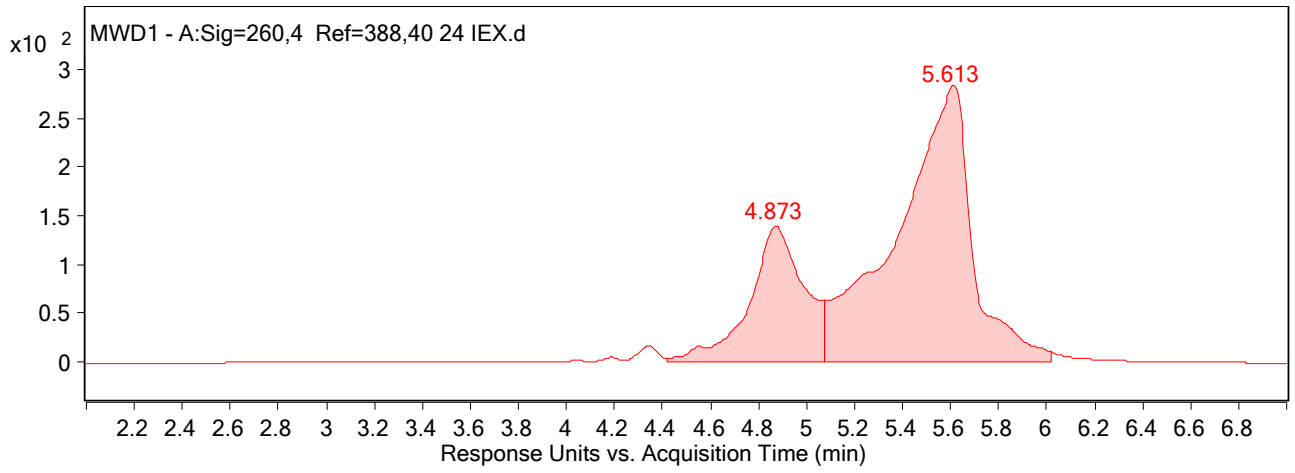
--- End Of Report ---

B.14 ASO 24

Qualitative Analysis Report

Data File	24 IEX.d	**Sample Name**	24 iex
Sample Type	Sample	**Position**	P2-F5
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column.m	**Acquired Time**	9/11/2016 3:43:58 PM
IRM Calibration Status	Success	**DA Method**	NEGATIVE MODE A1B1 PORT 1-2.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

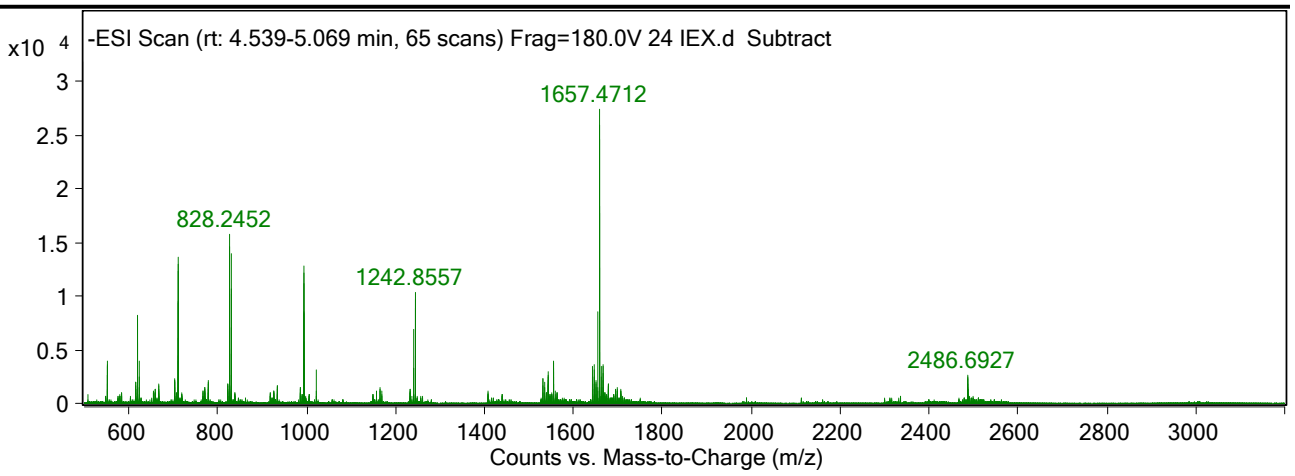
User Chromatograms



User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

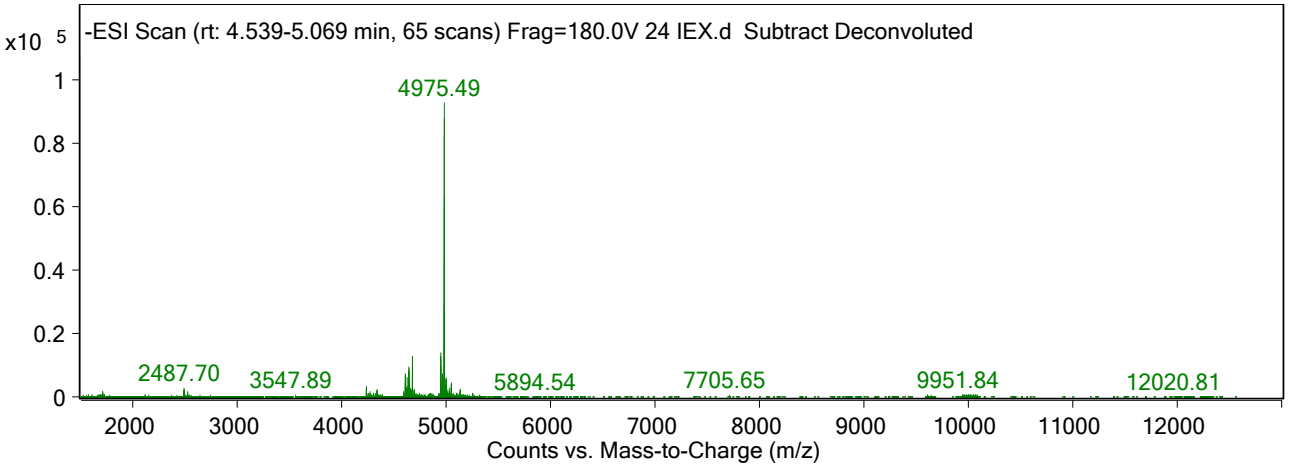
Qualitative Analysis Report



Peak List

m/z	z	Abund
709.6419	7	12923.09
709.7849	7	13593.55
828.0782	6	14614.56
828.2452	6	15721.9
828.4118	6	13921.6
1656.8041	3	17792.95
1657.1377	3	25941.03
1657.4712	3	27456.43
1657.8043	3	24681.97
1658.1369	3	18452.11

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI

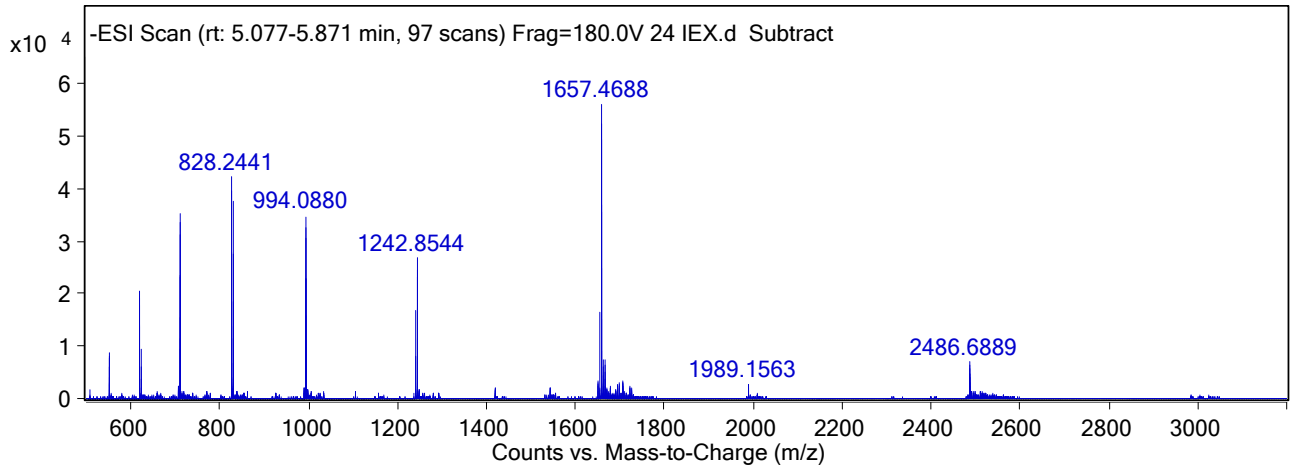


Peak List

m/z	Abund
4937.53	13677.05
4972.49	29528.09
4973.49	59035.06
4974.49	87404.84

4975.49	92700.01
4976.49	82284.13
4977.49	62622.62
4978.49	42120.09
4979.49	26711.77
4980.49	15911.89

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (2) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI



Peak List

m/z	z	Abund
709.784	7	35344.13
828.0773	6	39440.28
828.2441	6	42419.32
828.4104	6	37449.5
994.088	5	34737.64
1656.8019	3	35433.37
1657.1354	3	52458.21
1657.4688	3	56075.6
1657.8019	3	49461.24
1658.1348	3	36952.18

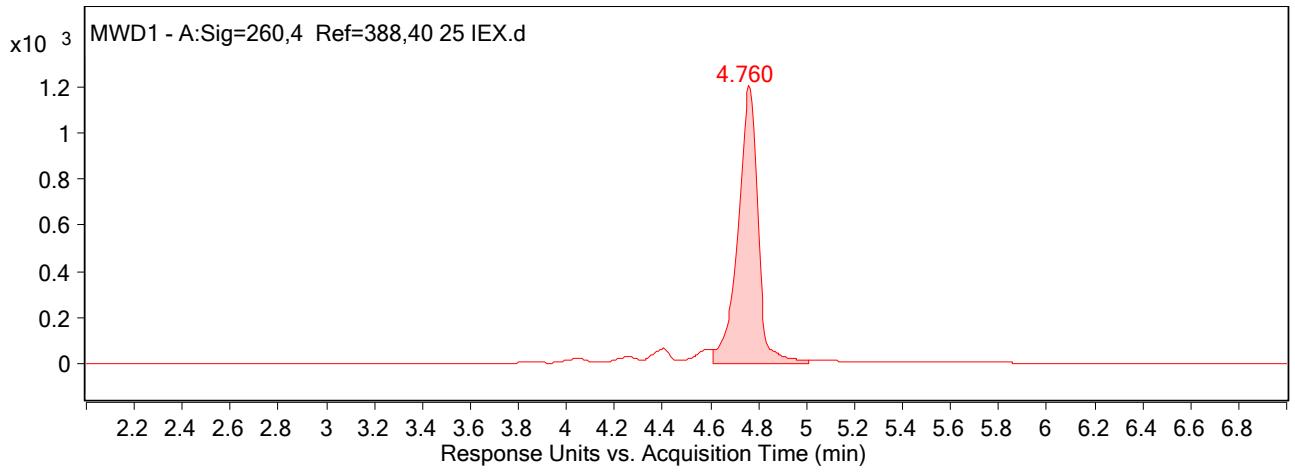
Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI

B.15 ASO 25

Qualitative Analysis Report

Data File	25 IEX.d	**Sample Name**	25 iex
Sample Type	Sample	**Position**	P2-F6
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column.m	**Acquired Time**	9/11/2016 4:00:01 PM
IRM Calibration Status	Success	**DA Method**	NEGATIVE MODE A1B1 PORT 1-2.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

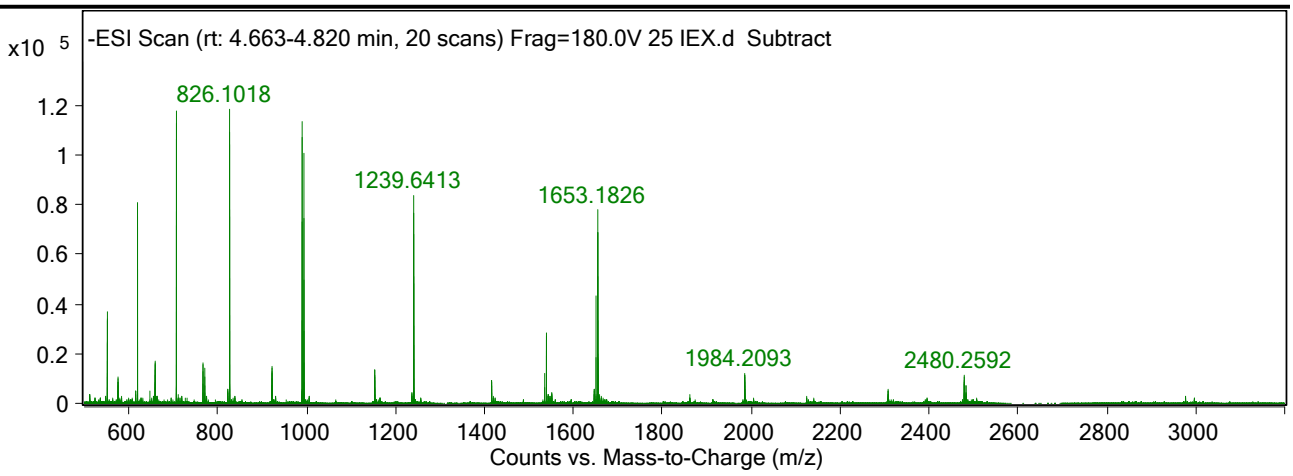
User Chromatograms



User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

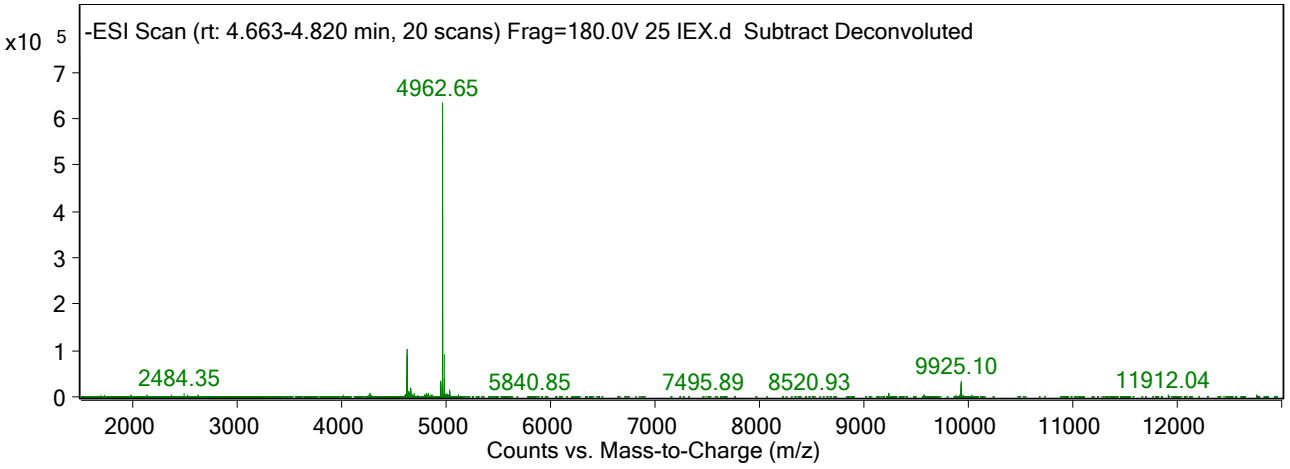
Qualitative Analysis Report



Peak List

m/z	z	Abund
707.8045	7	112750.52
707.9478	7	117516.98
708.0903	7	106369.54
825.9351	6	109173.9
826.1018	6	118372.06
826.2681	6	103868.72
991.317	5	106649.8
991.5168	5	113367.98
991.7169	5	100440.8
1239.6413	4	83529.86

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund
4615.6	101050.05
4616.6	103085.04
4959.65	187729.56
4960.65	403084.97

4961.65	590240.94
4962.65	635580.06
4963.65	558644.81
4964.65	406075.13
4965.64	266693.5
4966.64	160230.84

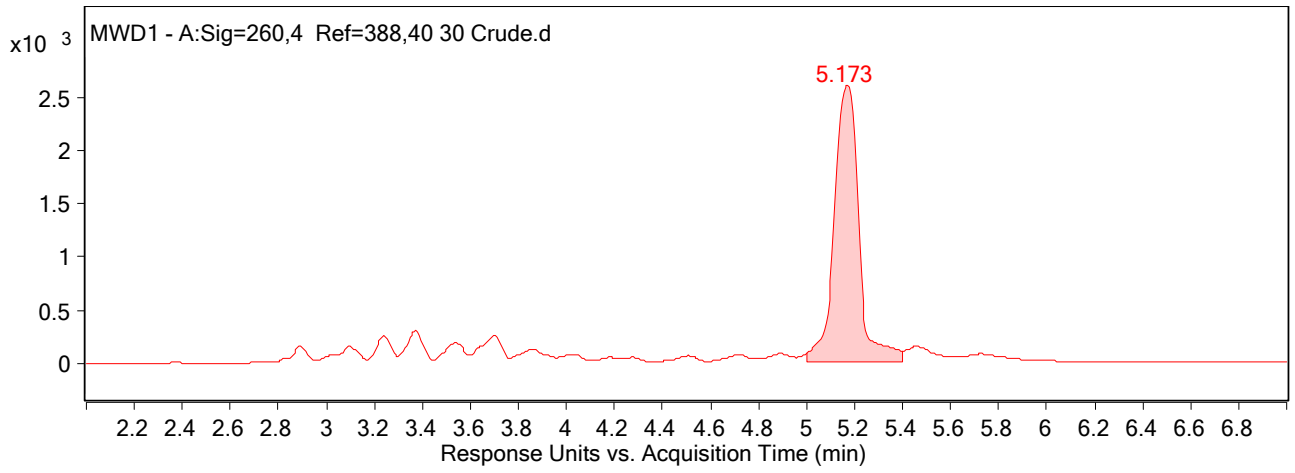
--- End Of Report ---

B.16 ASO 30

Qualitative Analysis Report

Data File	30 Crude.d	**Sample Name**	30 MOE
Sample Type	Sample	**Position**	P2-D2
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column.m	**Acquired Time**	9/28/2016 2:06:40 PM
IRM Calibration Status	Success	**DA Method**	NEGATIVE MODE A1B1 PORT 1-2.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

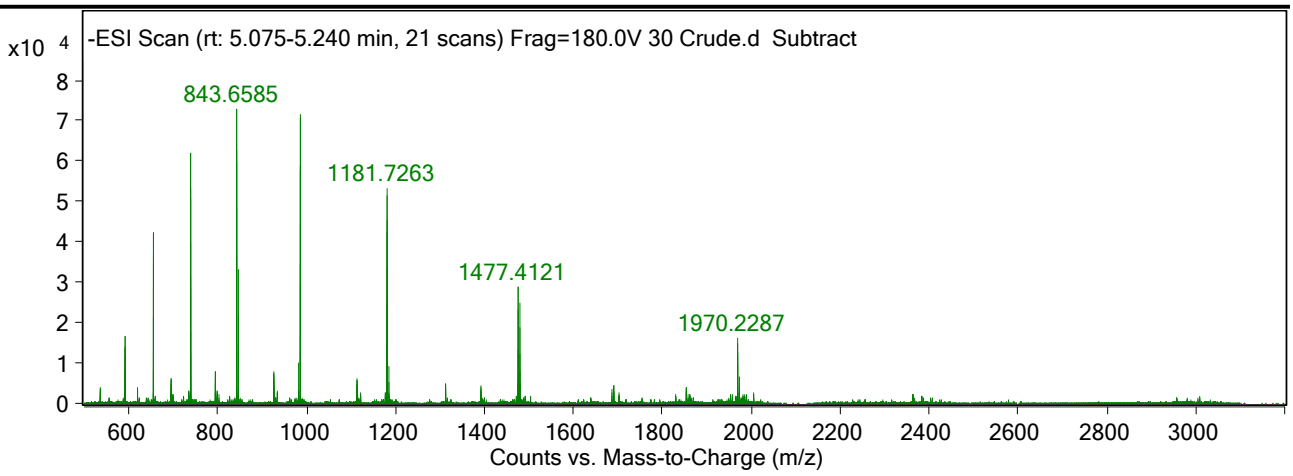
User Chromatograms



User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

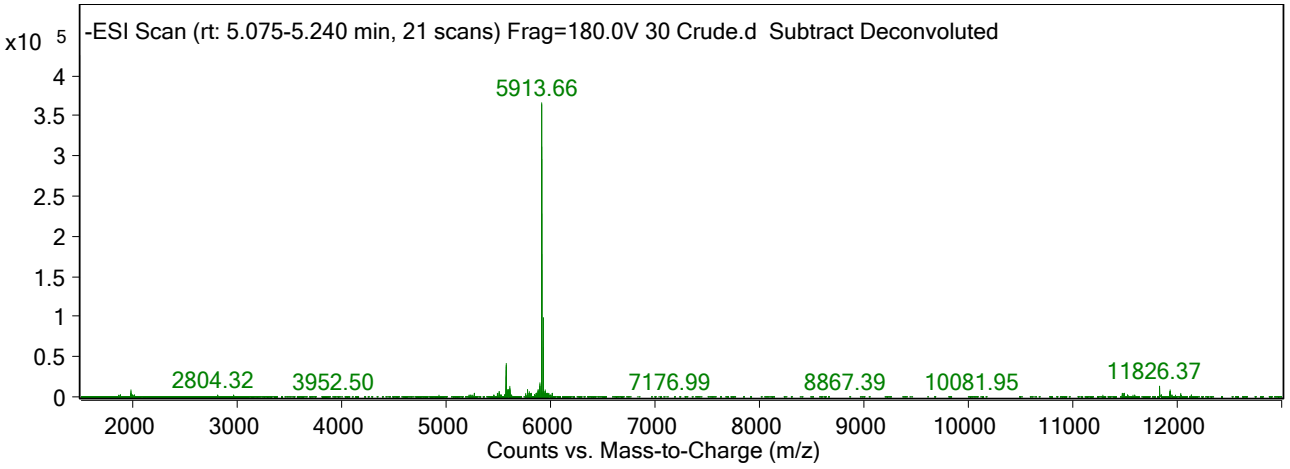
Qualitative Analysis Report



Peak List

m/z	z	Abund
738.0744	8	61894.88
738.199	8	62107.89
843.5156	7	59734.76
843.6585	7	72892.77
843.801	7	72849.21
843.9434	7	62814.93
984.2697	6	57631.79
984.4366	6	71564.68
984.6036	6	70715.67
984.7698	6	58913.63

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund
5909.67	70486.8
5910.66	178739.52
5911.66	295988.5
5912.66	363489.91

Agilent Technologies

5913.66	366473.94
5914.66	312231.94
5915.66	227813.91
5916.65	157124.69
5917.65	98653.34
5918.65	60497.9

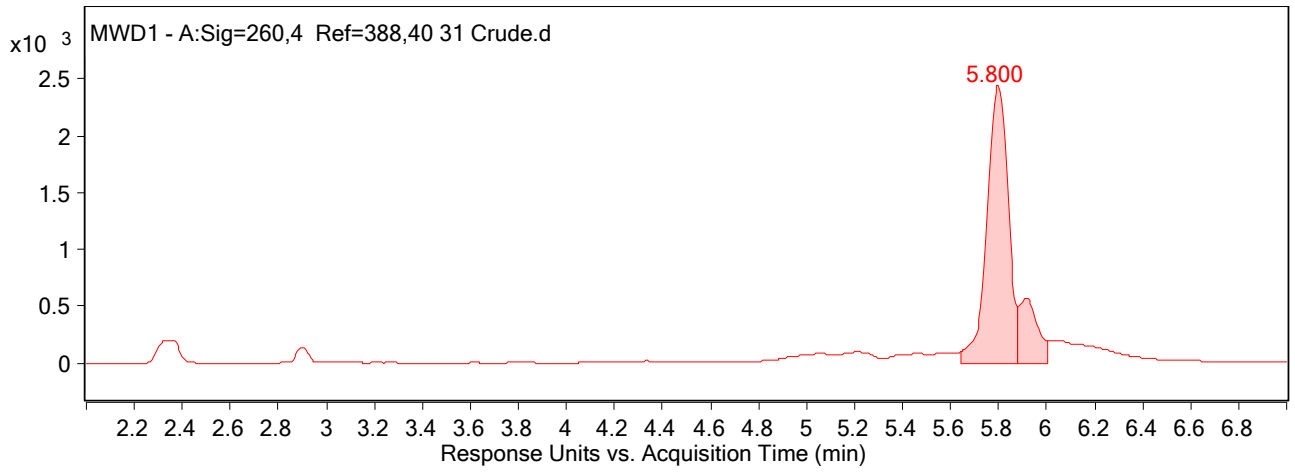
--- End Of Report ---

B.17 ASO 31

Qualitative Analysis Report

Data File	31 Crude.d	**Sample Name**	31 MOE
Sample Type	Sample	**Position**	P2-D3
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column.m	**Acquired Time**	9/28/2016 2:22:42 PM
IRM Calibration Status	Success	**DA Method**	NEGATIVE MODE A1B1 PORT 1-2.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

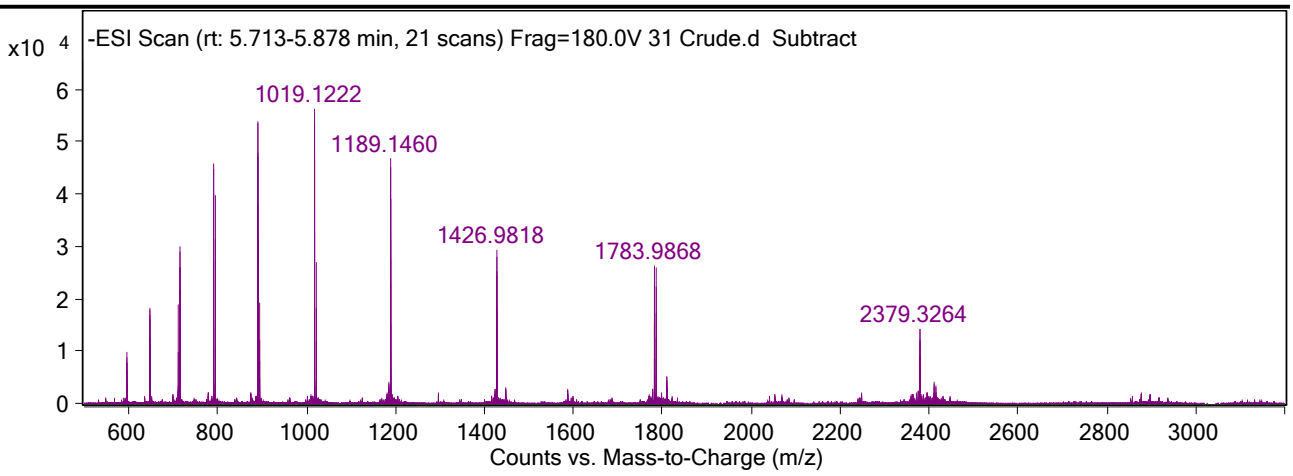
User Chromatograms



User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

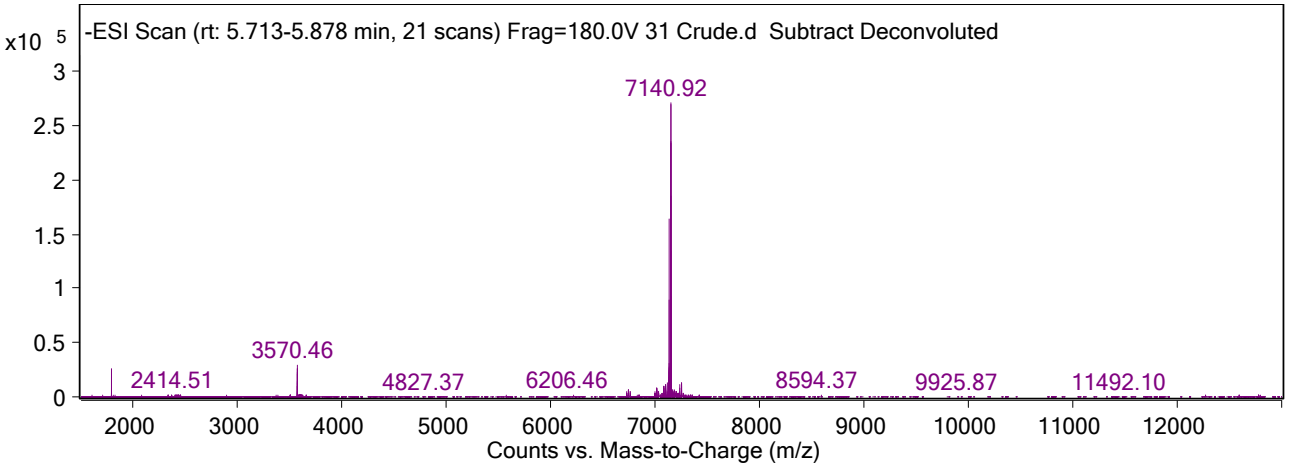
Qualitative Analysis Report



Peak List

m/z	z	Abund
792.3174	9	45623.81
891.358	8	47062.74
891.4827	8	53695.63
891.6077	8	53840.86
891.7321	8	47771.68
1018.8387	7	47097.68
1018.9807	7	53876.01
1019.1222	7	56259.79
1019.2657	7	46487.11
1189.146	6	46813.48

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund
7136.93	90257.39
7137.93	164921.67
7138.93	235900.02
7139.93	270718

7140.92	271777.81
7141.92	234189.34
7142.92	184914.23
7143.92	135741.91
7144.92	92445.11
7145.91	59921.01

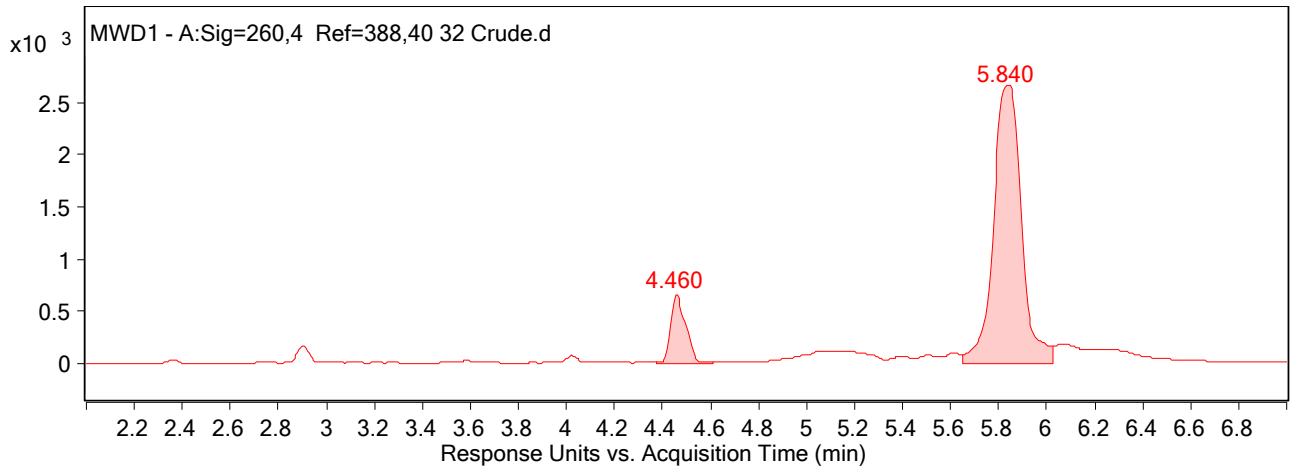
--- End Of Report ---

B.18 ASO 32

Qualitative Analysis Report

Data File	32 Crude.d	**Sample Name**	32 MOE
Sample Type	Sample	**Position**	P2-D4
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column.m	**Acquired Time**	9/28/2016 2:38:45 PM
IRM Calibration Status	Success	**DA Method**	NEGATIVE MODE A1B1 PORT 1-2.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

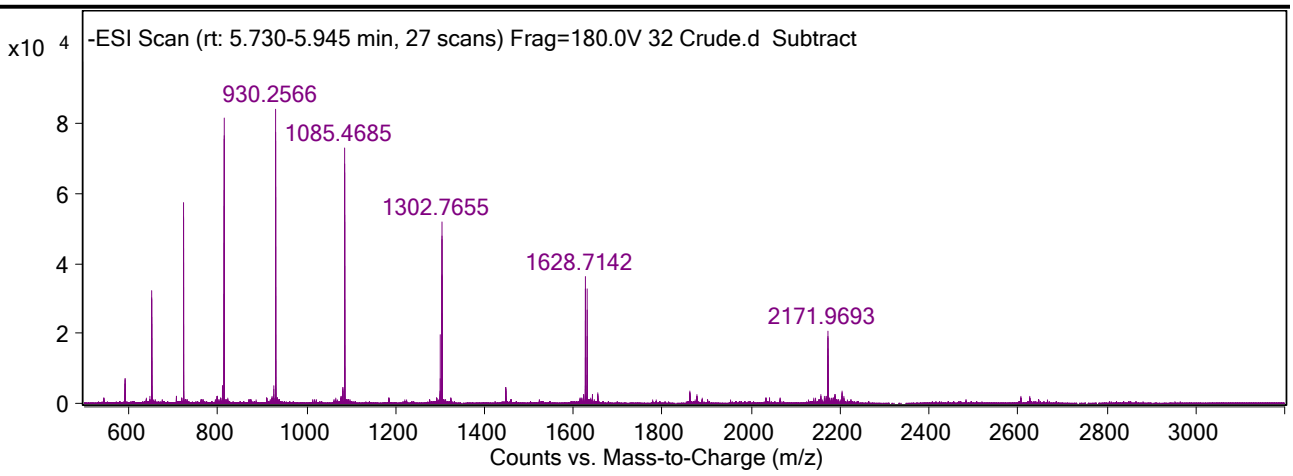
User Chromatograms



User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (2) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

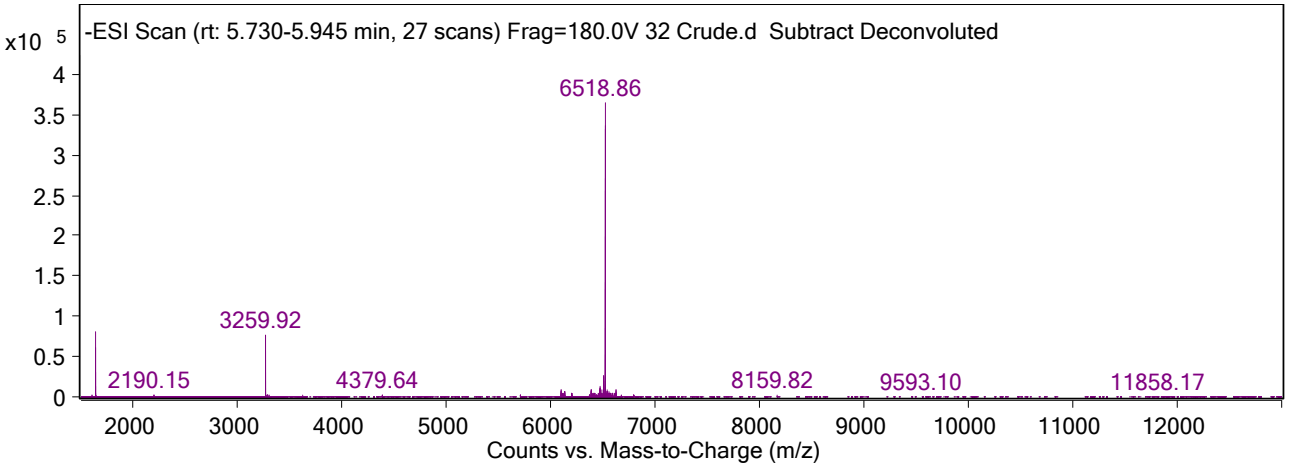
Qualitative Analysis Report



Peak List

m/z	z	Abund
813.7234	8	75729.79
813.848	8	81824.56
813.9723	8	76808.14
930.1138	7	77833.96
930.2566	7	84353.17
930.3992	7	75457.27
930.5412	7	62265.94
1085.302	6	68626.95
1085.4685	6	73390.02
1085.635	6	66329.65

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund
1629.71	81824.56
6515.86	143161.03
6516.86	254923.16
6517.86	337258.16

6518.86	365121.94
6519.86	332480.38
6520.86	271410.97
6521.85	198244.27
6522.85	133620.95
6523.85	84670.08

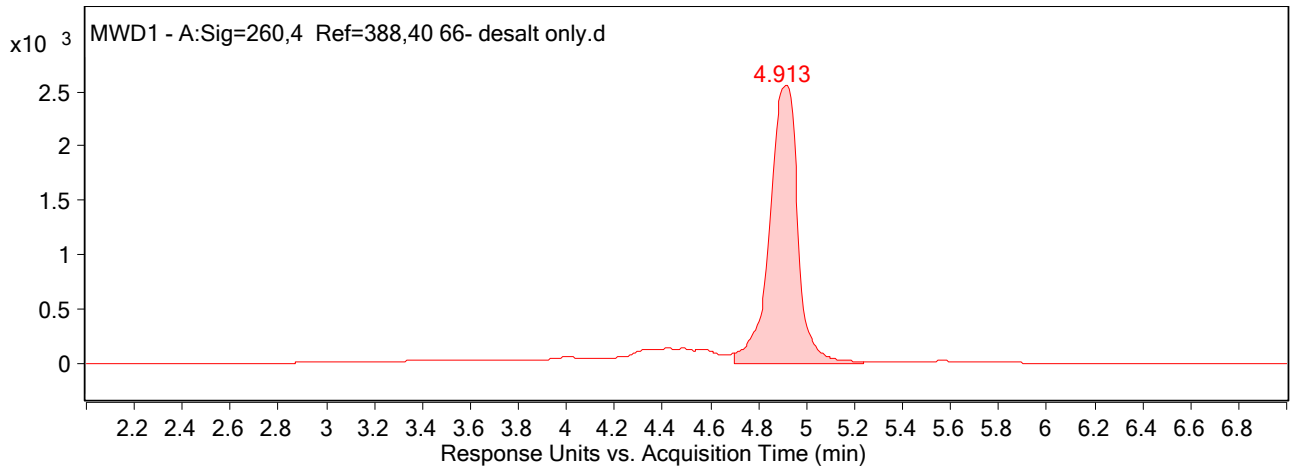
--- End Of Report ---

B.19 ASO 66

Qualitative Analysis Report

Data File	66- desalt only.d	**Sample Name**	66
Sample Type	Sample	**Position**	P2-A1
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column.m	**Acquired Time**	1/25/2017 6:59:22 PM
IRM Calibration Status	Success	**DA Method**	NEGATIVE MODE A1B1 PORT 1-2.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

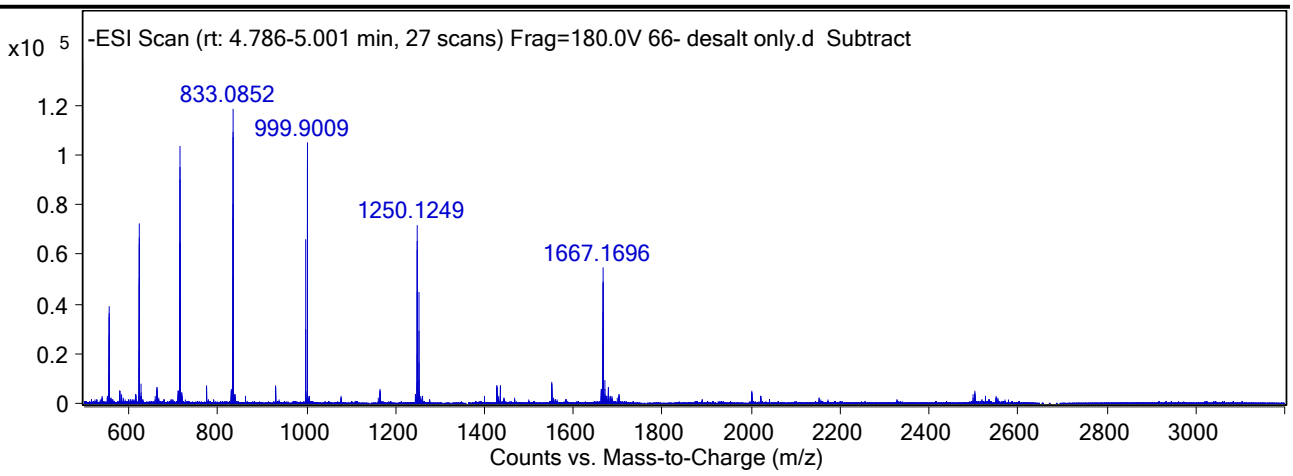
User Chromatograms



User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

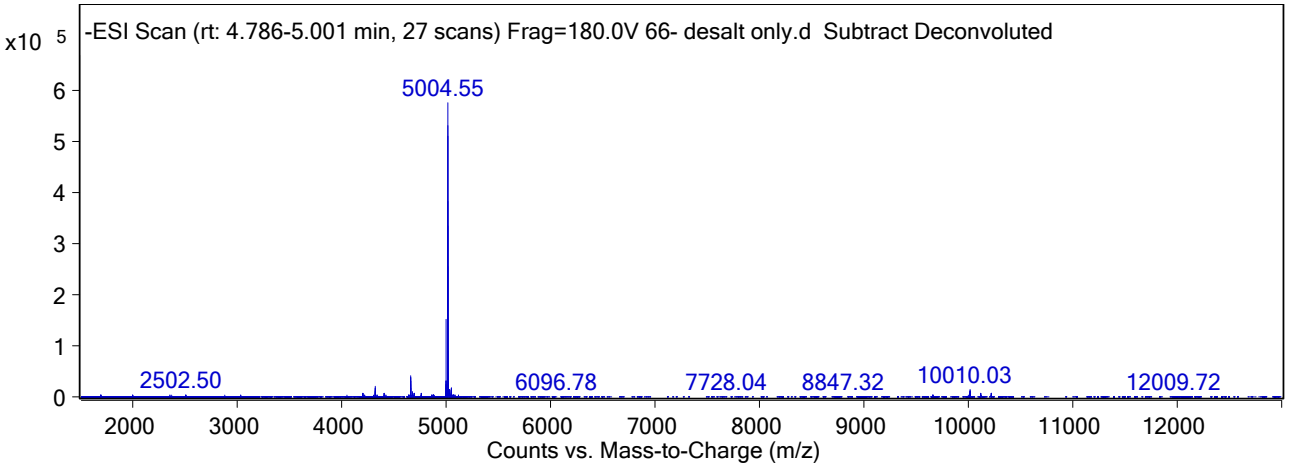
Qualitative Analysis Report



Peak List

m/z	z	Abund
713.7879	7	95356.6
713.9306	7	103564.3
714.0736	7	89762.63
832.9185	6	109018.73
833.0852	6	118531.36
833.2517	6	107016.48
833.4179	6	80118.59
999.7008	5	98157.79
999.9009	5	104813.45
1000.1005	5	94028.37

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund
5001.55	150625.22
5002.55	333178.31
5003.55	529396.31
5004.55	575719

Agilent Technologies

5005.55	510873.09
5006.55	383453.16
5007.55	254002.7
5008.55	154334.56
5009.54	89548.37
5010.54	47924.24

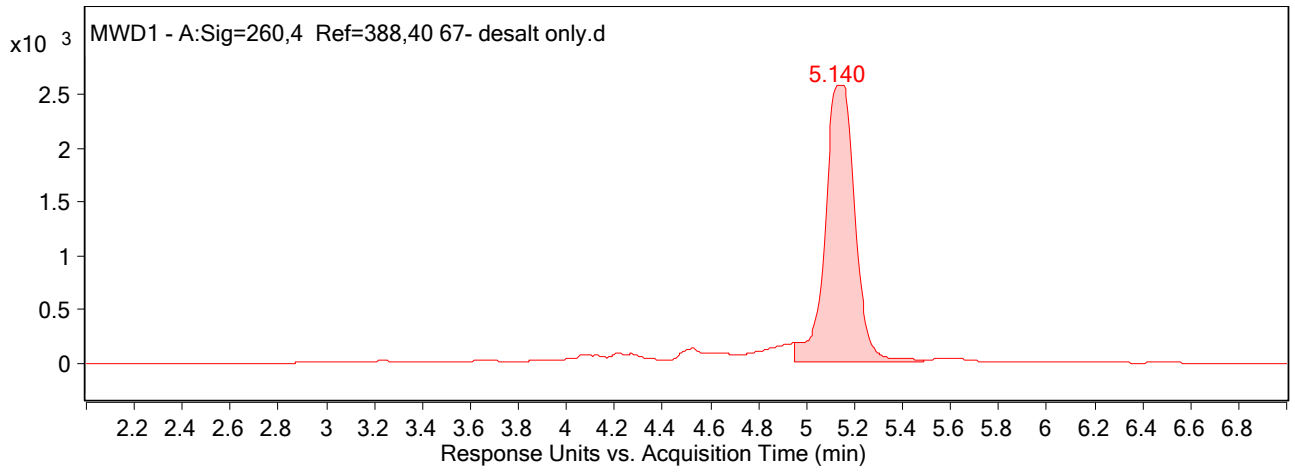
--- End Of Report ---

B.20 ASO 67

Qualitative Analysis Report

Data File	67- desalt only.d	**Sample Name**	67
Sample Type	Sample	**Position**	P2-A2
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column.m	**Acquired Time**	1/25/2017 7:15:23 PM
IRM Calibration Status	Success	**DA Method**	NEGATIVE MODE A1B1 PORT 1-2.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

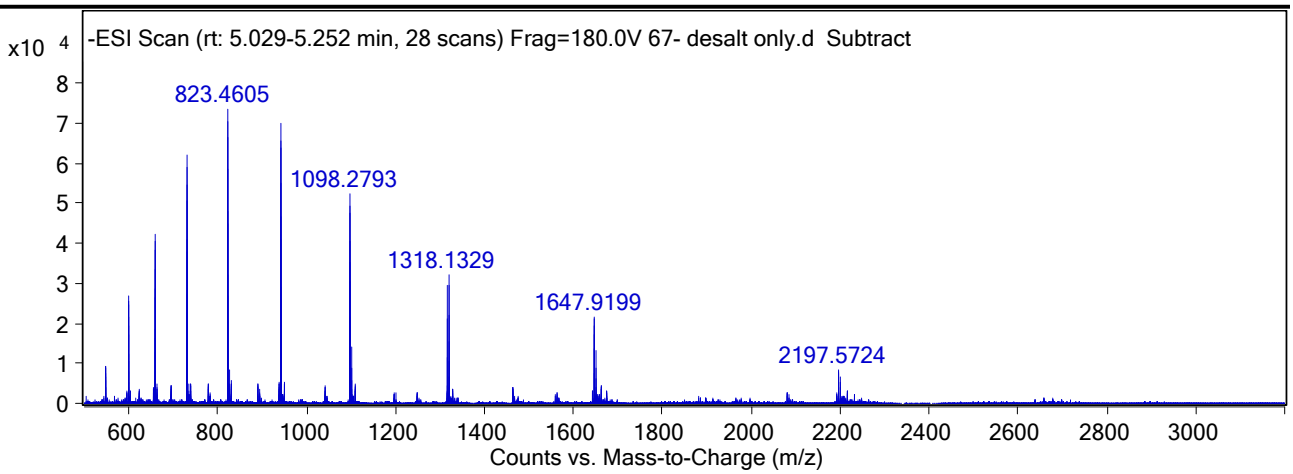
User Chromatograms



User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

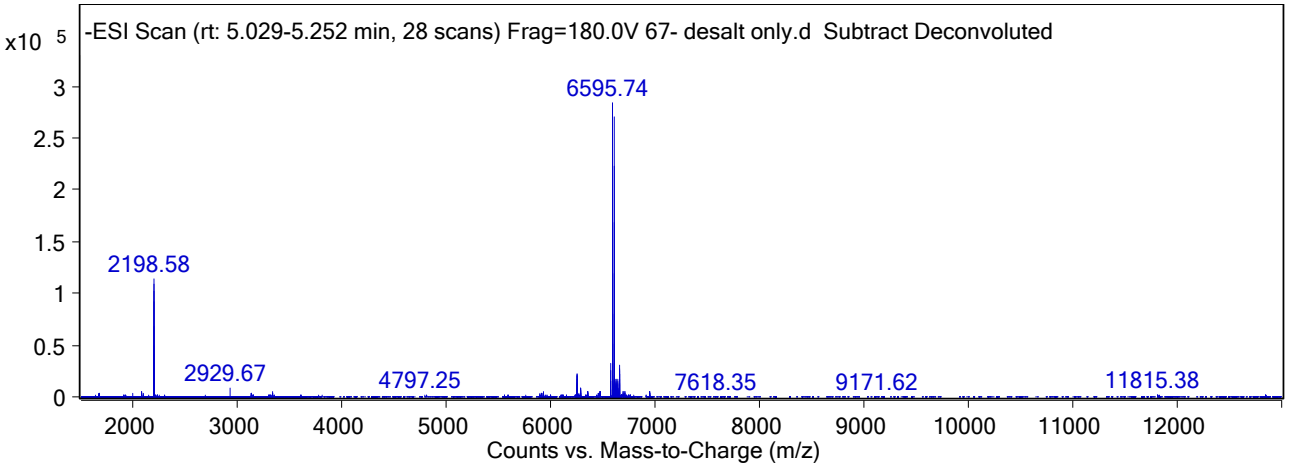
Qualitative Analysis Report



Peak List

m/z	z	Abund
731.8546	9	62275.54
731.9659	9	58967.45
823.3359	8	66431.59
823.4605	8	73559.35
823.585	8	70578.49
823.7093	8	58581.69
941.0978	7	63661.84
941.2403	7	69774.25
941.3823	7	65736.02
941.5248	7	55142.53

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund
2198.58	114454.62
2198.91	108346.48
6592.74	106280.27
6593.74	190025.41

6594.74	258298.64
6595.74	284313
6596.74	270759.81
6597.74	222504.16
6598.73	169224.59
6599.73	119664.34

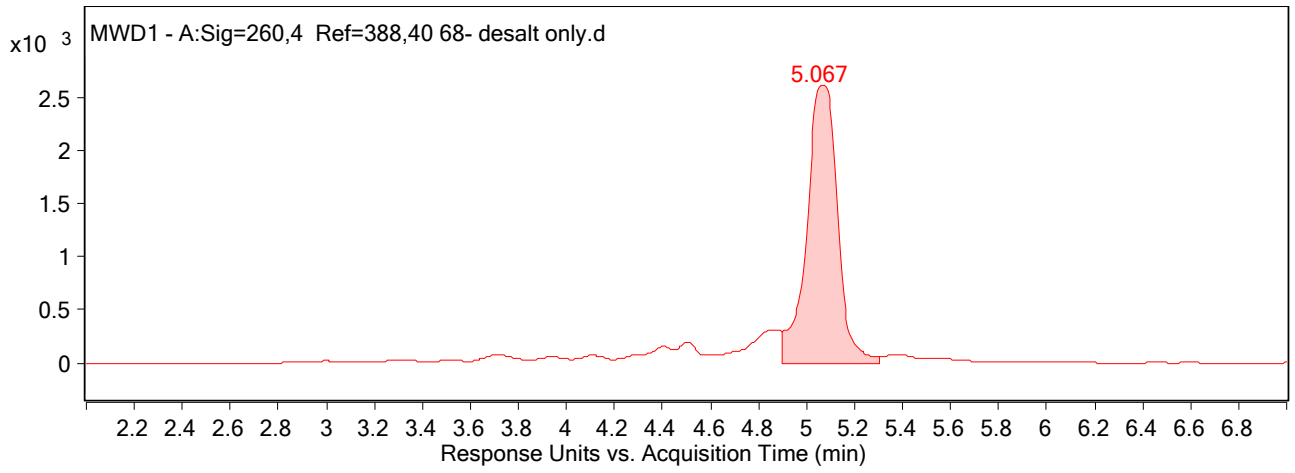
--- End Of Report ---

B.21 ASO 68

Qualitative Analysis Report

Data File	68- desalt only.d	**Sample Name**	68
Sample Type	Sample	**Position**	P2-A3
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column.m	**Acquired Time**	1/25/2017 7:31:22 PM
IRM Calibration Status	Success	**DA Method**	NEGATIVE MODE A1B1 PORT 1-2.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

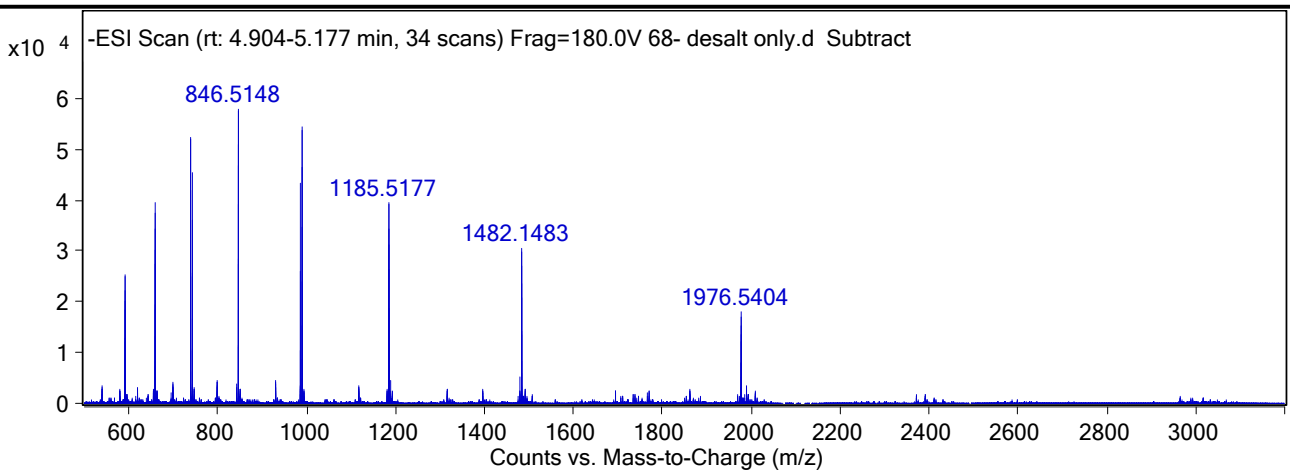
User Chromatograms



User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

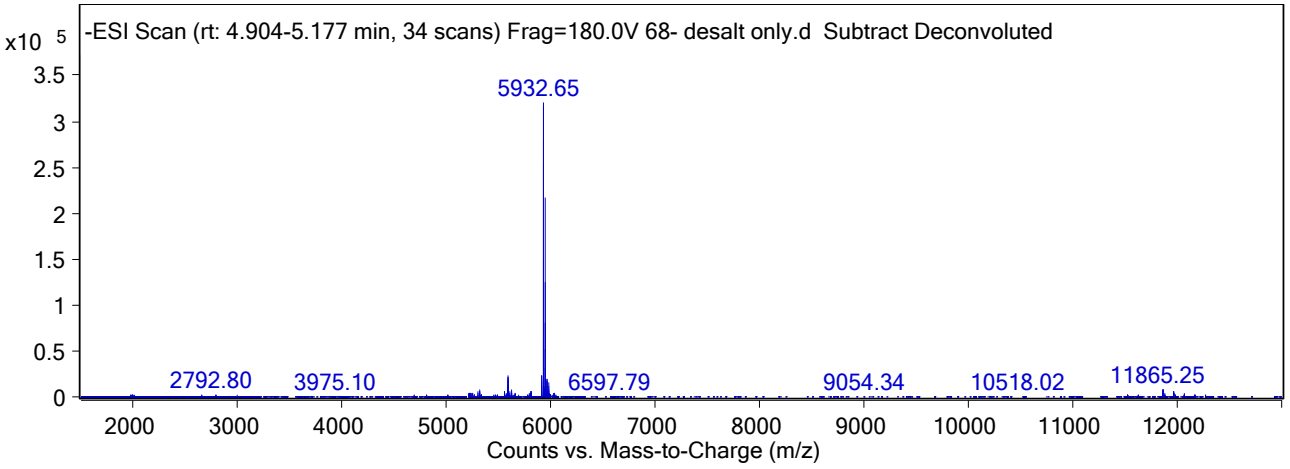
Qualitative Analysis Report



Peak List

m/z	z	Abund
740.4503	8	52099.13
740.575	8	52208.23
740.6996	8	45300.32
846.2292	7	46669.77
846.3725	7	57498.51
846.5148	7	57924.14
846.6571	7	51094.77
987.6002	6	54336.77
987.7667	6	53620.74
987.9336	6	46616.35

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund
5928.65	61536.74
5929.65	151986.86
5930.65	253456
5931.65	316023.91

Agilent Technologies

5932.65	320489.63
5933.65	279714.75
5934.65	217349.41
5935.64	125249.59
5936.65	80453.78
5937.64	51125.23

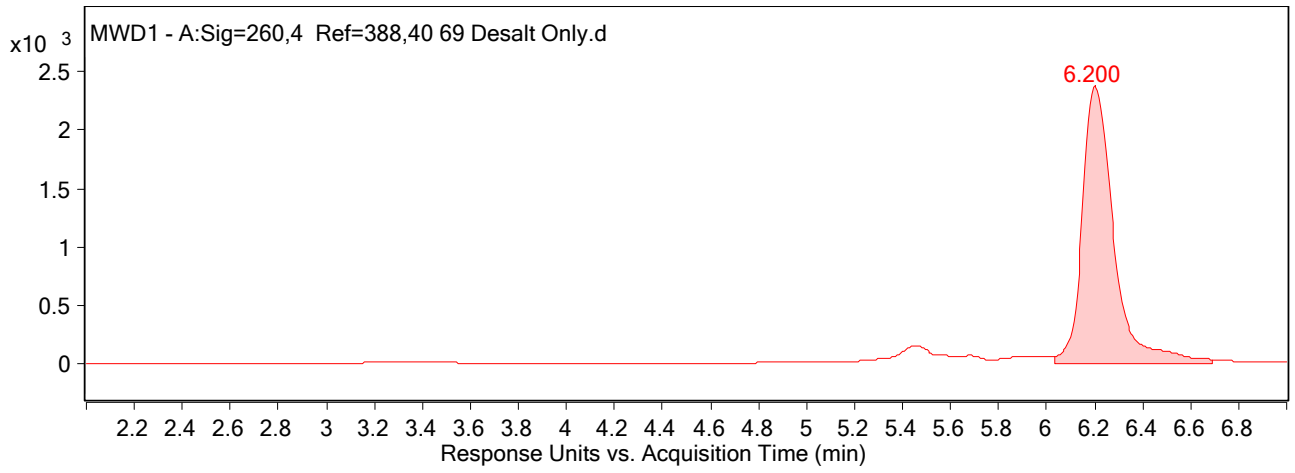
--- End Of Report ---

B.22 ASO 69

Qualitative Analysis Report

Data File	69 Desalt Only.d	**Sample Name**	69
Sample Type	Sample	**Position**	P2-A4
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column.m	**Acquired Time**	1/27/2017 3:45:12 PM
IRM Calibration Status	Success	**DA Method**	NEGATIVE MODE A1B1 PORT 1-2.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

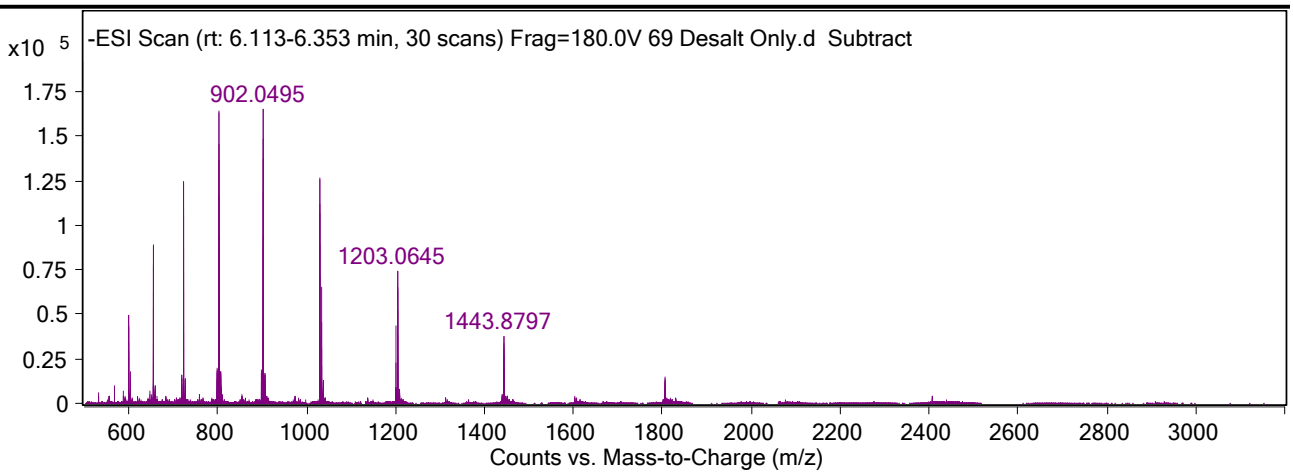
User Chromatograms



User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

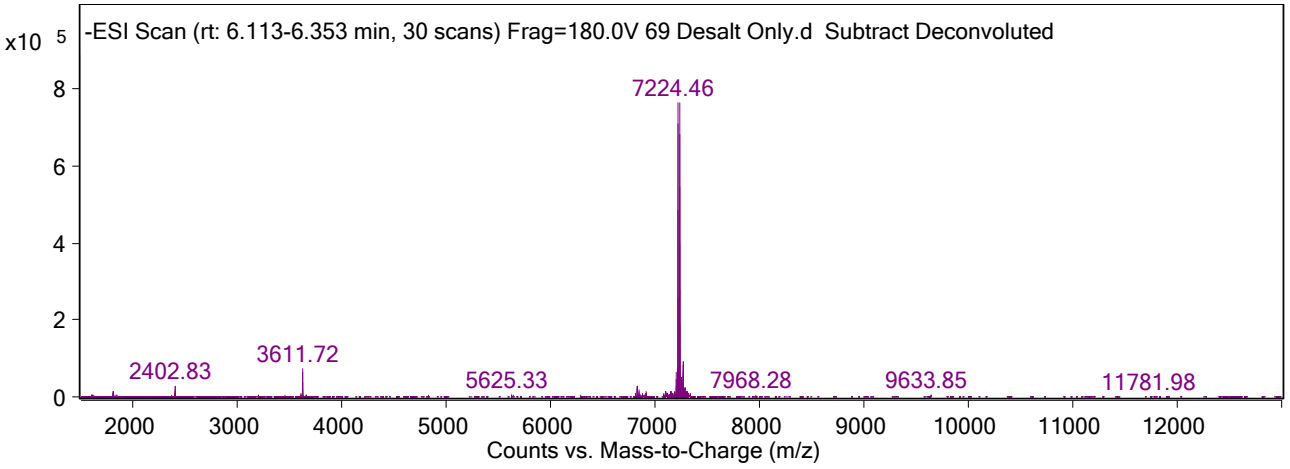
Qualitative Analysis Report



Peak List

m/z	z	Abund
801.4891	9	136349.8
801.6005	9	162760.2
801.7117	9	164393.45
801.8227	9	145837.81
901.7998	8	137691.56
901.9248	8	164836.91
902.0495	8	165274.83
902.1741	8	148498.42
1030.9129	7	125787.8
1031.0552	7	126141.52

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund
7220.46	255481.19
7221.46	487289.88
7222.46	709283.38
7223.46	761893.75

Agilent Technologies

7224.46	764487.19
7225.46	679791.63
7226.46	542818.81
7227.46	399198.31
7228.46	278879.66
7229.46	188442

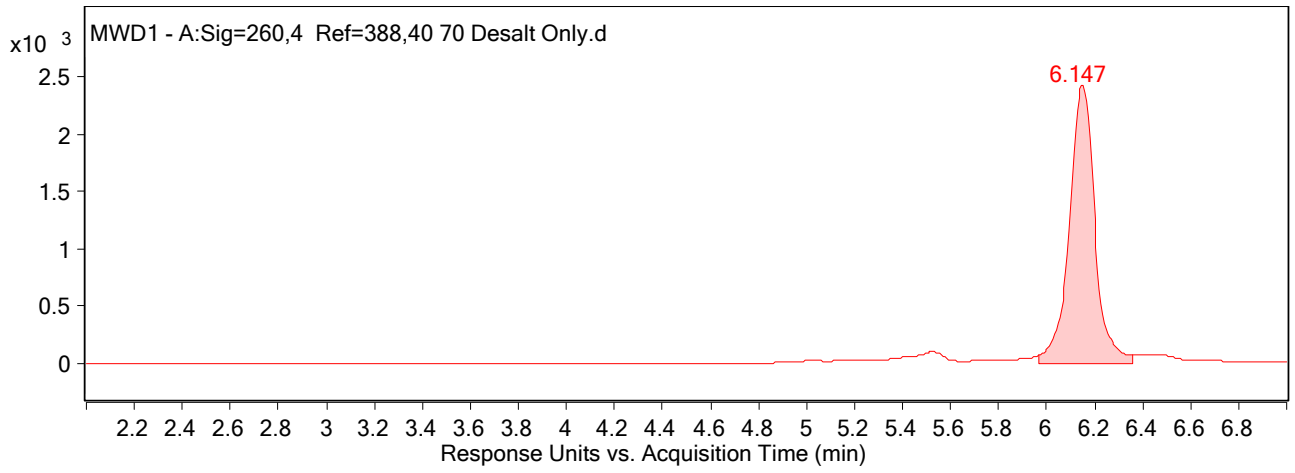
--- End Of Report ---

B.23 ASO 70

Qualitative Analysis Report

Data File	70 Desalt Only.d	**Sample Name**	70
Sample Type	Sample	**Position**	P2-A5
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column.m	**Acquired Time**	1/27/2017 4:01:13 PM
IRM Calibration Status	Success	**DA Method**	NEGATIVE MODE A1B1 PORT 1-2.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

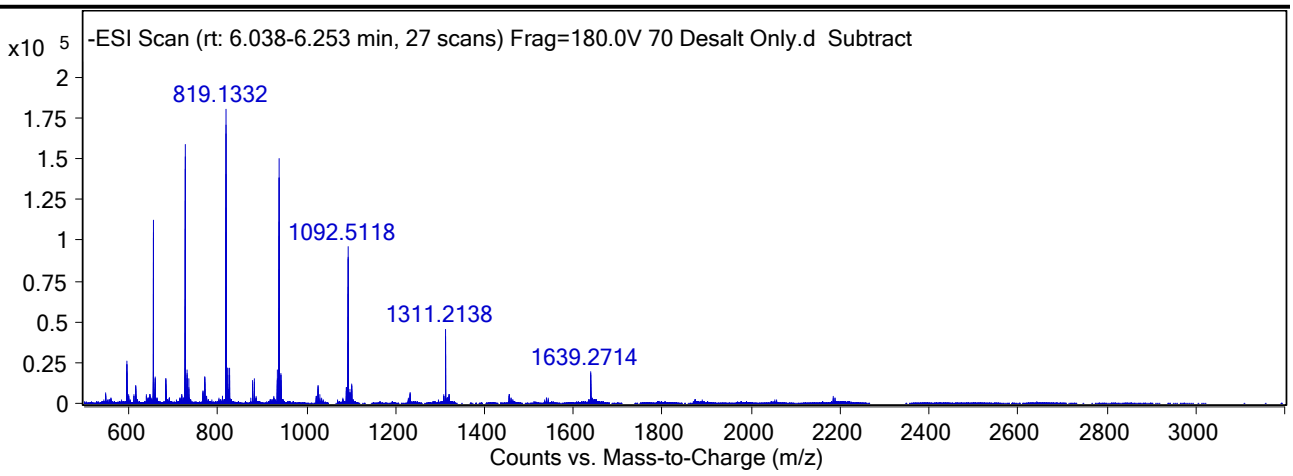
User Chromatograms



User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

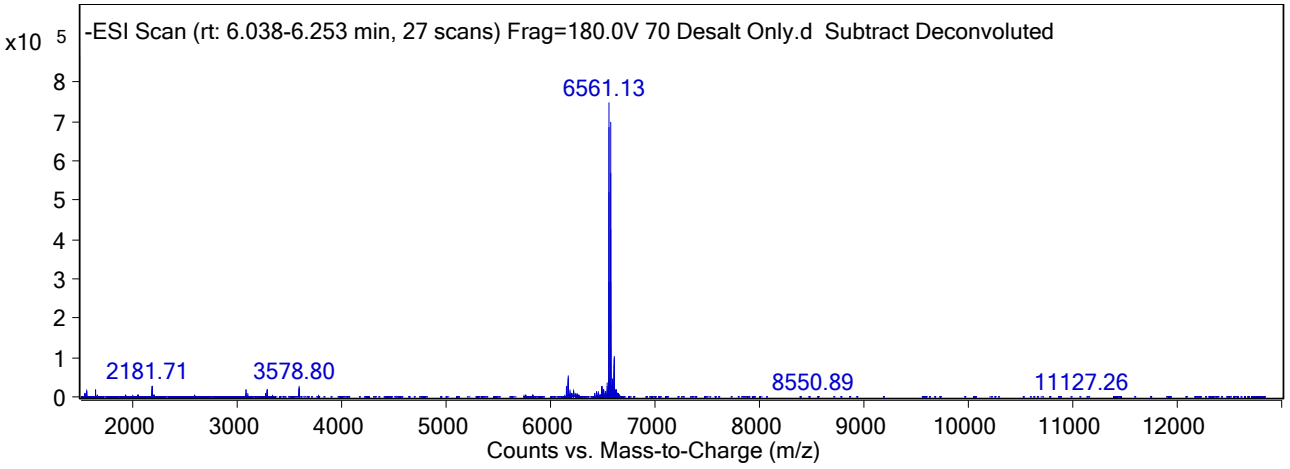
Qualitative Analysis Report



Peak List

m/z	z	Abund
727.897	9	143941.41
728.008	9	158801.48
728.119	9	151763.28
819.0089	8	165049.16
819.1332	8	180657.27
819.2575	8	170307.98
819.3817	8	140623.92
936.1524	7	138590.95
936.2949	7	150024.81
936.4375	7	138339.08

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund
6558.13	289947.63
6559.13	521084.22
6560.13	685541.31
6561.13	749002.81

6562.12	698522.63
6563.12	569664.75
6564.12	426139.13
6565.12	291132.47
6566.11	191452.2
6567.11	122698.39

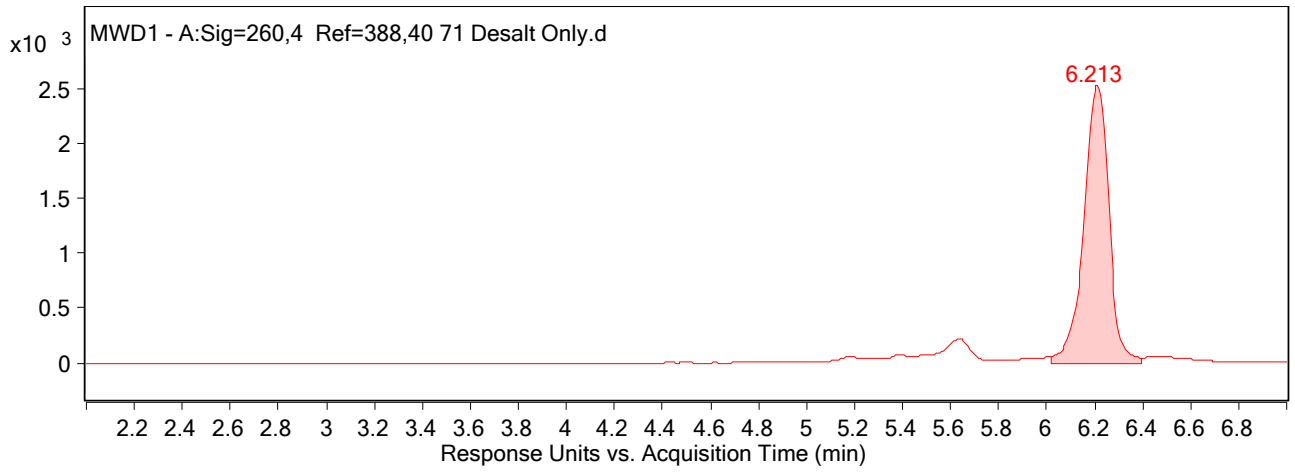
--- End Of Report ---

B.24 ASO 71

Qualitative Analysis Report

Data File	71 Desalt Only.d	**Sample Name**	71
Sample Type	Sample	**Position**	P2-A6
Instrument Name	Instrument 1	**User Name**	
Acq Method	Agilent AdvanceBio Column.m	**Acquired Time**	1/27/2017 4:17:14 PM
IRM Calibration Status	Success	**DA Method**	NEGATIVE MODE A1B1 PORT 1-2.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

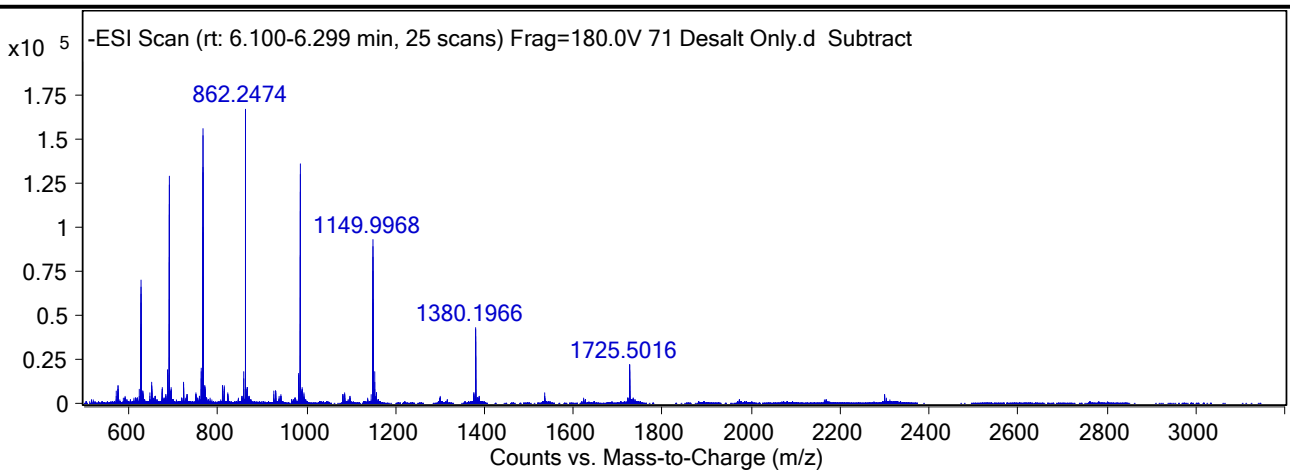
User Chromatograms



User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

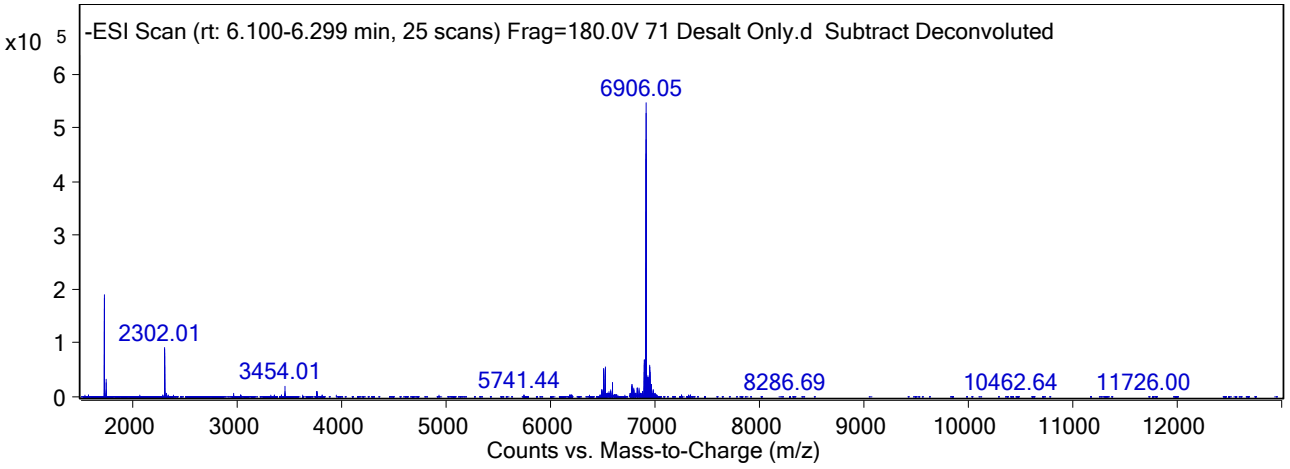
Qualitative Analysis Report



Peak List

m/z	z	Abund
766.221	9	134065.55
766.3321	9	155894.75
766.443	9	151659.36
766.5539	9	130856.98
862.1227	8	145273.88
862.2474	8	166990.44
862.3718	8	163483.06
862.4964	8	140058.39
985.5678	7	135664.8
985.7101	7	129545.7

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund
1726.51	189304.91
1726.76	185369
6903.05	238629.08
6904.05	444299.66

6905.05	479958.59
6906.05	547984.06
6907.05	528013.69
6908.04	451718.28
6909.04	344842.56
6910.04	247775.31

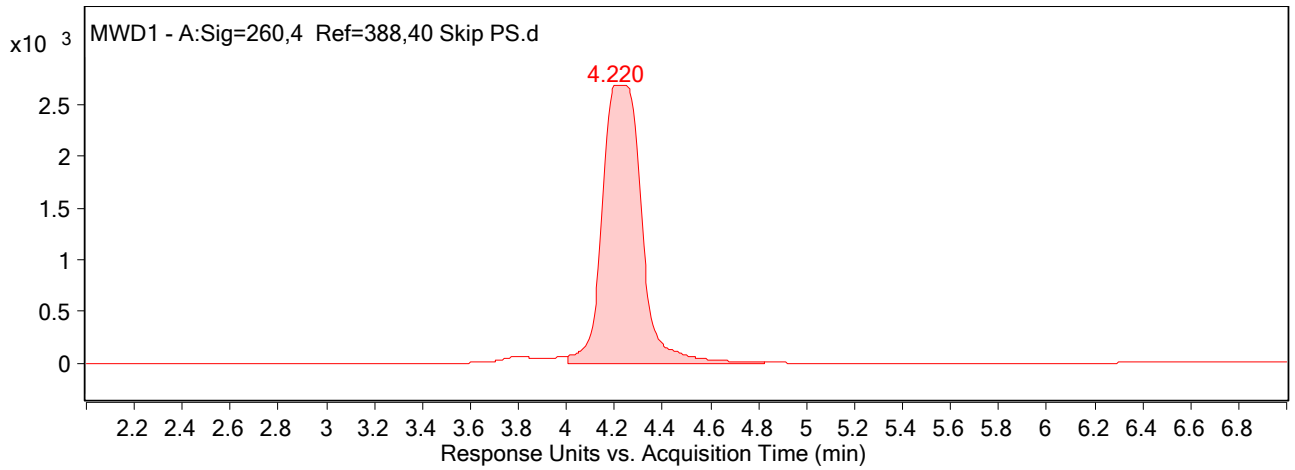
--- End Of Report ---

B.25 ASO 70- Skip

Qualitative Analysis Report

Data File	Skip PS.d	**Sample Name**	C9 skip PS
Sample Type	Sample	**Position**	P2-A2
Instrument Name	Instrument 1	**User Name**	
Acq Method	NEGATIVE MODE A1B1 PORT 1-2.m	**Acquired Time**	11/8/2017 2:06:32 PM
IRM Calibration Status	Success	**DA Method**	MS_Check_Oligos.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

User Chromatograms



Integration Peak List

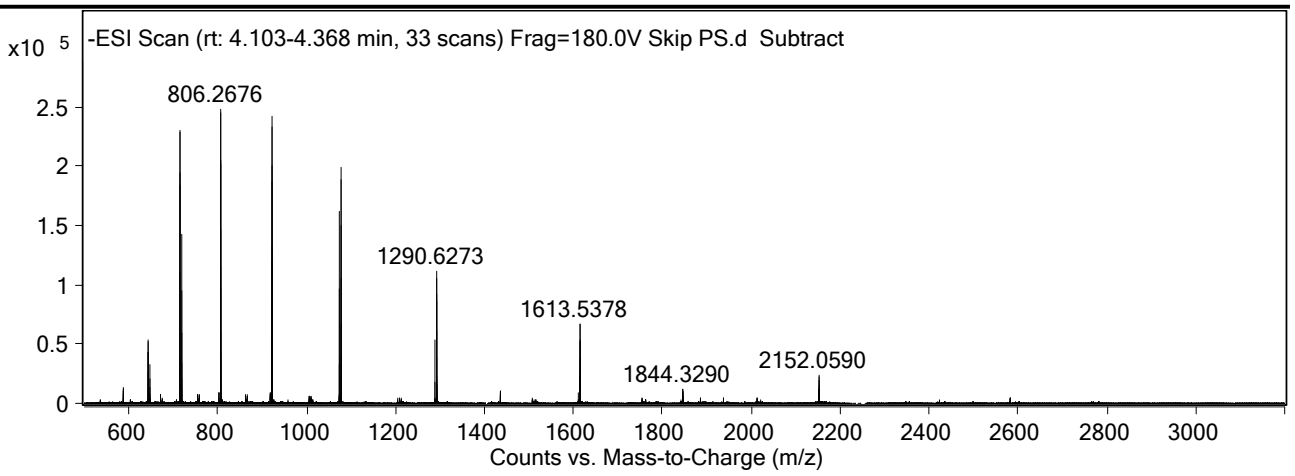
Peak	Start	RT	End	Height	Area	Area %
1	4.007	4.22	4.827	2686.44	30530.08	100

User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

Agilent Technologies

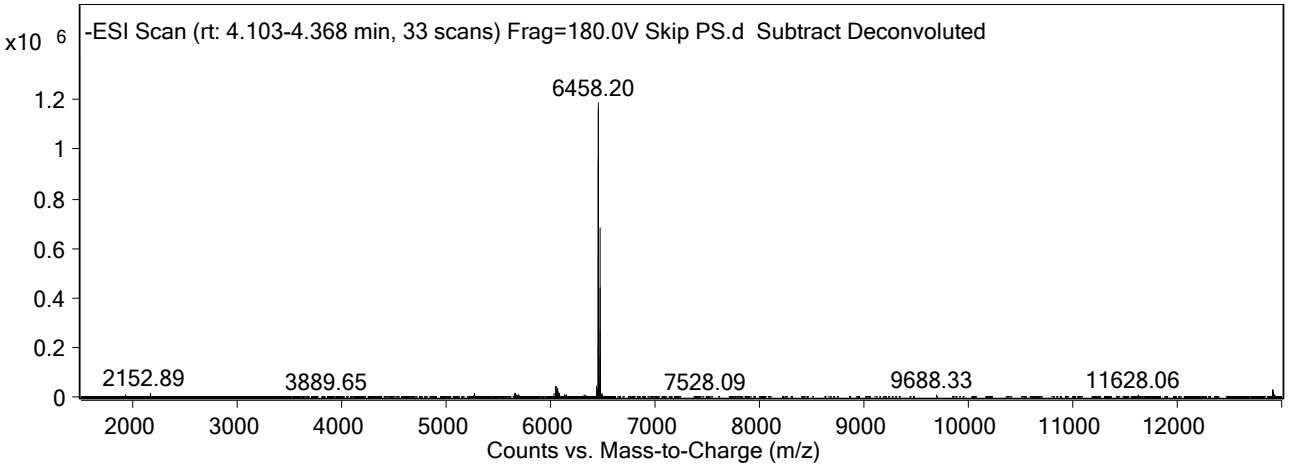
Qualitative Analysis Report



Peak List

m/z	z	Abund
716.572	9	230335.33
716.6829	9	228484.2
806.1429	8	202622.91
806.2676	8	248118.38
806.3918	8	244337.23
806.5157	8	204199.19
921.4487	7	200660.42
921.5913	7	241459.38
921.7334	7	232773.92
1075.3566	6	198612.91

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund
6455.19	197838.34
6456.19	566959.75
6457.2	964914.88
6458.2	1189131.25

Agilent Technologies

6459.19	1157976.5
6460.19	950003.25
6461.19	679838.13
6462.18	441737.38
6463.18	270560.59
6464.18	156937.7

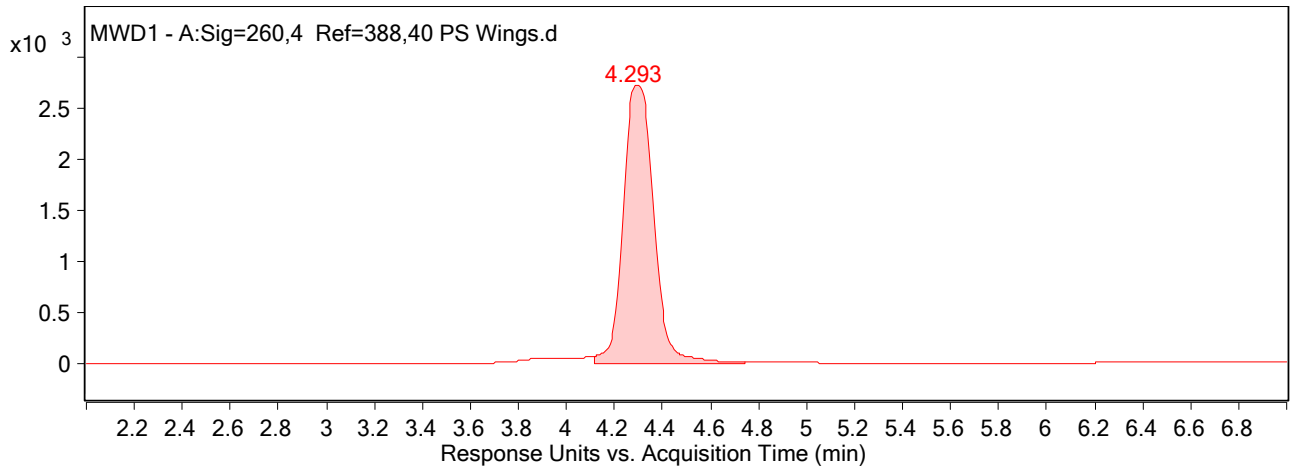
--- End Of Report ---

B.26 ASO 70- Wings 1

Qualitative Analysis Report

Data File	PS Wings.d	**Sample Name**	C9 Wings PS
Sample Type	Sample	**Position**	P2-A3
Instrument Name	Instrument 1	**User Name**	
Acq Method	NEGATIVE MODE A1B1 PORT 1-2.m	**Acquired Time**	11/8/2017 2:19:33 PM
IRM Calibration Status	Success	**DA Method**	MS_Check_Oligos.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

User Chromatograms



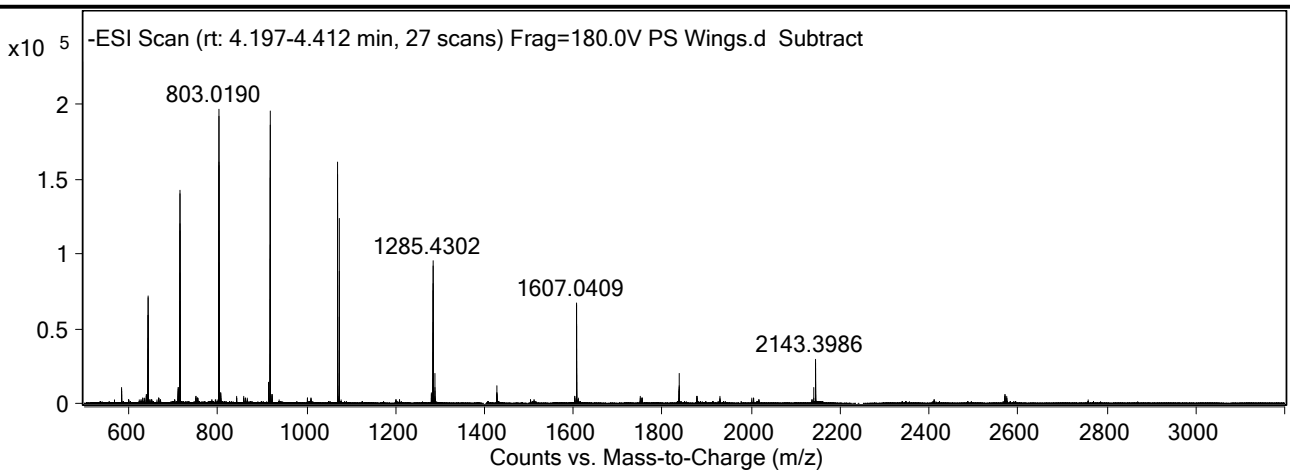
Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	4.113	4.293	4.74	2731.55	24170.76	100

User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	180	0	ESI

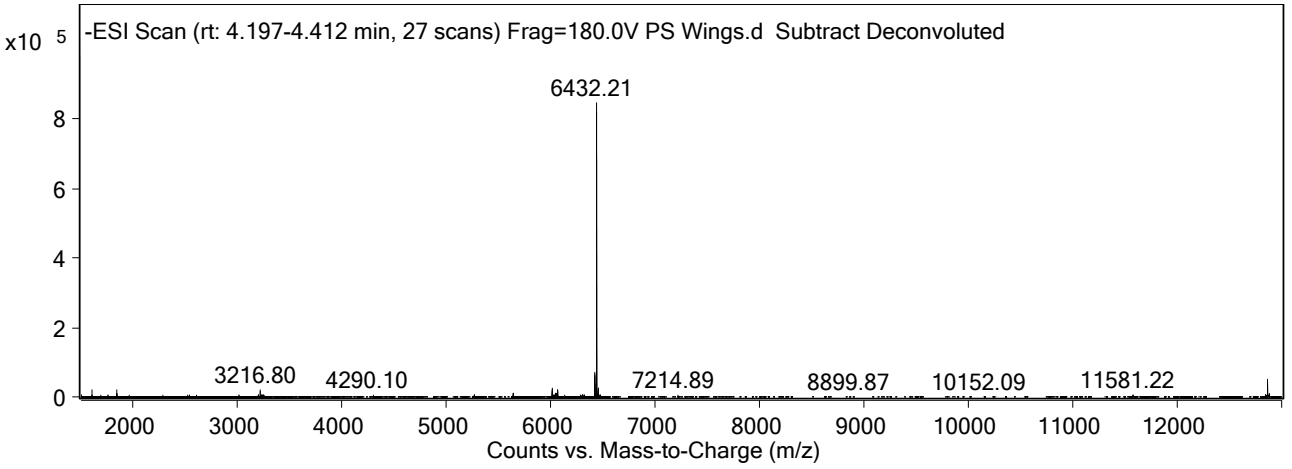
Qualitative Analysis Report



Peak List

m/z	z	Abund
802.8944	8	159362.38
803.019	8	196834.91
803.1433	8	192551.48
803.2673	8	160974.92
917.7361	7	162156.86
917.8787	7	195028.36
918.0208	7	186562.52
918.1632	7	150270.88
1071.0253	6	161347.5
1071.192	6	153666.55

Fragmentor Voltage	Collision Energy	Ionization Mode
180	0	ESI



Peak List

m/z	Abund
6429.21	143469
6430.21	410052.59
6431.21	691948.5
6432.21	846513.25

6433.21	821203.81
6434.2	677300.63
6435.2	485743.31
6436.2	319915.03
6437.2	196227.27
6438.19	117371.98

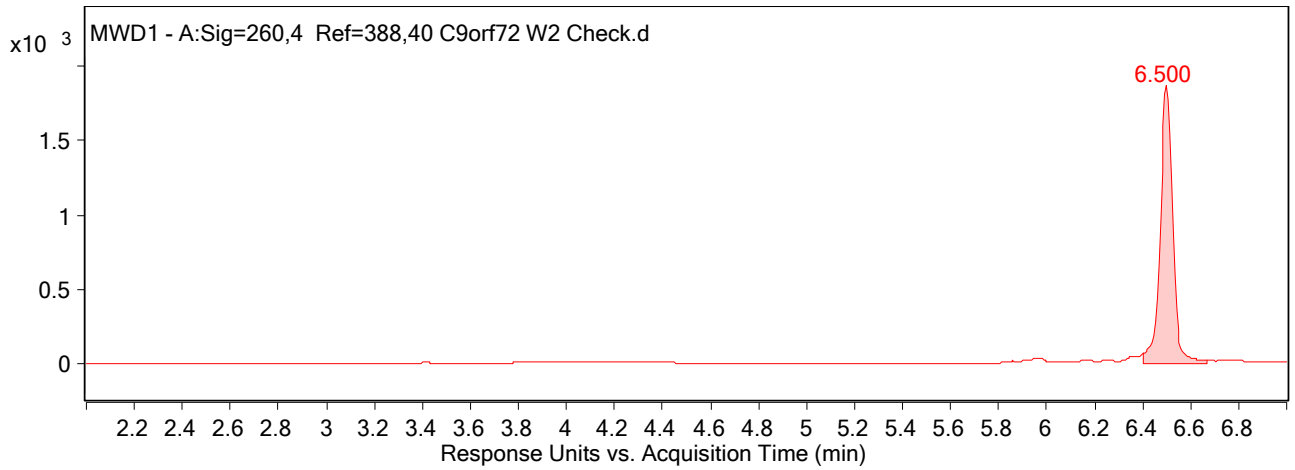
--- End Of Report ---

B.27 ASO 70- Wings 2

Qualitative Analysis Report

Data File	C9orf72 W2 Check.d	**Sample Name**	C9orf72W2Check
Sample Type	Sample	**Position**	P2-C3
Instrument Name	Instrument 1	**User Name**	
Acq Method	Negative Advance Column A1B1 port1-2 Longmer.m	**Acquired Time**	2/12/2018 12:45:11 PM
IRM Calibration Status	Success	**DA Method**	MS_Check_Oligos.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition SW Version**	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

User Chromatograms



Integration Peak List

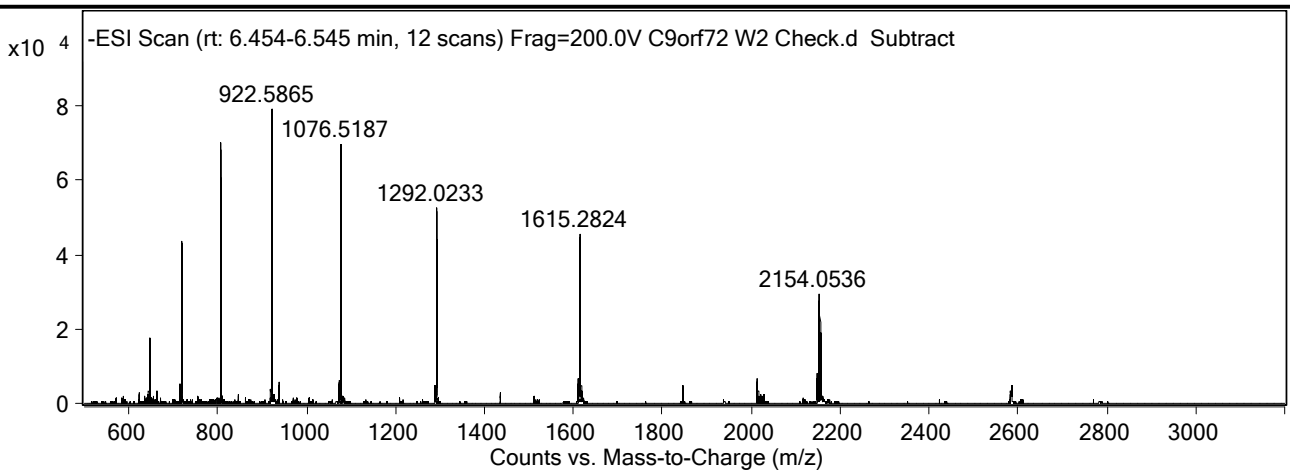
Peak	Start	RT	End	Height	Area	Area %
1	6.4	6.5	6.667	1870.18	6837.63	100

User Spectra

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "MWD1 - A:Sig=260,4 Ref=388,40"	200	0	ESI

Agilent Technologies

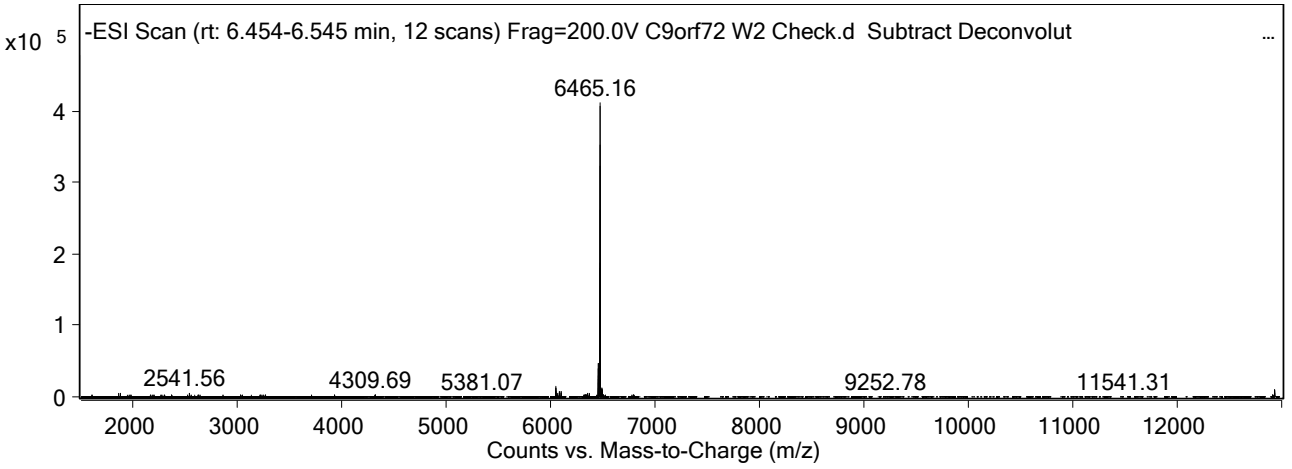
Qualitative Analysis Report



Peak List

m/z	z	Abund
807.0134	8	69413.9
807.1377	8	70650.84
807.2621	8	60868.91
922.302	7	62748.22
922.4438	7	80479.56
922.5865	7	79286.28
922.7287	7	67972.17
1076.3522	6	70235.55
1076.5187	6	71061.52
1076.6855	6	59508.15

Fragmentor Voltage	Collision Energy	Ionization Mode
200	0	ESI



Peak List

m/z	Abund
6461.17	67084.55
6462.17	186635.7
6463.17	323185.34
6464.16	407481.53

6465.16	412546.09
6466.16	354058.19
6467.16	262833.06
6468.15	180199.84
6469.15	114877.7
6470.15	71801.67

--- End Of Report ---

Appendix C

Regulatory Documents

C.1 IACUC Protocol 2551

University of Massachusetts Medical School
UMASS

Institutional Animal Care and Use Committee (IACUC)
Office of the Vice Provost for Research
University of Massachusetts Medical School
55 Lake Avenue North
Worcester, MA 01655-0002 USA
508.856.5416 (office) 508.856.5004 (fax)

Notification of IACUC Amendment Review and Approval

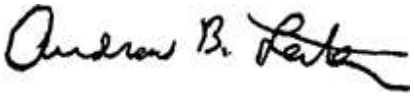
Docket #: A-2551-16-4 **May 05, 2017**

Principal Investigator: **Jonathan Watts, Ph.D.**
Department: **RNA Therapeutics Institute**
Title: **Development of RNA-based Therapeutics**

Project Approval Period: **02/12/2016 to 02/11/2019**
Date of Submission: **02/13/2017**
Date of Approval: **05/05/2017**

Description:

To modify analgesia and euthanasia procedure.
To add PBS and human tumor dissociates.
To add Timed Pregnancy for embryo or tissue culture from Immunocompromised Animals to screen compounds for Glioblastoma.



Andrew B. Leiter, M.D., Ph.D.
Chair, Institutional Animal Care and Use Committee

cc: Animal Medicine **IBC reference: 656-16** **Review type: Major Amendment - DMR**

UNIVERSITY OF MASSACHUSETTS MEDICAL SCHOOL

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Animal Study Protocol

PI: Watts Docket # A-2551-16

Instructions for IACUC Animal Study Protocol application:

1. Complete the Animal Study Protocol application form if you are applying for a new, 3-year renewal or *major amendment.
2. If you need assistance in developing an Animal Study Protocol or have any questions, please contact IACUC@umassmed.edu
3. Save your file as [Your last name-IACUC] and e-mail the completed document as an attachment to IACUC: IACUC@umassmed.edu
4. Print a copy and collect signatures where appropriate: send the hard copy to IACUC, Office of Research, S1-859

All Principal Investigators are required to fill out the application face page (next page) and the sections I through X of the Animal Study Protocol. In addition, please complete appropriate forms and check the boxes below.

Forms: Double click on checkboxes to mark		
A	☒	Transporting animals for use outside of or to another area of the animal facilities
B	☒	If animals are going to bred for the use in this protocol
C	☒	Using anesthesia
D	☒	Doing survival surgery
E	☒	Administering substances other than anesthetics (therapeutic agents, toxins, cells, cell lines, cell/tissue extracts, viruses and other infectious agents, purified biological products, etc.)
	☐	Will rodent cell lines, serum, or tissues be introduced into live animals?
F	☐	Food or water restriction, prolonged physical restraint or animal care done by lab staff.
G	☒	If using radioactive agents, hazardous chemicals, recombinant DNA, breeding transgenic animals carrying viral genes or synthetic nucleic acids (see form G for details), infectious agents, biotoxins, human or non-human primate materials and live non-human primates
H	☒	To indicate adverse effects of procedures and experiments/monitoring and managements
I	☒	If using any of the UMMS Core Facilities
J	☐	If using sites or vendors outside UMMS
K	☐	If you are conducting a training course
Worksheet 1	☐	Animal number worksheet
Worksheet 2	☐	Breeding worksheet

*Major amendment: Any change that could affect the well being of animals (addition of surgical procedures, use of hazardous agents as infectious agents or radioisotopes), change of animal species.
Please send a cover letter indicating what part of the protocol you are changing, and justify the reason for requesting the change. In the protocol-form use a *different font* or different color for the section that you wish to amend or to change.

Docket No. A-2551-16

APPLICATION TO USE VERTEBRATE ANIMALS IN RESEARCH OR INSTRUCTION

UMMS Institutional Animal Care and Use Committee

Principal Investigator	Name and Highest Academic Degree(s)	Jonathan Watts, PhD
	Faculty Title	Associate Professor of Biochemistry and Molecular Pharmacology
	Department \ Division or Company Name	RNA Therapeutics Institute
	Mailing Address or UMMS Building	AS4-1051
	E-mail Address	jonathan.watts@umassmed.edu
	Contact Phone Numbers	Office: (774) 455-3784 Home: 774-243-7931 Pager #:
Faculty Sponsor (if required) or co-investigator	Name and Degree	
	Faculty Title	
	Department/Division	
Primary Contact Person	Name	Mike Moazami
	Telephone No.	Office 774-455-3765 Home 774-666-0180
	Mailing Address Building	AS4-1006
Project Title	Development of RNA-based therapeutics	

☐ New ☐ 3-Year Renewal ☒ Major Amendment (state changes in a cover letter)

☒ Check this box if you like to receive an informal technical review and feedback from the IACUC office. The intent of this service is to assist investigators in improving the protocol and minimizing concerns during the formal review (veterinary pre-review and IACUC committee review). You may not require this option, if you are experienced in writing IACUC protocols.

USE OF THIS APPLICATION FORM

- DO NOT change formatting of this document. Forms submitted to IACUC MUST include shading. If attachments are used, they should be minimum and not replace answers to any section.
- If you have questions regarding the completion of this form, please contact the IACUC office (508.856.5384).
- Please note that upon request the University may be required by law to release a copy of this application to the public

Signature of Department Chair Date	Name (Please Print or Type) Phil Zamore, PhD

For IACUC USE ONLY:

Approved: Yes No

Approved with conditions: Yes No

Date Chair/Vice Chair, IACUC

Section I. OBJECTIVES OF PROPOSED RESEARCH OR INSTRUCTION

a. Check the box below that describes the type of animal use being proposed.

☒ Basic Research ☐ Service (Cores, Sentinels, etc.) ☐ Instruction or Training

☐ Field Research ☐ Testing (Biologicals, Toxicity, etc.) ☐ Applied Research

☐ Other_____

b. Lay Summary: In clear, concise, *non-technical,* language (i.e., that could be understood by someone at a high school level), summarize the background and objectives of your studies involving animals.

Genes are responsible for how a cell functions by instructing the cell what proteins to make and how those proteins are made. Changes (mutations) in a gene can cause disease by altering the proteins being made and how much of those proteins are made by a cell. Some proteins within a cell are able to carry out chemical reactions responsible for life. Current drug therapies reduce the function of a protein by interfering with its ability to perform chemical reactions that lead to disease. However, not all proteins involved in disease are able to be targeted by traditional small-molecule drugs. Oligonucleotides (small, synthetic strands of modified RNA) can be used to reduce production of overly abundant or mutated proteins that cause disease. Oligonucleotides represent a new class of drugs, which have the promise to become a major class of future therapeutics. There are several classes of oligonucleotides which can efficiently reduce protein production in various tissues with acceptable level of toxicity. We will compare the biodistribution (the distribution of a given structure throughout the tissues of an organism), silencing activity and specificity of oligonucleotides modified with different modifications as well as those assembled into various clusters, conjugates and other structures in animal models.

The overall survival for patients with glioblastomas, the most aggressive and lethal form of brain tumor, is less than 1 year and less than 5% of patients survive for 5 years or longer. This dismal clinical picture is the result of a number of characteristics unique to these tumors. Chief among the causes that make these tumors resistant to current treatments are the single cells that these tumors send into the brain. These tumor cells that invade the brain are responsible for the re-appearance of tumors after surgery and result in the patient's death. We plan to use our described oligonucleotide drugs to target tumours grafted into immunocompromised mice with the intention of increasing survival time.

c. Scientific Summary: Include an abstract of the study briefly describing the specific aims and scientific approaches to achieve these specific aims. This should serve as a scientific summary of the proposed study. Please note that the detailed procedures need not be included here.

The overall goals of our work are to develop oligonucleotide therapeutics for several disease indications as well as use oligonucleotides to study and validate gene function *in vivo*.

Oligonucleotides formulated as clusters or dendrimers have been shown to exhibit increased cellular uptake. We will conduct the first systematic and methodical study investigating the effect of cluster size and shape on *in vivo* distribution, uptake and activity. In addition, the specific oligonucleotide formulation, or if the co-administration of a drug will enhance various in vivo parameters, will also be tested.

Glioblastomas remain one of the deadliest forms of cancer despite numerous advances in surgical and radiological technologies, or the introduction of new chemotherapeutic agents that show promising results in other cancers. With the ability to silence oncogenes using oligonucleotide drugs we hope that we will be able to explore new therapeutic options for this disease. We are interested also in the MGMT gene as described later. MGMT (O-6-methylguanine-DNA methyltransferase) upregulation in GBM is strongly correlated with a poor prognosis. MGMT repairs the DNA lesions that the first line chemotherapeutic, temozolomide, induces thus vastly reducing efficacy.

d. Briefly explain the relevance of the proposed research or instruction to human or animal health and/or to the advancement of scientific knowledge.

The proposed research has direct relevance to human health, since our objective is to develop oligonucleotide therapeutics for several disease indications. It is conceivable to consider that the majority of targets currently believed to be 'undruggable' could be targeted with oligo therapeutics to effect improvement in the patient. With a GBM diagnosis remaining a terrible prospect for all affected patients, we hope that we can make progress towards finding a useful therapeutic compound.

e. Has this project undergone scientific peer-review?

☒Yes ☐ No

Section II. RATIONALE FOR USING ANIMALS AND ALTERNATIVES TO THE USE OF ANIMALS

a. Briefly explain why animals are required for your studies.

Animal models will be used to test toxicity, tissue distribution, pharmacokinetic/pharmacodynamic (PK/PD) parameters, and efficacy. Animal models are needed in order to test oligonucleotides to determine if they are able to reach the diseased tissue, if the oligonucleotide is able to enter the cell and reduce protein production, and to determine any effects on the expression of non-targeted genes. In order to test the oligonucleotides identified in the laboratory with the ultimate goal of developing treatments for

several diseases, the use of mice is required prior to clinical trials in humans. At present no mathematical models, computer simulations or in vitro biological systems represent suitable alternatives to live animals for testing gene therapy approaches for brain tumors. Experiments in cell culture do not model appropriately the migratory behavior of tumor cells in the brain.

b. Briefly explain why the species you propose to use is/are the most appropriate.

Mice and rats are mammals and provide an organism which models human physiology and pathology well enough to make our observations potentially relevant to human diseases for which the lab will design oligonucleotide therapeutics. It is anticipated that oligonucleotide therapeutics will be developed for several murine diseases models indeed we feel that GBM is an ideal target for this.

c. Describe the steps you have taken to reduce the usage of animals and to minimize the lethality of procedures in your experiments (e.g., using cell culture, computer simulations, or non-living models; doing pilot studies, using most specific assays possible).

Chemically modified oligonucleotides designed to specifically and selectively target a gene involved in a disease are screened and tested *in vitro* before animal models are used, also we have designed our experiments to include an initial step of fluorescence-based distribution study. This experiment gives statistically meaningful data using low numbers of animals. Only in subsequent steps will we test the knockdown efficacy of a smaller subset of oligonucleotides using larger numbers of animals (using a power calculation to determine the appropriate number of animals)

Section III. NUMBER OF ANIMALS REQUESTED: PAIN / DISTRESS LEVEL

a. List the number of animals you will use over the duration (to a maximum period of 3 years) of this protocol.

By NIH policy, you will still have to renew your protocol every 3 years. *This requirement is independent of the need for the IACUC to initially review all animal activities described in your grant proposal, irrespective of the grant's time period.*

All animals must be accounted for, including E-17+ embryos (rats and mice) and neonates. The total for each row should equal the sum of the values in that row.

The total should include all animals bred for use in experiments listed in categories C, D, and E in section IIIa plus all animals generated by breeding that are discarded due to the wrong genotype and/or sex, (Category C).

Species	Number in Pain / Distress Level B (to be used only for USDA - covered species)	Number in Pain / Distress Level C	Number in Pain / Distress Level D	Number in Pain / Distress Level E	Total (should agree with numbers in worksheets I and II, if used.)
Mouse	0	1000	1048 to 2221 (+1734)	0	2048 to 3582
Rat	0	1000	1400	0	2136

Pain / Distress level indicates maximum pain or distress level to be experienced by animal(s):
B = being bred but not used in testing, teaching or experiments (USDA covered species only)
C = negligible;
D = pain / distress relieved by appropriate drug use;
E = pain / distress not relieved by appropriate drug use. See the IACUC Instructions for definitions and examples.

- No Category B is to be used for rodents. Rodents that were once considered to be Category B should be listed under Category C.
- Induction of tumors is Category C if no distress is anticipated or if you monitor the status of animals and euthanize sick animals when detected.
- *Mice used for Ascites Production are always Category "E".*

b. If you have animals in category E, use this space to provide a description of the procedures producing pain or distress, and list the reasons why pain-relieving drugs cannot or will not be used to relieve pain or distress. If pain/distress relief would interfere with test results, justify why that is true.

N/A

c. Are you planning to reuse animals that were previously used for any other experiments or procedures under a different animal protocol?

☐Yes ☒ No If the answer is "yes," please provide more details (number of animals, protocol number, what previous procedures done in these animals)

d. If there are federal guidelines or regulations that *require* the use of laboratory animals then use this space to cite the agency, CFR title, number and specific section (e.g., Food and Drug Administration, 21:CFR1030.110).

Section IV. JUSTIFICATION OF THE NUMBER OF ANIMALS REQUESTED

NIH rules require that animal use must be kept to the minimum consistent with a sound scientific outcome. Please use the space below to justify that the number of animals requested is appropriate for the goals of the experiments.

a. Write a brief description of experimental design. In a table below (for large number of animals, please attach an animal number worksheet in the appendix), show all of the experimental groups and the number of animals per group. Be sure that the totals in the table match the totals shown in section III (Number of Animals Requested). Provide a science-based justification for why you chose the number of animals needed per group or other division of your research.

For each experiment, we will inject the oligonucleotide *via* at least two routes including intravenous via tail vein (iv), subcutaneous (sc), intraperitoneal (ip), intrathecal (it), intracranial (IC) or intracranial with a pump, we will also deliver oligos by nebulisation for targeted lung delivery in order to assess the effect that different administration routes (ROA) have on oligonucleotide pharmacokinetics. In addition, we will assess at least 2 doses within the range of 1μM to 1mM.

Since the oligonucleotides and conjugates might have toxicity that we are not expecting, we are requesting 10% additional mice to compensate for this possibility.

Anaesthetic Pilot: We intend to use an atypical injectable anaesthetic in mice and rats. Fentanyl/Midazolam/Dexmeditomidine has been shown in the literature* to be a safe, effective and completely reversible anaesthetic cocktail in several animal models, across several routes of administration. We want to use this coctail because having the capability to precipiate termination of anaesthesia is extremely beneficial in reducing animal mortality and allows for return to the home cage environment sooner. As this is an uncommon cocktail to use in mice, we would like to conduct a small scale pilot study in order to determine appropriate dosing regimes for different strains of mice before using this as our standard anaesthetic for invasive surgery.

Experiment #1: **Characterization of the pharmacological properties of the oligonucleotides**
The objectives are to conduct the initial characterization of the pharmacological properties of the oligonucleotides in vivo and require fewer mice that are wild-type. An immunology study to determine the immunogenicity of the clusters will be also performed.
We will determine the effects on biodistribution of conjugation of a clinically validated oligo to our clusters using in vivo fluorescent imaging. Different clusters designs are conjugated to a near IR cyanine dye to enable detection in vivo.

Experiment #2: **Sequence screen, timecourse, dosing, pharmacokinetics and pharmacodynamics and timecourse of sequences**
The objectives are to to establish active oligonucleotide sequences and characterise their biological effects before incorporation in to the clusters/dendrimers we have designed. This provides a baseline of activity/ cell permeability/toxicity against which the conjugated versions can be compared.

Experiment #3: **Novel structure tests**
Using the best sequences identified in experiment 2, we will build clusters/dendrimers that incorporate these oligonucleotides and compare their efficacy, immunogenicicty and PK/PD to the unconjuagted version.

Glioblastoma
The objectives are to implant immunocompromised animals with human GBM tumour cells and to allow the tumours to growWe will then give a series of different combinations of drugs in order to try to reduce tumour volume and increase survival as described below.

1) Tumour Kinetics
We first need to determine the ideal strain to use for tumour engraftment and then to continue with for the remainder of the study. We have been afforded 3 evaluation strains by Charles River: NCI Athymic NRc-nu/nu, Fox Chase SCID and Fox Chase SCID Beige.
Once this has been assessed, we would need to determine the kinetics of tumour growth while under Temezolomide (TMZ) therapy, the current chemotherapeutic of choice for GBM. This is because one of our target genes is responsible for modulating the efficacy of TMZ and we need to be able to have TMZ only group to control that study and to determine the best dose of TMZ to use.

2) Distribution within the Brain
We also need to test the variety of chemistries available to us to determine which distributes best within the brain. Oligonucleotide drugs come in a variety of different chemistries, and it's important to screen these in vivo as many of them have wildly variable distribution profiles that are not obvious in vitro.

3) Efficacy of gene silencing
When we have determined which chemistries distribute most effectively throught the brain, we need to evaluate our best gene targets found in vitro, in vivo. We intend to use a survival metric to determine this.

4) Survival Studies
Survival Studies are required in order to determine the actual efficacy of the oligo drugs given.
a) Survival of animals with one oncogene being targetted
Several different oncogenes are individually targetted and survival examined in treated animals
b) Survival of animals with a single gene being targetted with TMZ co-treatment
TMZ is given along with the top 2 candidates from 4a to determine whether it promotes survival
c) Survival of animals with 2 genes being targetted using different oligo drugs
Combinatorial survival of animals following knockdown of 2 separate gene targets is assessed here in order to detemrine whether silencing two oncogenes improves over knocking down one.
d) Survival of animals with MGMT targeting and TMZ co-treatment
MGMT is silenced and TMZ given to determine the extent to which MGMT knockdown affects survival with TMZ administration.
e) Survival of animals with MGMT targetting, TMZ co-treatment AND targetting of a separate oncogene
MGMT is silenced, TMZ is given AND a supplemental oncogene is knocked down.

	Number/group	ROA	design	Replicates	Timepoints	Category C	Category D
Anaesthetic Pilot (mice)	8	1	1	5 (different strains)	1	0	40
Anaesthetic Pilot (rat)	8	1	1	1	1	0	8
Exp #1 (mice)	2	4	9	7	1	144	144
Exp #1 (rat)	2	4	9	7	1	144	144
Exp #2 (mice)	2	4	25	2	3	600	600
Exp #2 (rat)	2	4	25	2	3	600	600
Exp #3 (mice)	8	4	10	2	1	320	320
Exp #3 (rat)	8	4	10	2	1	320	320

Tumour Kinetics	3 strains	n=4/ time point	36 mice	
TK with TMZ administration	best strain, latest timepoint from TK	n=3/ time point./TMZ dose	3 doses (25, 50 and 100 mg/kg)	54 mice
Chemistry sceen-distribution	n=2/ ROA, 3 ROA's	1 time point	10 different chemistries	60 mice
Efficacy Screen	3 best chemistries, 2 Doses	n=8/group aCSF, NTC, Dose 1, Dose 2	10 different genes	960 mice
Single gene KD survival	best chemistry, best ROA	5 different genes (best from ES)	n=20/group, 2 groups (aCSF and oligo)	200 mice
Single Gene KD with TMZ	top 2 gene targets (1 administered only), TMZ added	n=12/group, 4 groups: aCSF, TMZ only, oligo, oligo + TMZ	1 TMZ dose only	96 mice
2 Gene KD Survival	2 genes silenced	n=12/group, 6 groups: aCSF, Gene 1, Gene 2, Gene 1 + Gene 2, Gene1+Gene2 (same molecule) NTC for		96 mice

		Gene 1 + Gene 2 (same molecule)		
MGMT KD with TMZ	MGMT knocked down, TMZ given	4 groups, n=12/group: aCSF, TMZ only, MGMT, MGMT+TMZ		48 mice
MGMT + 2nd oncogene KD with TMZ	MGMT Knocked Down, 2 other oncogenes knocked down, TMZ given	5 groups. n=12/group: aCSF, Gene 1+2, Gene 1+2 with TMZ, Gene 1+2 +MGMT, Gene 1 + 2 + MGMT with TMZ		60 mice

Sample size justification:

We will carry out sample size calculations based on the actual knockdown we observe in earlier aims. For the purposes of this protocol, we make the conservative assumption that we will be able to obtain a knockdown of 50±30%. In this case we calculate that we will require n=8 to obtain 95% power for a type I error rate of 0.05. (http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html). If greater knockdown is observed, we will be able to reduce the number of mice accordingly.

b. Describe, in general terms, the statistical tests required for the study.

Our outcome is percent knockdown, to be transformed to achieve normality (arcsine). We will compare our three groups (PBS, test compound, non-silencing control) using 2 one-way ANOVA followed by Tukey-Kramer post-hoc comparisons.

* http://link.springer.com/article/10.1186%2Fs12917-014-0198-3
http://www.ncbi.nlm.nih.gov/pubmed/22439839
http://onlinelibrary.wiley.com/doi/10.1111/j.1439-0442.2004.00632.x/full
http://www.sciencedirect.com/science/article/pii/S0165027006003347

Section V. ANIMAL SPECIFICS

a. Describe the age/weight, sex, and source of each animal species/strain.

SPECIES / STRAIN	AGE / WEIGHT	SEX	*VENDOR
Mus musculus (healthy phenotype wild type), immunocompromised mice	4 weeks – 6 months	both	Approved vendors
Rattus Norvegicus (healthy phenotype wild type)	4 weeks – 6 months	both	Approved Vendors

*Note that the UMass Transgenic Core should be listed as a vendor if animals are produced there for you. If you are receiving animals from another investigator, indicate the name of the PI and protocol number.

b. If any of your animals have special needs, use the space below to list needs for special handling or housing. If animals cannot be socially housed, please explain why

Mice that have been catheterized are housed separately in order to maintain the catheter placement.

c. Specify what parameters you will assess to ensure that the animals are healthy before your experiments begin. Check all boxes that apply.

Activity	x	Appearance	x	Appetite	x	Behavior	x
Excreta		Respiratory Pattern	x	Temperature		Weight	x
Laboratory tests or other observations (specify)	Chemistry profile will be obtained after injection of the agents weekly to monthly. Animals will be weighed every week unless it appears to be particularly thin, in which instance it will be weighed as noticed. All animals will be weighed to obtain a baseline weight before experimentation.						

Section VI. EXPERIMENTAL PROCEDURES: (*except* any survival surgery and procedures that may lead to a permanent physical or physiological handicap)

IACUC reviewed procedures

a. Basic chronology (please use a flow chart or a sequential list of activities)

Experiment #1

1) injections 2) assays in animals as needed (live imaging 1hr to 1 week after injection, up to once a day; blood draws minutes to days after injection, up to the limits specified in section (b) below) 3) perfusions for neuropathology/organs harvested for biochemistry or real-time PCR or bDNA assay (1-14 days post injection).

Experiment #2

1) injections or cannula implantation followed by oligonucleotide infusion 2) assays in animals as needed (blood draws, minutes to days after injection, up to the limits specified in section (b) below) 3) perfusions for neuropathology/organs harvested for biochemistry or real-time PCR or bDNA assay (1-14 days post injection).

Experiment #3

Injections or cannula implantation followed by oligonucleotide infusion 2) assays in animals as needed (blood draws, minutes to days after injection, up to the limits specified in section (b) below) 3) perfusions for neuropathology/organs harvested for biochemistry or real-time PCR or bDNA assay (1-14 days post injection).

Experiment #4

1) Injection of the GBM cells into the striatum 2) imaging of the tumours by IVIS or MRI or CT 3) injection of the therapeutic compound at various time points into the tumour, or IC 4) imaging of tumours OR tissue harvest and assessment of drug distribution by fluorescence imaging of fixed tissue 5) co-administer temozolomide OR monitor weight of animals until they meet criteria for termination 6) sacrifice the animals, remove the brain for immunohistochemistry, fluorescence imaging and/or bDNA assay.

b. Detailed description of procedures (do not include details of in vitro procedures): Describe in narrative form all procedures to be carried out on living animals from initial contact to euthanasia.

Anaesthetic Pilot

Successful anaesthesia will be assessed by toe pinch. We will monitor the time to non-withdrawal, the duration of anaesthetic depth (by repeated toe pinch every 10 minutes) and the total time to recovery without administration of reversal agents. We will also reverse anaesthesia chemically after 30 minutes in order to assess the effect on animals and to ensure no adverse effects.

Intravenous via tail vein (iv), subcutaneous (sc), intraperitoneal (ip), or intracranial (IC) or intracranial with a pump, in order to assess the effect that different administration routes have on oligonucleotide pharmacokinetics.

Injections:

1) **Subcutaneous Injections:** The mice are scruffe and the needle is advanced through the skin and a bleb raised over the nape or flank. The mouse is returned to the cage and the cage to the rack. This is repeated as needed. Similar process for rats, except that two people are required to safely scruff and restarin the rat.
2) **Tail Vein Injections:** Animals will be restrained using a mouse-compatible device for tail vein injections, and the tail will be cleaned by wiping with 70% ethanol pads. Oligonucleotide preps, or PBS will be infused using an insulin syringe. The needle will be removed and compression will be applied to the injection site with sterile gauze to ensure complete hemostasis. Animals will be returned to their cages and observed for 10 min. The entire procedure will take approximately 20 minutes.
3) **IP Injections:** One mouse is removed and scruffed and injected laterally to the midline at the line of the knee (bottom left quadrant of the thorax to avoid the cecum). The mouse is returned to the cage and the cage is returned to the rack. Similar process for rats, except that two people are required to safely scruff and restarin the rat.
4) **Intraarterial infusion**: Animals will be catheterized as described in (FORM D). Intraarterial infusion will be performed after carotid artery cannulation. Lock solution to maintain patency will be flushed from the system and an infusion pump or a peristaltic pump will be used to perfuse drugs and control solutions.

Bleeding

< 10% of the total blood from animals will be collected in a 24 hour period. If blood draws are needed after the 24 hour period, we will wait 2 weeks to allow the mouse to recover completely. For serial bleeding (PK/PD studies), 20 uL up to 10 times in the course of one day, no more than 10 times in total.

5) **Tail vein (IACUC 6.02)):** Animal is made comfortable in a mouse restrainer and maintained at a temperature of 24-27^{O}C. Tail may be warmed in 40^{o}C water or by using a specifically designed restrainer. A needle is used to incise the tail vein resulting in the formation of a drop of blood which is collected via capillary tube. Pressure is applied to ensure haemostasis.
6) **Saphenous vein:** Animal is restrained as for tail vein. Back of the hind leg is trimmed. Hind leg is immobilized and slight

pressure is applied gently above the knee joint. The vein is punctured using a lancet or needle and blood is collected.

7) **Mandibular bleed (IACUC 6.01):** Open the sterilized individual lancet for the prospective mouse. The mouse is scruffed and restrained and the mandibular bleed undertaken as descibed in the SOP.

Following any non-terminal bleeds, site of blood draw will be treated with first pressure and animals will be monitored.

Terminal bleed: Animals are euthanized (double killed) before an incision in the thorax is made to collect the blood via cardiac puncture. Or, the animal is deeply sedated and blood drawn through the chest from the heart, once no more blood is available, the animal will be euthanized by cervical dislocation and/or bilateral thoracotomy.

Live Animal Imaging (IVIS or Pearl): The mice are carried to the imaging room in their own cages. We will follow the IACUC guidelines on IVIS and Pearl imaging http://inside.umassmed.edu/IACUC/Policies/Pearl-Imaging/ and http://inside.umassmed.edu/IACUC/Policies/IVIS-Imaging/
After imaging the mice are placed back into their cages where they wake up. Then they are carried back to the animal room (outback).

Timed Pregnancy for embryo or tissue culture from Immunocompromised Animals to screen compounds for Glioblastoma
Prior to animal studies we will assess the potency of the oligonucleotide in *in vitro* cultured primary cells from mice. This will allow us to design the *in vivo* animal studies more efficiently and also to progress with the best oligonucleotide candidate.

Experimental Design: Mouse primary neurons and neural stem cells (NSCs) will be isolated from E17-18.5 pups. The mother will be checked regularly for a plug after introduction of a male, once the plug is found (day 0) we will observe her for 17-18.5 days, euthanise her according to Section VII and harvest tissue from her pups.

Totals: We are expecting to run several time breeding experiments, where we will be using the whole litter (expected litter size of 6) plus the mother. These animals are category C. We are not requesting any more animals for this at this stage because we feel breeding the immunocompromised animals will provide sufficient cells for our *in vitro* requirements.

Lumbar intrathecal injection: Lumbar intrathecal (IT) injection will be performed in anesthetized animals via a modified protocol based on Fairbanks (Fairbanks 2003) and Hylden et al. (Hylden and Wilcox 1980). The lower hind of the animal is shaved and the skin prepared. The site of injection will be between L5 and L6. This is an area close to the terminus of the spinal cord and the inception of the cauda equina. The L5 and L6 site is used to maximize vertebral accessibility and minimize any possible spinal damage to the animal. Each animal is manually restrained by firmly grasping the pelvic girdle in the non-dominant hand and gently pulling upward from the pelvis. The needle is held at an approximate 20° angle from the spinal column. The needle is inserted into the tissue laterally to the spinous process of L5. The needle will gently slide into a groove between the spinous and transverse processes. Once the needle has entered this groove, the angle is dropped from 20° to 10° and the needle is gently guided posteroanteriorly into the intravertebral space until approximately 0.5 cm (mouse) of the needle resides in the spinal column. Puncture of the dura will be indicated by a reflexive lateral flick of the tail or formation of an "S" shape by the tail. The solution is injected slowly, without stopping. At the end of the injection, the needle is rotated approximately ¼ hand turn upon removal (to ensure proper maintenance of tissue as well as minimization of any puncture damage). The volume delivered is well under the volume that could cause volumetric-related discomfort in the animal. Animals will be observed until they are able to maintain sternal recumbency. An analgesic will be administered following intrathecal injection (Form E1).

Mouse: Up to 10 µl per mouse will be administered by IT administration with a total dose ≤1mmol.
Rat: Up to 10 µl per rat will be administered by IT administration with a total dose ≤1mmol.

Orotracheal intubation and direct instillation of agents (Mice):
All recipient animals will be anesthetized (fentanyl/midazolam/dexmedetomidine). Mice with then be placed in dorsal recumbency on a rodent workstand (Braintree Scientific, Braintree MA) with head extended using an incisor loop. The larynx will be visualized using a Welch Allen otoscope and a 20 gauge 1.25in catheter placed with the assistance of a guide wire. The intubation catheter will be used to instill experimental agent diluted in 1x sterile saline (agent will be concentrated such that the total volume instilled will not exceed 75ul). Following instillation, mice will receive 10 seconds of ventilation using an artificial ventilator with a tidal volume of 10mL/kg per breath and then the catheter will be removed. Mice will be monitored closely post-intubation, until they have fully recovered from anesthesia. The entire intubation and agent delivery should take less than 1 minute, allowing inhalation anesthesia to be used. Animals will be monitored daily for the next 48 hours.

Orotracheal intubation and nebulization of agents (Mice):
All recipient mice will be anesthetized (fentanyl/midazolam/dexmedetomidine). Mice are intubated using a sterile 20g as described above. When inserted, the cannula will be advanced ≈5 mm. The fiber optic cable is then withdrawn and the cannula is attached to the ventilator of the flexiVent via Y-tubing. No more than 50 uL of agent will be administered. The ventilator settings are pre-configured and the cannula is calibrated prior to intubation. To ensure proper placement once the mouse is ventilated, a deep inflation is administered to ensure bilateral chest rise. In addition, during the deep inflation, a pressure of 30 cmH_20 over a 3 second period is applied and this also ensures the absence of a leak.

Once adequate tube placement is confirmed, the Aeroneb ultrasonic nebulizer will be used to deliver aerosols into the lung. The catheter will then be removed and mice will be monitored closely post-intubation, until they have fully recovered from anesthesia and daily for the next 48 hours.

Terminal perfusion:
Animals are transported to the lab. Animals are anesthetized. When the anesthetic has taken effect, they are laid on a towel and their fur is rinsed with 70% alcohol. An incision is made in the abdomen and thorax to expose the heart. The perfusion needle is inserted into the apex of the heart and the right atrium cut . PBS followed by fixative is perfused throughout the body using a pump.

c. Indicate how you will identify animals

Animals will be identified by ear tagging or ear notching.

d. Will the studies result in adverse effects on animal health or well being?

☒ Yes ☐ No If yes, please complete form H

Section VII. TERMINATION OF STUDY / EUTHANASIA

Unless specified otherwise by the Principal Investigator, Department of Animal Medicine personnel who are euthanizing animals on behalf of the Principal Investigator will do so using any method listed as acceptable for the species in the most recent edition of the euthanasia guidelines of the American Veterinary Medical Association. Neonatal and pregnant rats and mice will be euthanized using methods approved by the UMMS IACUC and posted on the IACUC intranet site.

a. Is death used as an endpoint in this study? Death as an endpoint means that the animal is permitted to die as a result of experimental manipulation, i.e. exclusive of planned euthanasia. If yes, explain why an earlier end point is not acceptable. (Studies using death as an endpoint are Category E)	☐ Yes	☒ No

b. What criteria will be used to perform euthanasia earlier than planned?

Animals will be euthanized if they display signs of pain and distress such as not eating, not making nests, or losing more than 15% of body weight comparing to the maximum weight. All animals will be checked every day including weekends and holidays. Weight will be determined every other day.
Animals involved in the survival study will be removed and euthanized when they appear moribund, typically displaying hunched posture, tremor and inability to move well.

c. Other Use – Will animals be available for further use by other investigators? Link to the Animal Medicine Exchange Board at http://i.umassmed.edu/ExchangeBoard/messagepage.aspx

No

d. Describe the method(s) of euthanasia for each species or procedure. For injectable drugs, give name, dose and route below. US Government policy requires the use of pharmaceutical grade drugs[1].
Must comply with the American Veterinary Medical Association (AVMA) Guidelines on Euthanasia.

Species	Method/Drug	Dose (mg/kg body wt.)	Route
Mouse and rat	CO2 asphyxiation	To follow IACUC 5.01	Inhalation
Mouse and rat	Pentobarbital overdose (As Fatal Plus or diluted fatal plus, 1:10 in sterile saline or PBS)	>150mg/kg	IP
Mouse and rat	Thoracotomy/exsanguination/Cervical Dislocation under anesthesia following overdose of anaesthetic (more than 1.5 x the anaesthetic dose)	See Form C	Inhalation or injection (of anaesthesia)
Mouse	Conscious Cervical Dislocation (Mike Moazami only)	To effect as per http://inside.umassmed.edu/IACUC/Policies/Converted-pdfs/502_EUTHANASIA-OF-MICE-AND-RATS-BY-CERVICAL-DISLOCATION-CD/	Physical
Mouse embryos	Decapitation (following: http://inside.umassmed.edu/IACUC/Policies/Converted-pdfs/503_EUTHANASIA-OF-SMALL-LABORATORY-RODENTS-Mice-Rats-Hamsters-Gerbils/)or Fatal Plus as per IACUC 5.04.02	FP >800mg/kg	Physical, IP

[1]A pharmaceutical grade compound is a drug, biologic, or reagent that is approved by the Food and Drug Administration (FDA) or for which a chemical purity standard has been established by the United States Pharmacopeia-National Formulary (USP-NF), or British Pharmacopeia (BP).

e. Current rules require that after euthanasia, death be confirmed by using a second method. For example bilateral pneumothorax is done after euthanasia using CO_2. Indicate below how you will double kill your animals.
Cervical dislocation, decapitation or bilateral thoracotomy with/without exsanguination followed by 4% paraformaldehyde. Animals euthanised with conscious cervical dislocation will be double killed either by bilateral thoracotomy or major organ (heart and/or brain at the minmum) harvest

Section VIII. DATABASE SEARCHES

In the space below, document that you have searched databases

1. To determine that you are not unnecessarily duplicating previous experiments,
2. To determine that alternatives to animal use are either not available or not appropriate,
3. To determine that procedures involving animals will avoid or minimize discomfort, distress and pain, AND
4. To determine that alternatives to procedures that may cause more than momentary or slight pain or distress to the animals have been considered

Sources of information regarding possible duplication and alternatives include:

- Medline (http://www.ncbi.nlm.nih.gov/pubmed)
- Animal Welfare Information Center (awic.nal.usda.gov/)
- UMMS veterinarians (508.856.3151)
- National Agricultural Library (www.nal.usda.gov/)
- WWW Virtual Library of Veterinary Medicine (http://netvet.wustl.edu/vetmed.htm)
- Altweb (http://www.jhsph.edu/~altweb)

a. Dates of Searches:
29-Nov-2016
b. Name of the database(s) you searched:
Pubmed, Reaxys
c. Years Covered by Searches:
1966-2016
d. Keywords Searched:
oligonucleotide dendrimer in vivo, oligonucleotide dendrimer distribution, fluorescent oligonucleotide dendrimer, alternative to animal use, glioblastoma, GBM, glioblastoma model, glioblastoma end point, humane end point, surrogate end point, intracranial injection, intrastiratal injection, intrastriatal tumour mouse, oligo inhalation, oligonucleotide inhalation, antisense lung delivery
e. Other Sources of Information:

Section IX. PARTICIPATING PERSONNEL (including PI)

a. Identify all personnel working with animals at the time of this application.

Name	UMMS Phone	Emergency Phone	What are this person's responsibilities with animals on this study?	Years of experience with the technique to be used	Years of experience with the technique in species to be used	[2]First time animal user at UMMS (Yes/NO)	[3]On-line training courses completed (Yes/No)	[4]Occupational health requirements completed (Yes/No)
Jonathan Watts	774-455-3784	774-243-7931	PI	0	0	Yes	Yes	No
Mike Moazami	774-455-3765	774-666-0180	All procedures	1	1	Yes	Yes	Yes
Andrew Coles		508-243-5813	All procedures, excepting conscious cervical dislocation	14	14	No	Yes	Yes
Bruno Godinho			All procedures, excepting conscious cervical dislocation	4	4	No	Yes	Yes
Maire Osborn		508-654-6215	All procedures, excepting conscious cervical dislocation	0	0	Yes	Yes	Yes
Mai ElMallah			Training in orotracheal intubation and lung-delivery methods			No	Yes	Yes
Alisha Gruntman			Training in orotracheal intubation and lung-delivery methods			No	Yes	Yes

b. Briefly describe how the individuals with less than one year of experience with the technique(s) in species will be trained and/or supervised. Identify the individual(s) who would be responsible for training and/or supervising new or inexperienced personnel. Note that hands-on training can be given by an experienced personnel or designated Department of Animal Medicine staff. Those who perform survival surgery must receive aseptic Survival Surgery Training from the Department of Animal Medicine before approved as a surgeon. Please contact Van Gould (extension 66811) or Suzanne Wheeler (extension 62363) for training.

All personnel with limited experience will be trained and supervised by Andrew Coles (Khvorova Lab) or trained lab members in mouse handling and other procedures.
All inhalation procedures will be learned from the labs that currently maintain the inhalation apparatus. Protocols A2519 and A2494.

For survival surgery training, we will follow the training outlined below prior to any survival surgeries.
1. Observing others doing the procedure, 2. Practice surgery on dead animals, 3. Non-survival surgery on live animals, 4. Survival surgery supervised by a trainer. The first few surgeries will be conducted side-by-side with Andrew Coles or other trained surgeons.

ADDITIONAL NOTES ON PARTICIPATING PERSONNEL:

IACUC training webpage IACUC new personnel webpage

- Before other new personnel perform any procedures, a written minor amendment request must be submitted to and approved by the IACUC. All personnel new to UMMS or those who never worked with animals will have to complete all the Training and Occupational Health and Safety Requirements before added on an IACUC protocol.

- The principal investigator is responsible for ensuring that all personnel adhere to the conditions approved by the IACUC.

Section X. APPLICANT'S CERTIFICATION

IACUC is charged with carrying out the rules and regulations of the Federal Government's Animal Welfare Act governing the care and use of animals in research and instruction. The Act stipulates that (a) Principal Investigators must give written assurance that the activities do not unnecessarily duplicate previous experiments; (b) procedures involving animals must avoid or minimize discomfort, distress, and pain to the animals; (c) Principal Investigators must consider alternatives to procedures that cause more than momentary or slight pain or distress to the animals and give a written description of methods used to determine that alternatives are not available; and (d) paralytic agents cannot be used in unanesthetized animals. Accordingly, the Applicant, who must be a member of the faculty holding Principal Investigator status, is required to read and sign the following certification:

BY SIGNING BELOW, I CERTIFY THE FOLLOWING:

1. I am thoroughly familiar with the literature in the field of research proposed in this application, and I have determined that the research does not unnecessarily duplicate experiments, that appropriate non-animal models are not available, and that the research must be conducted on living animals.
2. I will abide by all UMMS policies and procedures regulating use of animals in instruction and research, by the provisions of the PHS/NIH Guide for the Care and Use of Laboratory Animals, and by all other applicable laws, policies, and regulations governing the use of animals in instruction and research.
3. I will supervise all experiments involving live animals. Furthermore, I will ensure that all listed participants are qualified or will be trained in proper procedures, including animal handling, anesthesia, surgery, post-procedural management, and euthanasia. Also, I will ensure that individuals not listed in the application will not have responsibility in experiments involving animals.
4. All listed personnel will read the IACUC-approved Application to Use Vertebrate Animals in Research or Instruction before undertaking any procedures on laboratory animals.
5. Survival surgery will be performed using standard aseptic procedures.
6. Animal Medicine clinical veterinary staff will be consulted as needed to ensure satisfactory veterinary care.
7. In the event of an animal health emergency, my staff or I will contact the Department of Animal Medicine. We will not attempt animal treatment by ourselves, unless it is a life-threatening emergency.
8. If I cannot be contacted, and animals in this project show evidence of illness or pain, emergency care, including euthanasia, may be administered at the discretion of the Animal Medicine veterinary staff.
9. Significant changes in study objectives and procedures require IACUC approval.
10. Unanticipated adverse events will be reported to the IACUC as required by UMMS IACUC policy
11. This application meets all animal use and care requirements of the funding agencies that have been asked to support the research.
12. By signing below, I certify that all animal studies described in grant proposals using this protocol are described in this animal use application.

PI's Signature: [signature] Date: May 4, 2017

GRANTS ASSOCIATED WITH THIS ANIMAL PROTOCOL

Sponsor	Funding period	Grant ID #

FORM A TRANSPORTING ANIMALS FOR USE OUTSIDE OF OR TO ANOTHER AREA OF THE ANIMAL FACILITIES Animal Transfer Policies		
A1. Will animals be used in areas, e.g., laboratories, outside one of the general animal facilities? (A Level, BioTech II, BNRI 1st floor, LRB 1st floor, Rose Gordon, ASC Facility)	☒ Yes	☐ No
If "yes", list the building and room number(s) where animals will be housed or used outside the animal facility.	Sherman Center AS4-1006/ AS5.2004A for inhalation work	
A2. Indicate which of the following procedures will be used outside of the animal facility: ☒ Fluid Collection/Tissue Harvesting ☐ Non-Surgical Procedure ☒ Non-Survival Surgery ☐ Survival Surgery ☐ Irradiation ☒ Core Service/Imaging ☐ Other:		
A3. Will animals be held, housed, and/or used in study areas outside of the animal facility for more than 12 hours for USDA regulated species or 24 hours for non-USDA regulated species? If "Yes", in the space below list the building and room number(s), and justify scientifically the need to hold animals.	☐ Yes	☒ No
A4. Use this space to describe how you will transport animals from the animal facility to a study area outside of the animal facility		
Before transport to the laboratory animals will be transferred to fresh containers and placed within plain paper bags for hand carrying to the laboratory. For the simultaneous transport of several cages, the cages will be placed on a cart after being placed in paper bags. All transport to the laboratory will be done via the freight elevators in the Sherman Center.		
A5. Is a patient procedural area to be used for animal studies? If "Yes", use the space below to provide room number and/or location of patient area. If animals are to be transported to the patient area, you will require the approval of the Infectious Disease Committee (ICC). Please fill out the ICC Approval form and send to Mack, Deborah Ann (Infection Control) (UMMHC) DeborahAnn.Mack@umassmemorial.org. Contact Deborah Ann Mack at 508-856-5843, if you have questions.	☐ Yes	☒ No
A6. If "Yes", describe any special animal transport or facility procedures that will be followed to assure health and safety of both animals and patients.		
A7. Will animals for your use be transported to a different area of the animal facility or to a different UMMS animal facility? ☐ Yes. If yes see below.		
☐ I agree to use the Web Animal Ordering System for all transfers within the animal facility, except as noted below. ☐ Our work requires that we transport of animals to different areas of the animal facility for activities approved by the IACUC e.g. irradiator, biocontainment suite. Explain very briefly below.		

FORM B
BREEDING

Use this section if you are breeding animals for this project.

B1. Describe the species, strain, and source of each animal that you plan to breed

SPECIES / STRAIN	AGE	WEIGHT	VENDOR
Immunocompromised Mice*	All	All	Approved Vendors
*strain not yet determined, evaluation animals from Charles River to be trialed			

B2. Specify what parameters you will assess to ensure that the animals are healthy. Check all boxes that apply.

Activity	X	Appearance	X	Appetite	
Excreta		Respiratory Pattern		Temperature	
Laboratory tests or other observations (specify)					

B3. In this space describe how many animals you anticipate breeding over the next 3 years. Briefly explain your calculations below.

1734
This is the total number of immunocompromised mice required for the study. We intend fully to breed all of the mice required for this work, however it is not unreasonable to imagine that we might need to purchase animals for studies that require a large number of mice at very similar ages. Nonetheless we want the flexibility to breed up to the total number of mice required.

B4. Anticipated health problems in the animals being bred. If there are known health or well being issues associated with the animals you are proposing to breed, or their offspring, please describe them in detail.

B5. Male:Female Ratio

☐ A ratio of 1:1 will be used. No continuous breeding

☐ A ratio of 1:1 with continuous breeding will be used. See below

☒ A ratio of 1:2 or 1:3 will be used (harem breeding). See below

☒ I have read the IACUC Mouse Breeding Policy that prohibits more than one litter per cage. I agree to separate pregnant females to other cages prior to their giving birth in harem breeding and to wean continuously bred litters at day 21 if the female is pregnant.

Mouse Breeding Policy

B6. If your animals have special needs, use the space below to list needs for special handling or housing.

B7: Describe how you will identify your animals (e.g. ear tags):

No need to identify beyond a cage card

B8. Use this space to describe what will be the disposition of retired breeders or any excess animals.

Excess animals will be incredible few as the genetic modification is homozygous.

B9. Will you require special caging? (If so it is your responsibility to provide it)

No

B10. How often will each male and female be bred?

As often as necessary to generate the numbers of mice required for individual studies.

B11. If you will be using inbred or outbred animals, describe how you will ensure that they remain in- or outbred:

Backcrossing

B12. Describe any special handling that you anticipate:

None

B13. Will males be removed from cages before birthing?

☒Yes ☐ No for continuous breeding (if no, explain why not)
B14. What nesting materials will you use? (For rodents, consider Nestlets or similar material)
Nestlets and/or mouse houses and/or paper
B15. At what age will the young be weaned?
3 weeks
B16. Will the offspring be genotyped, and if so, at what age and by what method?
No, no need.
B17. Will mice be bred and transferred to other investigators? ☒YES ☐NO
If yes please check the attestation ☒ I agree that all transfers to another investigator whether within the Animal facility or directly to a laboratory must use electronic Web Animal Ordering system. I understand that failure to do so represents unapproved animal use under the donating investigator's (my) protocol and I will be responsible for all regulatory issues, violations, etc arising from such use. Use of animals not on the recipient's protocol also represents a protocol violation of his/her protocol.

FORM C
ANESTHESIA
Anesthesia and Analgesia

C1. PRE-ANESTHETIC AGENTS (e.g., tranquilizers, narcotics) and ANESTHETIC AGENTS: Describe how these agents will be used in your studies. If none will be used, enter "none" in the "Agents" column.

Frequency of administration	Species	Pre-anesthetic Agents & Anesthetic Agents	Dose	Route/Volume
For crainial or vascular surgery or intrathecal injections or bilateral pneumothorax/perfusion	mouse and rat	Fentanyl/Midazolam/Dexmedetomidine	0.01-0.1 /0.5-10/0.025-2.5 mg/kg (initial trial dose, subject to change based on pilot study results)	intraperitoneal
For intracranial injections/live imaging	mouse and rat	Isoflurane	Up to 5 % (in a chamber) To 2 % (nose cone)	Inhalation
For crainial or vascular surgery or intrathecal injections or bilateral pneumothorax/perfusion	mouse and rat	Ketamine/Xylazine	100mg/kg / 10mg/kg redose at 25% of original ketamine (only) dose if the animal appears to be regaining consciousness during surgery or is not completely unconscious in the anticipated time	intraperitoneal
For crainial or vascular surgery or intrathecal injections or bilateral pneumothorax/perfusion	mouse and rat	Ketamine/Midazolam	100/5 mg/kg redose at 25% of original ketamine (only) dose if the animal appears to be regaining consciousness during surgery or is not completely unconscious in the anticipated time	intraperitoneal

C2. MONITORING OF ANESTHESIA: Describe (a) what will be monitored (e.g., corneal reflex, heart rate, respiration, response to noxious stimulus) and (b) how frequently each of these variables will be monitored.

A form (suggested template) for recording Rodent Anesthesia and Analgesia can be obtained from the IACUC website: RODENT ANESTHESIA RECORD FORM

Watch for slowed, regular respiration (55-65 breaths/min), pink mucus membranes, non-response to noxious stimuli (toe pinch withdrawal every 10 minutes) and blink reflex. Non response to toe-pinch will be confirmed before any incision or depilation.

C3. Use of Isofluorane: It is IACUC policy to ensure that animals do not contact liquid isofluorane. Please see the IACUC policy on isoflurane use.

Animals never come into contact with the isoflourane liquid by using a vaporizer.

C4. Describe the anesthetic gas scavenging system you will use to eliminate waste anesthetic gas. Follow the link for the policy on scavenging anesthetic gas.

Facility evacuation system.

C5. Are any of the anesthetic or pre-anesthetic agents listed in C1 non-pharmaceutical grade? A pharmaceutical grade compound is a drug, biologic, or reagent that is approved by the Food and Drug Administration (FDA) or for which a chemical purity standard has been established by the United States Pharmacopeia-National Formulary (USP-NF), or British Pharmacopeia (BP).

☐Yes ☒No If the answer is "yes," please explain
US government policy requires the use of pharmaceutical grade drugs especially for anesthetics or analgesics. Exceptions include unavailability of pharmaceutical grade drug and lack of a suitable alternative. Exceptions require scientific justification.

FORM D

SURVIVAL SURGERY INCLUDING LAPAROSCOPY, ENDOSCOPY, and any procedure that has a *reasonable* potential of causing a permanent physical or physiological handicap.

Complete this section if any animals will recover from local or general anesthesia after a surgical or other major operative procedure.

D1. In the space below, explain why it is necessary for the animals to recover from surgery/anesthesia.

We are evaluating of oligonucleotide to determine if they are able to knockdown the gene expression. Typically, organs are harvested 5-7 days post injection meaning that the animals need to recover from their surgery and continue living. Also in order to determine any negative effects.

D2. In the space below, describe pre-operative care (including physical examinations, lab tests, and any preconditioning apparatus). All anesthetic agents and pre-operative medications should be listed in Form C.

We inspect the animal's weight, movement, and nesting.

D3. Use the space below to describe in detail the surgical procedure(s)/other major procedure to be used.

Animal pre-surgical preparation: IACUC 6.12
They are anesthetized. For intracranial injections, animals are positioned on a stereotaxic apparatus and we will use vetbond to remove fur. The glue is applied carefully to the hair on the top of the head with a brush and when it is dry it is gently peeled off the fur. There is no damage to the skin and it is safer than depilatory near the eye. For non-cranial surgeries, the animals are positioned as appropriate on an operating surface.

Instrument pre-surgical preparation: IACUC 6.10
Instruments are autoclaved before surgery and are placed on a sterile tray during surgery. We also use a dry sterilizer between surgeries. Instruments from the dry sterilizer are allowed to cool before use. Areas used are wiped down with clidox and stereotaxes are cleaned with 70% alcohol.

Surgeon's pre-surgical preparation: IACUC 6.12
There are canisters of Alcare Surgical Hand Scrub in the procedure rooms which will be used for this.

Details of Techniques:

Intracerebral injection
The animals will be anesthetized and placed in a stereotactic instrument, positioned, and the incision site appropriately prepared.

A 1 cm incision is made and the surface of the skull is exposed. The needle used for injection is positioned over the bregma and this is used as the zero position. Using the stereotax to measure, the needle is then positioned at the appropriate co-ordinates rostrocaudally and mediolaterally and it is lowered to the surface of the skull to pinpoint the spot for drilling. A micro drill (diameter <1 mm) is used to make a hole. The needle is then lowered into the brain using the stereotax to measure and guide the needle.

The oligonucleotide, or tumour cells will be delivered by pump (microinjection pump) at an appropriate rate, depending on the site of infusion. This takes 1-30 minutes depending on the volume. The needle is withdrawn and the skin is sutured or closed by clips.

Pump implantation:
The pump, which has been prefilled and primed, is implanted. To do this, a pocket is made through the incision opening at the intrascapular space beneath the skin, toward the animals back by tunneling with sterile blunt forceps. The pump is inserted into the pocket using sterile forceps, and the cannula, which is already attached to the pump by tubing, is lowered through the previously drilled hole. The cannula is attached by gluing it in place on the skull surface with cyanoacrylate gel. The skin is sutured closed using sutures and simple interrupted stitches over the cannula so the pump, tubing and cannula are all subcutaneous. The pump (Alzet 2004) is 1.5cm long and 0.6 cm wide. It holds 100uL of fluid when full and weighs 0.4g. Pumps can be used for up to 4 weeks without being changed. None of our studies require changing the pumps.

After 7-10 days the sutures/staples are removed.

Cannulation: Cannulation is done on the tail vein, femoral vein, carotid artery or jugular vein depending on mouse or rat and how much blood is required. Animals will be housed in an individual cage following procedure in order to prevent grooming behaviors that may cause decannulation.

Tail vein
Prepare the catheter for insertion by preloading it with an appropriate lock solution (Form D). Position the anesthetized rodent on its side so the lateral tail vein is uppermost or the femoral vein is uppermost. One of the keys to successful catheter insertion is to have the animal, particularly the tail, warmed to dilate the vein, which may be partially constricted due to the anesthetic. It can be helpful to place the tail in a small plastic tube of warm water (105° – 115°F). Equally important is to match the angle of the needle with the angle of the vein. To achieve this, the tail is bent slightly so the approach angle of the needle is almost parallel to the vein. Use the dominant

hand to hold the catheter, the other to grasp the tail between the thumb and index finger, thumb on top, then pull back slightly so the tail is firm. The catheter needle is held with a hemostat or forceps and the needle, bevel up, is introduced through the skin at a flat angle and advanced for several mm. Blood will usually enter the catheter if the needle is in the vein. If no blood enters the catheter but you believe it was inserted correctly, using slight pressure with your thumb and sweeping back along the tail will flush blood into the catheter if it is properly inserted. Also, to verify correct insertion and that the vein is not perforated, use a small saline flush into the catheter. If the needle is in the vein, the plunger moves easily and the dark colored vein clears as the saline is injected. If the plunger is hard to push or the tail around the injection site blanches white, the needle tip is not in the vein, so the needle should be withdrawn. If needed, secure the catheter in place with tape (eg, transpore tape). It is critical to keep the needle in the same position once in the vein so it doesn't poke through the vessel wall. Because there is always the possibility that the insertion will be unsuccessful, the first attempt should be made in the end third of the tail, so that subsequent tries can be done on parts of the tail closer to the body, downstream from previous puncture sites. Cannula is held in place by adhesive dressing and sutures. In the instance of self-decannulation or cannula occlusion, the mouse will be recannulated on the opposite tail vein.

Femoral vein

With the animal laying prone (on its stomach), make approximately a ½ inch (12 mm) horizontal incision on the back of the neck at the level of the shoulder blades with scissors or a scalpel, then blunt dissect a subcutaneous "pocket" in the back approximately the size of a quarter. This will be used as an area to place a small amount of tubing that will compensate for animal growth and/or movement. Place the animal onto its back (supine position) and make an incision in the inguinal area approximate ½ inch (12 mm) incision along the natural angle of the hind leg. Blunt dissect to separate the connective tissue until the femoral artery and vein are exposed. Using fine tip forceps gently separate the nerve (whitish in color) that is along the femoral artery away from the artery and vein. Separate the artery and vein as one unit, trying to expose an approximately ¼ inch (5-7 mm) length section of artery/vein. Repeat the process in separating the vein from the artery. Keep your surgical instruments (i.e. fine tip forceps) perpendicular to the vessels and separate the vessels in parallel. This assists in avoiding tearing, pricking or damaging the vessels. By placing the fine tip forceps gently between the artery and vein from underneath and slowly opening the forceps and repeating this, you will slowly separate the vessels. Using the micro-dissecting scissors, make a small incision in the vein approximately ¼ thru and at a 45 degree angle. Place fine-tipped forceps (45 forceps) into the incision and using another pair of forceps; carefully feed in the vein catheter. Gently open the forceps that are placed into the vein, as this will allow the researcher to gently place the venous catheter under the forceps and into the vein. When the catheter is fully inserted (approx. 6-7 cm) tighten the anterior ligature around the vein and catheter, tying a triple knot. Use the silk suture near the leg (posterior ligature) to again secure the catheter (triple knot) and ensure placement. Slowly draw back the syringe until there is a little blood visible in the catheter, which helps to ensure that the suture knots are not too tight and that the catheter is functional. Make a dime to quarter sized loop in the catheters and place on the inside of the leg, secure them with 1-2 stitches of surgical suture into the muscle layer. Close the incision with sutures.

Maintenance of the catheters

Catheters will be maintained for as long as is necessary for the pharmacokinetic experiments, ranging from 5 hours to 5 days. To maintain patency the following will be performed every 2-3 days: clamp the catheter with padded forceps. Remove the catheter plug. Unclamp the forceps. Flush the catheter with 0.05mL sterile saline using a syringe. Place a blunt tipped syringe with lock solution onto the catheter. Fill the catheter with the lock solution (volume is pre-determined-typically 0.3 ml for rat and 0.01mL for mouse). Cannula ports will be wiped clean using alcohol pads before flushing.

Alternative to heparin lock solution

The oligonucleotides we are testing bind to heparin, making the use of this agent in maintenance of the catheters problematic. We will use alternative lock solutions (http://www.nc3rs.org.uk/preventing-thrombosis) such as

1) Sodium Citrate glycerol: This is a 4% sodium citrate and 30% glycerol with pH adjusted to 6.2 (range 6.0 to 6.5) with citric acid.
2) 0.9% saline
3) 40% dextrose
4) 50% sucrose

All lock solutions will be of animal grade. If none of animal grade can be purchased then we will make them according to: http://inside.umassmed.edu/IACUC/Policies/Converted-pdfs/301_USING-NON-PHARMACEUTICAL-GRADE-CHEMICALS-IN-ANIMALS/

Carotid artery cannulation

The description of the following procedure has been adapted from Harlan (HUS-QREC-PRD-921, Issue: 01, Revision: 04) and Martin et al. 2012)

Anesthetized animal is placed in dorsal recumbency. The ventral cervical area is shaved and the skin is swabbed with surgical scrub (iodine and alcohol). A 1-1.5 cm skin incision is made over the intersection of the omohyoid and sternohyoid muscles with a scapel. The sternohyoid muscle is separated by blunt dissection to locate the carotid artery. The vagus nerve is carefully separated from the carotid artery. Supporting the carotid artery using forceps, two pieces of equal length of sterile silk suture are passed beneath the carotid artery. In reference to the heart, the more distal silk suture is tied tightly to occlude blood flowing from the head region. Irrigate the artery with 1 % lidocaine to relax the artery. The proximal silk suture is tied loosely around the carotid artery. A microvascular clamp is placed on the artery to stop blood flowing from the heart. A small cut is made using microscissors into the carotid artery between the two ligatures. A catheter filled with saline solution is inserted into the carotid artery. The proximal ligature is tightened to prevent dislodgement. The microvascular clamp is removed and the catheter is threaded further into the artery to a pre-determined distance. The proximal ligature is tightened further around the artery and catheter. The ends of the distal ligature are tied between the cuffs on the catheter for additional anchorage. After confirming patency, the catheter is tunneled subcutaneously and exteriorized at the nape of the neck or infusions are carried out straight away. The site where the catheter tubing exits the skin of the nape should also have the

hair removed and be aseptically prepared. The catheter is filled with locking solution. The initial surgery incision is closed with non-absorbable suture. To maintain arterial catheter patency, the lines will be flushed with lock solution every 24 hours. Catheters will remain in place for 1-3 days.

Jugular vein cannulation

The description of the following procedure has been adapted from (Feng et al. 2015).
Anesthetized animal is placed in dorsal recumbency. The ventral cervical area is shaved and the skin is swabbed with surgical scrub (iodine and alcohol). Using a hemostat, bluntly dissect the jugular vein, separate out the salivary and lymphatic tissues to visualize and isolate a 5 mm section of the vessel. Using silk suture, place a loose tie on both cranial and caudal ends of the vessel to maximize the exposure of the vessel. Using a micro surgical scissor make an incision large enough to pass the catheter, in line with the vessel between the two ligatures and tie the cranial ligature around the vessel. Insert the venous catheter into the vessel towards the heart with the assistance of the micro dissecting hook and forceps and advance the catheter until all of the PU 3F segment is in the vessel. Use the ligatures at the cranial and caudal ends of the vessels to secure the catheter to the vessel. Non-dissolvable sutures will be used to close the incision. Catheters will remain in place for 1-3 days.

D4. List all participating surgeons and assistants, and indicate the number of years of experience with the particular species and surgical or other major operative procedures to be used. Those who perform survival surgery must receive Surgery Training from the Department of Animal Medicine and submit individual form (download). Please contact Van Gould (extension 66811) or Suzanne Wheeler (extension 62363) for training.
Policy for Survival Surgery Training

Name	Role in surgery	Are you performing survival surgery?	Years of experience with the role
Jonathan Watts	Supervisor	No	0
Mike Moazami	Surgeon	Yes	1 year
Andrew Coles	Surgeon	Yes	14 years
Bruno Godinho	Surgeon	Yes	4 years
Maire Osborn	Surgeon	Yes	0

D5. Describe immediate postoperative care, and provide dosage, route, and frequency of administration of specified analgesics (pain relieving drugs) for the first 48 hours.

Note that "As needed" or "PRN" do not constitute an acceptable schedule for analgesia.

Species	Analgesic Agents	Dose	Route/Volume	Frequency of Administration
Mouse/Rat	Oxymorphone	0.2-0.4 mg/kg	sc (100-500 uL)	Breakthrough pain only (q2-4h)
Mouse/Rat	Buprenophrine	0.2-2 mg/kg for mice, 0.05-0.2 mg/kg for rats	sc (50-250 uL)	Once, after intracerebral surgery. Redose if the animal seems in pain after 8-12 hours. Mandatory redose for any non-cerebral surgery.
Mouse/Rat	Morphine	10 mg/kg	sc (200-400 uL at 1mg/mL)	Breakthrough pain only (q2-4h)
Mouse/Rat	Meloxicam	1-5 mg/kg	ip (100-500 uL)	q24 h for 48 h post op as an alternative to Buprenorphine. Sole use of meloxicam is only for use after intracerebral injection.

Describe the immediate postoperative care for the animal until it is able to maintain a sternal position (e.g., maintaining body temperature, fluid administration, bandaging, vital signs monitoring and monitoring frequency).	The mice are placed in a warmed cage (by a heating pad underneath) and allowed to recover. After surgery we check them continuously until they are able to maintain sternal recumbency. When the animal is awake it is returned to the mouse room with a hand warmer left in the cage. Food pellets soaked in ensure are placed in the cage to allow for easy reach. Analgesics: In all instances of survival surgery, buprenorphine will be administered before the animal regains consciousness. In the instance of vascular surgery, where a higher level of post-operative pain can be anticipated, animals will be monitored especially closely and administered either morphine or oxymorphone if symptoms of pain are noticed, using http://inside.umassmed.edu/AnimalMedicine/signs-of-pain/ as a guide.

D6. List the names of individual(s) who will check animals during recovery.

Name	AREA CODE/TELEPHONE#
MIke Moazami	774-666-0180
Jon Watts	774-455-3784

Andrew Coles	
Maire Osborn	

D7. In the space below describe any expected or potential postoperative complications and describe how you will handle them.

If the animal fails to wake up in the expected time period: we keep the animal warm and gently stimulate it by turning it from side to side at short intervals. We put hand warmers under the bedding to help keep the mice warm after surgery even if they seem to be awake as we have noticed that they may come in and out of sleep, and mice that do not move tend to lose body temperature quickly. Any mouse that does not seem normal on the day after surgery will be euthanized. After surgery they will be checked 2-3 times a day and monitored for weight loss by weighing them biweekly and for infection by taking the animals temperature using a rectal thermometer. Potential problems include ataxia, seizures, circling, and head tilt.

D8. Where will surgery/procedure be performed?	Rm AS2.1054A
D9. Where will animals be housed during recovery?	Rm AS2.1054A
D10. Where will animals be housed after recovery?	Rm AS2.1054

D11. ARE MULTIPLE SURVIVAL SURGERIES PERFORMED ON THE SAME ANIMAL?

☒Yes ☐No

D11a: Justify the need for multiple survival surgeries

Multiple survival surgeries will only be comprised of intracranial injections. The first surgery is required in order to first establish the brain tumour and the second to inject the therapeutic oligos into the brain. The first surgery will be deep in to the brain parenchyma, in order to allow for tumours to grow appropriately. The second surgery will be in to the CSF (lateral ventrical, or 3rd or 4th ventricles), this requires drilling a second burr hole and is nesecessary in order to better recapitulate the delivery options in a human. Intratumoural injections are not commonly used in humans whereas injection in to the CSF is done. To our knowledge, there is no alternative to direct intracranial injection of GBM cells to generate xenograft GBM tumours. No animal would be subjected to more than two (2) separate intracranial injections.

D11b: Give the species and number of animals that will have multiple survival surgeries.

Mus musculus, immunocompromised n= 1122

D11c: Specify the time intervals between the surgical procedures

Longer than 3 weeks.

FORM E
ADMINISTRATION OF SUBSTANCES OTHER THAN ANESTHETICS

Non-Anesthetic Agents

List all 1) Therapeutic , 2) Cell lines or Cell/Tissue extracts and 3) Other Experimental/Study non-anesthetic agents that will be administered to the animals, including but not limited to: 1) drugs such as antibiotics, analgesics or local anesthetics used to minimize post-procedural pain, distress, or discomfort, 2) isolated cells, cell lines, cell or tissue/cell extracts and 3) drugs, infectious agents such as viruses or other substances under study. For drugs under study in the experimental component of your protocol, drug type or group (e.g., non-steroidal anti-inflammatory agents, α-adrenergic receptor blockers) will suffice; however specific drugs should be indicated if known.

E1. Therapeutic agents:

Species receiving the agent	Agent Name	Dose	Route	Volume	Frequency & Total Duration
Mouse/Rat	Morphine	10mg/kg	sc	200-400 uL	Breakthrough pain only (q2-4h)
Mouse/Rat	Buprenophine	1.0 mg/kg	sc	125-250 uL	Once post operatively, as needed after that if the animal appears to be in pain.
Mouse/Rat	0.9% Saline	0.5-5mL	IV or SC	0.5-5 mL	As needed to prevent shock from blood loss after surgery
Mouse/Rat	Lidocaine	1-10 drops	Skin rub as cream & injectable solution before incision closure	1-10 drop	Before tail vein injections and after opening the head wound for intracranial injections
Mouse/Rat	Flumazenil	10 nmol	IP	30 uL	As needed to reverse anaesthesia (benzodiazepine induced)
Mouse/Rat	Naloxone	1-2 mg/kg	SC	100-200 uL	As needed to reverse anaesthesia (opioid induced)
Mouse/Rat	Temezolomide	25-100 mg/kg	IV/IP/Oral Gavage	100-200 uL	As indicated in GBM Study
Mouse/Rat	Atipamezole	1-2.5 mg/kg	IP	100-200 uL	As needed to reverse anaesthesia (xylazine/ dexmedetomidine induced)
Mouse/Rat	Meloxicam	1-5mg/kg	IP	100-500 uL	Every 24 h for 48 h following surgery as an alternative to buprenorphine. Sole use of meloxicam is only for use after intracerebral injection.
Rat	Oxymorphone	0.2-0.4 mg/kg	SC	100-500 uL	Breakthrough pain only (q2-4h)
Mouse/Rat	Sodium Citrate/glycerol pH 6.0-6.5	NA	cathater lock solution (IA or IV)	Volume of Cathater	To maintain cathater and prevent clot.
Mouse/Rat	0.9% Saline	NA	cathater lock solution (IA or IV)	Volume of Cathater	To maintain cathater and prevent clot and to dissolve test substances

Mouse/Rat	40% Dextrose	NA	cathater lock solution (IA or IV)	Volume of Cathater	To maintain cathater and prevent clot.
Mouse/Rat	50% Sucrose	NA	cathater lock solution (IA or IV)	Volume of Cathater	To maintain cathater and prevent clot.
Mouse/Rat	3% Hydrogen Peroxide	1-10 drops	Applied to exposed skull	1-10 drops	To better visualize bregma before intracranial injection
Mouse/Rat	0.25 % Bupivacaine	1-10 drops	Applied with lidocaine to extend the duration of local anaesthesia	1-10 drops	After opening the head wound for intracranial injections
Mouse/ Rat	Phosphate Buffered Saline	1-1000 uL	Used as a vehicle for dissolving test substances	1-1000 uL	With injected substances

E2. Cells or Cell/Tissue Extracts (do not include purified cell products here) Rodent cell lines, serum and cell/tissue products can only be used in animals after confirming the absence of rodent infectious agents by MAP or PCR testing.
Please review the IACUC policy on cell line testing.

Species receiving the agent	Cells or Cell/Tissue extracts		Dose	Route	Volume	Frequency and Total Duration
	Name of cell line or tissue type	Species of origin				
Mus. musculus	GBM8	Human	1-5E5 cells	Intracranial	1-5 uL	Once-tumour will grow until termination
Mus. musculus	GBM6	Human	1-5E5 cells	Intracranial	1-5 uL	Once-tumour will grow until termination
Mus. musculus	U87	Human	1-5E5 cells	Intracranial	1-5 uL	Once-tumour will grow until termination
Mus. musculus	Primary Human tumour dissociates	Human	1-5E5 cells	Intracranial	1-5 uL	Once- tumour will grow until termination
Mus Musculus	E98 (unmodified and mCherry/Fluc transfected)	Human	1E5-1E7 cells	Intracranial and/or subcutaneous	1-10 uL IC 1-200 uL SC	Once- tumour will grow until termination.

E3. Experimental / Study Agents

Species receiving the agent	Agent/Substance Name	Dose Range	Route	Volume	Frequency & Total Duration
Mice (post bilateral pneumothorax)	Phosphate buffered saline	0.1M	intracardial	15mL	Once for 2 minutes
Mice (post bilateral pneumothorax)	paraformaldehyde	4%	intracardial	50mL	Once for 5 minutes
Mice	oligonucleotide	10uM-1mM	intracranial	1-3uL	1 injection 30 minutes
Mice	oligonucleotide	10uM-1mM	i.p, s.c, i.t, i.n, tail vein, im, inhaled	50uL-1mL	One time or 4 times (once every other week)
Mice	Cy3/5/5.5/7/7.5 labeled oligonucleotide	10uM-1mM	intracranial	1-3uL	1 injection 30 minutes
Mice	Cy3/5/5.5/7/7.5 labeled oligonucleotide	10uM-1mM	i.p, s.c, i.t, i.n, tail vein, im, inhaled	50uL-1.5mL	One time or 4 times (once every other week)
Mice	Vetbond	na	On fur	2 brush strokes	One time one minute

E4. Are any of the therapeutic agents listed in E1 or drugs listed in E3 are non-pharmaceutical grade? A pharmaceutical grade compound is a drug, biologic, or reagent that is approved by the Food and Drug Administration (FDA) or for which a chemical purity standard has been established by the United States Pharmacopeia-National Formulary (USP-NF), or British Pharmacopeia (BP). More information for the use of non-pharmaceutical compounds.
☒ Yes ☐ No If the answer is "yes", please explain. US government policy requires the use of pharmaceutical grade drugs especially for anesthetics or analgesics. Exceptions include unavailability of pharmaceutical grade drug and lack of a suitable alternative. Exceptions require scientific justification. There is no pharmaceutical grade for these oligonucleotides. We will follow IACUC 3.01.

FORM F

PROLONGED PHYSICAL RESTRAINT OR STRESS OF CONSCIOUS ANIMALS FASTING, FOOD RESTRICTION, ANIMALS UNDER THE CARE OF THE INVESTIGATORS STAFF RATHER THAN ANIMAL MEDICINE PERSONNEL

Complete this section if any unanesthetized animals will be restrained, except when the restraint is for a brief examination, sample collection, or injection. Also complete if noxious stimuli will be administered, if food or water will be withheld, etc.

F1. Explain rationale for use of restraint or induction of stress:

F2. Describe device, dimensions, etc.:

F3. Duration and frequency animal will be confined to device:

F4. Observation intervals during confinement:

F5. Qualified faculty or staff making observations:

Name	AREA CODE/TELEPHONE#

F6. Will pain or discomfort be induced? If yes, describe in detail using the space below.	☐Yes	☐No
F7. Will stimulation, including light and sound, be used to modify animal behavior? If yes, describe in detail.	☐Yes	☐No
F8. Will animals be fasted (food, approx. 24 hours and/or water, approx. 12 hours) or placed on a diet deficient in one or more nutrients? If yes, provide the details below. How long animals will be restricted? How will the general well-being of the animal be determined How often will the animal be weighed? ☐ I have read the IACUC policy on fasting and agree to label all cages with start dates, stop dates, and telephone contacts.	☐Yes	☐No
F9. Animal medicine staff will be restricted from caring for animals ☐ I have read the IACUC policy on investigators caring for their animals and agree to label all cages with start dates, stop dates, and telephone contacts. Please explain why the restrictions are required below:	☐Yes	☐No
F10. Will analgesics, sedatives, or tranquilizers be used to provide additional restraint? If yes, make sure that the agent(s) are listed in Form C.: ANESTHESIA	☐Yes	☒No

FORM G HAZARDOUS AGENT INFORMATION		
Protocol may require ancillary reviews by the Institutional Biosafety Committee (IBC), the Environmental Health and Safety (EH&S), the Radiation Safety Office prior to the IACUC approval. IACUC will notify you if ancillary reviews are required based on the information provided in the protocol. Omission of appropriate information may delay the approval process.		
G1. Will this project require the use of infectious biological agents? (pathogenic to man or animal) If Yes, IBC approval is required.	☐Yes	☒No
G2. Will this project require the use of recombinant DNA technology in live animals? If Yes, IBC approval is required.	☐Yes	☒No
G3. Will this project require creation of new transgenic animals in any UMMS laboratory or transgenic core facility? If yes, IBC approval is required	☐Yes	☒No
G4. Will this project require the breeding of transgenic animals that contain transgene encoding more that 50% of the genome of an exogenous eukaryotic virus from a single family or express transgene under the control of gammaretroviral long terminal repeat? If Yes, IBC approval is required.	☐Yes	☒No
G5. Will this project require the use of materials of human origin? If Yes, IBC approval is required.	☒Yes	☐No
G6. Will this project require working with live non-human primates or materials of non-human primate origin (tissues, cells, etc.)? If Yes, IBC approval is required.	☐Yes	☒No
G7. Will this project require the use of biotoxins? If Yes, IBC approval is required. Please see examples of biotoxins that require IBC approval:	☐Yes	☒No
G8. Will this project require IBC approval for the use of synthetic nucleic acids (e.g. siRNA, synthetic DNA)? Please see the footnote below for synthetic nucleic acids which require IBC approval[1].	☒Yes	☐No
G9. Will your research activities potentially expose humans, other than researchers and Animal Medicine personnel, to zoonotic infections (e.g., Q fever from sheep) [2]? If yes, IBC approval is required.	☐Yes	☒No
G10. Will this project require the use of ionizing radiation in live animals? If Yes, Radiation Safety approval is required	☐Yes	☒No
G11. Will this project require the use of hazardous chemicals in live animals? If Yes, Chemical Safety Review is required. Please see the footnote below for Occupational Health and Safety Administration (OSHA) definition of a hazardous chemical[3].	☐Yes	☒No
G12. If you will be using any of the above agents, use this space to describe briefly what you will be doing.		
Modified and unmodified RNA oligonucleotides will be used to knockdown in vivo proteins involved in disease. GBM cells will be injected into the striata of immunocompromised animals in order to generate tumours that we will quantify and attempt to treat using oligonucleotide therapy.		

[1] Synthetic nucleic acids

If the answer to ANY of the following questions is "yes," IBC approval is required for synthetic nucleic acids:

1. Will synthetic nucleic acid molecules be modified, or used in a vehicle that is intended to enhance cell membrane penetration *in vivo* (e.g. bound to nanoparticles)?
2. Will synthetic nucleic acid molecules have ANY of the following characteristics?
   - *Able to express a transgene*
   - *Able to replicate in vivo*
   - *Able to integrate in cellular genome or modify genomic sequences*
   - *Prolonged (not transient) biologic effects if introduced into cells*

[2] The researchers and Animal Medicine personnel are required to be knowledgeable about the Occupational Health and Safety hazards of the animal species they will be working with. However, if there is the possibility of untrained individuals (e.g., if animals and patients are using the same imaging equipment) contracting serious zoonotic diseases by coming into contact with animals directly or indirectly , IBC has to review and approve procedures used for transporting, experimentation, decontamination, disposal of animal waste, etc.

[3] Chemical Safety Review

Purpose: Many chemicals used in conjunction with animals in research as part of the experimental test system have the potential to adversely affect the health and safety of researchers as well as animal care personnel. These 'hazardous chemicals' are broadly defined in the OSHA Hazard Communication Standard as chemicals that pose either a physical hazard or a health hazard to humans.

Definition: Among the chemicals that pose a 'physical hazard' are those that are combustible or flammable liquids, compressed gases, organic peroxides, oxidizers, and unstable or water-reactive. Chemicals that pose a 'health hazard' are those that may have acute or chronic health effects in exposed employees. Among these chemicals are carcinogens, toxic or highly toxic agents, reproductive toxins, sensitizers, hepatotoxins, nephrotoxins, neurotoxins, agents which act on the hematopoietic system, and agents which damage the lungs, skin, eyes, or mucous membranes. See 29 CFR 1900.1200(c) for the complete definition.

Process: Chemicals used with animals that are considered to be potentially 'hazardous' according to OSHA need to be listed in the IACUC Animal Study Protocol application, and their use reviewed by EH&S. Material Safety Data Sheets may be consulted for guidance regarding whether or not a given chemical may pose a chemical or physical hazard to researchers and/or animal care personnel. You may contact EH&S for additional information.

G13. If you have IBC approval for the studies described in the protocol, please check the box below and fill in the requested information	
☒ I (PI of this IACUC protocol) have reviewed the IBC protocol and verified that all the hazardous biological agents used in live animals under this protocol have IBC approval. There is an IBC approved animal addendum for use of animals at animal biosafety level >ABSL-1.	IBC protocol number I-656-16 PI of the IBC protocol Jonathan Watts

C.2 IACUC Protocol 2591

University of Massachusetts Medical School

Institutional Animal Care and Use Committee (IACUC)
Office of the Vice Provost for Research
University of Massachusetts Medical School
55 Lake Avenue North
Worcester, MA 01655-0002 USA
508.856.5416 (office) 508.856.5004 (fax)

Approval Letter

February 01, 2017

Investigator: Jonathan Watts, Ph.D.
RNA Therapeutics Institute

Docket #: A-2591-16

Title: Development of Polyclonal Antibodies against Therapeutic Oligonucleotides

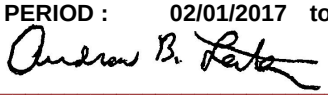
This is to certify that the project identified above has been reviewed by the Institutional Animal Care and Use Committee (IACUC), which has considered specifically the compliance with applicable requirements of the Animal Welfare Act and pertinent state and local laws, regulations, and adherence to the U.S. Public Health Service Policy and the NIH Guide.

The proposed study has been approved by the IACUC and complies with the institutional assurance certification of the University of Massachusetts Medical School. The University of Massachusetts Medical School is fully accredited by AAALAC and has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW). The Assurance number is: A-3306-01, effective 01/04/2017.

Total number of animals approved for this study.

Animals/Species	Category (B)	Category (C)	Category (D)	Category (E)
Rabbit	0	0	50	0

PROJECT APPROVAL PERIOD : 02/01/2017 to 01/31/2020



Andrew B. Leiter, M.D., Ph.D.
Chair, Institutional Animal Care and Use Committee

To the Investigator:

Please note the enclosed copy of your protocol is the final, approved protocol by the IACUC, and overrides all previous versions.
Annual review will be required for protocols in accordance with the Animal Welfare Act Regulations and Department of Defense (DoD) instruction for use of animals.

Investigators will not be allowed to exceed the approved number of animals without IACUC approval.

Modifications of any type (additional species; new procedures that may cause discomfort, stress or pain; use of new biohazardous materials or methods; and addition of new personnel or investigators) must be reviewed and approved by the IACUC before being initiated.

For your information see the link for "A Quick Reference to the Responsibilities and Functions of the Principle Investigator in Research Facilities under the Animal Welfare Act" at http://www.nal.usda.gov/awic/pubs/Legislat/awabrief.shtml#Q14 and “What Investigator Need to Know About the Use of Animals” at http://grants.nih.gov/grants/olaw/InvestigatorsNeed2Know.PDF.

C(pdf): Animal Medicine

IBC reference: 656-16

UNIVERSITY OF MASSACHUSETTS MEDICAL SCHOOL

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Animal Study Protocol

PI: Watts Docket # A-2591-16

Instructions for IACUC Animal Study Protocol application:

1. Complete the Animal Study Protocol application form if you are applying for a new, 3-year renewal or *major amendment.
2. If you need assistance in developing an Animal Study Protocol or have any questions, please contact IACUC@umassmed.edu
3. Save your file as [Your last name-IACUC] and e-mail the completed document as an attachment to IACUC: IACUC@umassmed.edu
4. Print a copy and collect signatures where appropriate: send the hard copy to IACUC, Office of Research, S1-859

All Principal Investigators are required to fill out the application face page (next page) and the sections I through X of the Animal Study Protocol. In addition, please complete appropriate forms and check the boxes below.

Forms: Double click on checkboxes to mark		
A	☐	Transporting animals for use outside of or to another area of the animal facilities
B	☐	If animals are going to bred for the use in this protocol
C	☒	Using anesthesia
D	☐	Doing survival surgery
E	☒	Administering substances other than anesthetics (therapeutic agents, toxins, cells, cell lines, cell/tissue extracts, viruses and other infectious agents, purified biological products, etc.)
	☐	Will rodent cell lines, serum, or tissues be introduced into live animals?
F	☐	Food or water restriction, prolonged physical restraint or animal care done by lab staff.
G	☒	If using radioactive agents, hazardous chemicals, recombinant DNA, breeding transgenic animals carrying viral genes or synthetic nucleic acids (see form G for details), infectious agents, biotoxins, human or non-human primate materials and live non-human primates
H	☒	To indicate adverse effects of procedures and experiments/monitoring and managements
I	☐	If using any of the UMMS Core Facilities
J	☐	If using sites or vendors outside UMMS
K	☐	If you are conducting a training course
Worksheet 1	☐	Animal number worksheet
Worksheet 2	☐	Breeding worksheet

*Major amendment: Any change that could affect the well being of animals (addition of surgical procedures, use of hazardous agents as infectious agents or radioisotopes), change of animal species.
Please send a cover letter indicating what part of the protocol you are changing, and justify the reason for requesting the change. In the protocol-form use a *different font* or different color for the section that you wish to amend or to change.

Docket No. A-2591-16

APPLICATION TO USE VERTEBRATE ANIMALS IN RESEARCH OR INSTRUCTION

UMMS Institutional Animal Care and Use Committee

Principal Investigator	Name and Highest Academic Degree(s)	Jonathan Watts, PhD
	Faculty Title	Associate Professor of Biochemistry and Molecular Pharmacology
	Department \ Division or Company Name	RNA Therapeutics Institute
	Mailing Address or UMMS Building	AS4-1051
	E-mail Address	jonathan.watts@umassmed.edu
	Contact Phone Numbers	Office: (774) 455-3784 Home: 774-243-7931 Pager #:
Faculty Sponsor (if required) or co-investigator	Name and Degree	
	Faculty Title	
	Department/Division	
Primary Contact Person	Name	Mike Moazami
	Telephone No.	Office 774-455-3765 Home 774-666-0180
	Mailing Address Building	AS4-1006
Project Title	Development of Polyclonal Antibodies against Therapeutic Oligonucleotides	

☒ New ☐ 3-Year Renewal ☐ Major Amendment (state changes in a cover letter)

☒ Check this box if you like to receive an informal technical review and feedback from the IACUC office. The intent of this service is to assist investigators in improving the protocol and minimizing concerns during the formal review (veterinary pre-review and IACUC committee review). You may not require this option, if you are experienced in writing IACUC protocols.

USE OF THIS APPLICATION FORM

- DO NOT change formatting of this document. Forms submitted to IACUC MUST include shading. If attachments are used, they should be minimum and not replace answers to any section.
- If you have questions regarding the completion of this form, please contact the IACUC office (508.856.5384).
- Please note that upon request the University may be required by law to release a copy of this application to the public

Signature of Department Chair 12/6/16 Date	Name (Please Print or Type) Phil Zamore, PhD

For IACUC USE ONLY:

Approved: (Yes) No

Approved with conditions: Yes (No)

Date 2/1/17 Chair/Vice Chair, IACUC Andrew B. Lentz

Section I. OBJECTIVES OF PROPOSED RESEARCH OR INSTRUCTION

a. Check the box below that describes the type of animal use being proposed.

☒ Basic Research ☐ Service (Cores, Sentinels, etc.) ☐ Instruction or Training

☐ Field Research ☐ Testing (Biologicals, Toxicity, etc.) ☐ Applied Research

☐ Other_____

b. Lay Summary: In clear, concise, *non-technical,* language (i.e., that could be understood by someone at a high school level), summarize the background and objectives of your studies involving animals.

Antibodies are small proteins produced by the immune system in response to some challenge from a foreign molecule. Antibodies are often very specific for the thing that they are binding, the utility here is in that binding capability.
Oligonucleotides are a class of therapeutic molecule that act by preventing the production of deleterious proteins, we study these separately (A-2551-16). We are very interested in studying the effects of changing the shape and size of these oligonucleotides because there is good evidence that doing so enhances the potency and utility of these therapeutics. One of the big challenges in assessing the viability of these compounds is finding out where they go in the body when they're administered systemically. Often people do this by attaching a fluorescent dye to the oligonucleotide, indeed this has been the general methodology applied in the field. Unfortunately, fluorescent dyes are often different molecules to the oligonucleotides and attaching them can lead to distribution to organs they don't normally go to, in addition to potentially being toxic.
What we want to do is to raise antibodies against these oligonucleotides so that when we do our distribution studies in other animals, we don't have to use fluorescently tagged molecules and can use the antibody generated here to detect them.

c. Scientific Summary: Include an abstract of the study briefly describing the specific aims and scientific approaches to achieve these specific aims. This should serve as a scientific summary of the proposed study. Please note that the detailed procedures need not be included here.

The goals of our work are to develop antibodies against oligonucleotide therapeutics for several diseases to assess systemic distribution of oligonucleotides. Rabbit pAbs have been generated by a company in the field, as such we are confident that it is possible to do. If the naked Antisense Oligonucleotide (ASO) itself is not sufficiently immunogenic to yield antibodies, we will conjugate to Keyhole Limpet Haemocyanin.

We are particularly interested in polyclonal antibodies (pAb) as opposed to monoclonal Abs because of the enhanced variety of epitopes that can be targeted. What we are interested in is a pool of antibodies that are sequence-independent. One of the potential issues raising monoclonal antibodies (mAb) is that an Ab generated against the oligo could be targeting a certain base sequence/modification which might not be present in other sequences, hence we would have to raise a mAb to each individual sequence.

d. Briefly explain the relevance of the proposed research or instruction to human or animal health and/or to the advancement of scientific knowledge.

If we can know with some certainty that the drugs we are developing localise to a given tissue, we can develop sequences to target abberent gene expression in that tissue.

Oligonucleotides are a class of therapeutic molecule. One of the big challenges in assessing the viability of these compounds is finding out where they go in the body when they're administered systemically. Unfortunately, fluorescent dyes are often different molecules to the oligonucleotides and attaching them can lead to distribution to organs they don't normally go to, in addition to potentially being toxic.
Antibodies against these oligonucleotides will facilitate assess to the distribution profile of the compounds.
The only pAb that will be produced in the first instance is against monomeric ASO, which is a) commonly used and b) always screened in tissue culture (TC) before use in animals.
We hope that the pAb that is produced against monomeric ASO will be applicable for the multimeric dendrimers we are making and will check to see whether we can use it for these compounds before we start trying to generate pAbs against them.

e. Has this project undergone scientific peer-review?

☒Yes ☐ No

Section II. RATIONALE FOR USING ANIMALS AND ALTERNATIVES TO THE USE OF ANIMALS

a. Briefly explain why animals are required for your studies.

In order to raise polyclonal antibodies, it is completely necessary to use a live animal as a functioning immune system is required. In addition, it has been shown*** that polyclonal antibodies can be raised against our molecules of interest by a large company in the field, using rabbits. There are no available hybridoma cell lines available that produce the antibody we're interested in raising. We could generate a hybridoma, but this would involve the use of animals, and indeed potentially more animal suffering regarding

ascites production.

*Murray, S.F., Jazayeri, A., Matthes, M.T., Yasumura, D., Yang, H., Peralta, R., Watt, A., Freier, S., Hung, G., Adamson, P.S. and Guo, S., 2015. Allele-Specific Inhibition of Rhodopsin With an Antisense Oligonucleotide Slows Photoreceptor Cell Degeneration Allele-Specific Inhibition of Rhodopsin for adRP. *Investigative ophthalmology & visual science*, *56*(11), pp.6362-6375.

*Butler, M., Crooke, R.M., Graham, M.J., Lemonidis, K.M., Lougheed, M., Murray, S.F., Witchell, D., Steinbrecher, U. and Bennett, C.F., 2000. Phosphorothioate oligodeoxynucleotides distribute similarly in class A scavenger receptor knockout and wild-type mice. *Journal of Pharmacology and Experimental Therapeutics*, *292*(2), pp.489-496.

*Hung, G., Xiao, X., Peralta, R., Bhattacharjee, G., Murray, S., Norris, D., Guo, S. and Monia, B.P., 2013. Characterization of target mRNA reduction through in situ RNA hybridization in multiple organ systems following systemic antisense treatment in animals. *nucleic acid therapeutics*, *23*(6), pp.369-378.

b. Briefly explain why the species you propose to use is/are the most appropriate.

Rabbits are highly reactive to antigens and yield sufficient amount of serum samples for extensive analyses. The large quantity of antibody obtained from a single rabbit allows for the use of far, far fewer animals. A plethora of anti-rabbit secondary fluorophore-conjuagted antibodies are commercially available to use if we can raise primary antibody to detect our agents of interest.
We feel that rabbits represent a good medium between raising antibodies in mice and goats. We make a large number of different antisense oligos and routinetly need to assess distribution. Any purified antibody would be stocked in the freezer for future use, if not used immediately.

c. Describe the steps you have taken to reduce the usage of animals and to minimize the lethality of procedures in your experiments (e.g., using cell culture, computer simulations, or non-living models; doing pilot studies, using most specific assays possible).

We are using a small number for each compound of interest to limit the number of animals. There's no need for control animals, so we aren't wasting animals in untreated groups. We will follow known protocols* for raising polyclonal antibodies and seek advice from more experienced researchers. We will endeavor to design experiments logically and clearly to avoid the need to repeat them. From a search it seems as though most computer simulations model the growth of animal cells in culture in the context of Ab production, so far as we are aware there are no relevant computation simulations available for our work.

*Fodey, T.L., Delahaut, P., Charlier, C. and Elliott, C.T., 2008. Comparison of three adjuvants used to produce polyclonal antibodies to veterinary drugs. *Veterinary immunology and immunopathology*, *122*(1), pp.25-34.

Section III. NUMBER OF ANIMALS REQUESTED: PAIN / DISTRESS LEVEL

a. List the number of animals you will use over the duration (to a maximum period of 3 years) of this protocol.

By NIH policy, you will still have to renew your protocol every 3 years. *This requirement is independent of the need for the IACUC to initially review all animal activities described in your grant proposal, irrespective of the grant's time period.*

All animals must be accounted for, including E-17+ embryos (rats and mice) and neonates. The total for each row should equal the sum of the values in that row.

The total should include all animals bred for use in experiments listed in categories C, D, and E in section IIIa plus all animals generated by breeding that are discarded due to the wrong genotype and/or sex, (Category C).

Species	Number in Pain / Distress Level B (to be used only for USDA - covered species)	Number in Pain / Distress Level C	Number in Pain / Distress Level D	Number in Pain / Distress Level E	Total (should agree with numbers in worksheets I and II, if used.)
Rabbit	0	0	50	0	50

Pain / Distress level indicates maximum pain or distress level to be experienced by animal(s):
B = being bred but not used in testing, teaching or experiments (USDA covered species only)
C = negligible;
D = pain / distress relieved by appropriate drug use;
E = pain / distress not relieved by appropriate drug use. See the IACUC Instructions for definitions and examples.

- No Category B is to be used for rodents. Rodents that were once considered to be Category B should be listed under Category C.
- Induction of tumors is Category C if no distress is anticipated or if you monitor the status of animals and euthanize sick animals when detected.
- *Mice used for Ascites Production are always Category "E".*

b. If you have animals in category E, use this space to provide a description of the procedures producing pain or distress, and list the reasons why pain-relieving drugs cannot or will not be used to relieve pain or distress. If pain/distress relief would interfere with test results, justify why that is true.

N/A

c. Are you planning to reuse animals that were previously used for any other experiments or procedures under a different animal protocol?

☐Yes ☒ No If the answer is "yes," please provide more details (number of animals, protocol number, what previous procedures done in these animals)

d. If there are federal guidelines or regulations that *require* the use of laboratory animals then use this space to cite the agency, CFR title, number and specific section (e.g., Food and Drug Administration, 21:CFR1030.110).

na

Section IV. JUSTIFICATION OF THE NUMBER OF ANIMALS REQUESTED

NIH rules require that animal use must be kept to the minimum consistent with a sound scientific outcome. Please use the space below to justify that the number of animals requested is appropriate for the goals of the experiments.

a. Write a brief description of experimental design. In a table below (for large number of animals, please attach an animal number worksheet in the appendix), show all of the experimental groups and the number of animals per group. Be sure that the totals in the table match the totals shown in section III (Number of Animals Requested). Provide a science-based justification for why you chose the number of animals needed per group or other division of your research.

We are interested in raising polyclonal antibodies against 10 different therapeutic oligonucleotides. One cannot be certain that any given animal will raise potent antibodies against the administered compounds, hence the need for the use of multiple animals.

With 10 compounds, we intend to use a maximum of 5 rabbits per compound, this is in order to maximise our chances of raising a suitable antibody for a total of 50 rabbits. It is possible that we will generate an antibody that will recognise specific chemical moieties across the range of oligonucleotides we intend to use, in which instance there would be no further need for any more rabbits. We will not immunise more than 2 animals at any one time with a given compound and will screen serum to determine whether we have the antibody we're looking for before we immunise any more rabbits.

We came to this number of animals by reading this paper:
Fodey, T.L., Delahaut, P., Charlier, C. and Elliott, C.T., 2008. Comparison of three adjuvants used to produce polyclonal antibodies to veterinary drugs. *Veterinary immunology and immunopathology*, *122*(1), pp.25-34.
Ideally, we want to use as few animals as possible. We request up to 5 to i) allow for re immunization should a rabbit die from some non-immunisation cause ii) to allow for multiple rabbits to be used to ensure a constant supply of antibody iii) to allow for best chances of antibody production.
We would start with 2 animals, if we have good Ab production and don't need more than is being producted, we would simply maintain those two animals. If we need more Ab, we would add animals.

b. Describe, in general terms, the statistical tests required for the study.

No statistical tests are required for this work.

Section V. ANIMAL SPECIFICS

a. Describe the age/weight, sex, and source of each animal species/strain.

SPECIES / STRAIN	AGE / WEIGHT	SEX	*VENDOR
New Zealand White Rabbit	8-10 weeks, 2 kg	female	Approved vendors

*Note that the UMass Transgenic Core should be listed as a vendor if animals are produced there for you. If you are receiving animals from another investigator, indicate the name of the PI and protocol number.							
b. If any of your animals have special needs, use the space below to list needs for special handling or housing. If animals cannot be socially housed, please explain why							
c. Specify what parameters you will assess to ensure that the animals are healthy before your experiments begin. Check all boxes that apply.							
Activity	x	Appearance	x	Appetite	x	Behavior	x
Excreta		Respiratory Pattern		Temperature	X	Weight	x
Laboratory tests or other observations (specify)	Temperature and weight will be monitored when the animals are sedated for bleeding or immunization/booster.						

Section VI. EXPERIMENTAL PROCEDURES: (*except* any survival surgery and procedures that may lead to a permanent physical or physiological handicap)

IACUC reviewed procedures

a. Basic chronology (please use a flow chart or a sequential list of activities)

1) Animal will be sedated
2) Peripheral blood collection as specified below
3) Animal will be recovered/reversed from sedation

OR
1) Animal will be sedated
2) Fur trimmed as necessary and skin wiped down with alcohol
3) Animal will receive immunizations subcutaneously

Following the recommendations found here (https://www.thermofisher.com/us/en/home/life-science/antibodies/custom-antibodies/custom-antibody-production/custom-polyclonal-antibody-production/custom-rabbit-polyclonal-antibody-production-protocols.html) we propose:

70-day Antibody Production Protocol:
2 Rabbits
Day 0: Pre-Immune bleed
Day 1: Immunizations
Day 14: 1st booster and small bleed (3.5 mL)
Day 28: 2nd booster and small bleed (3.5 mL)
Day 35: Production Bleed (10 mL-10%cbv)
Day 42: 3rd booster
Day 56: Production Bleeds (10 mL-10%cbv)
Day 60: decide to continue or terminate, if to continue- boost every 1-3 months and bleed 10 days after the booster (10 mL-10%cbv) and a maximum of every 2 weeks thereafter depending on the lab's need for antibody.

Animals will be euthanized or put up for adoption after one year of antibody production or in the instance that no more antibody is required

Total: 6 boosters after initial immunisation, max 22 bleeds

If we observe no production of suitable antibodies after the day 56 bleed we will remove the animal from the study and attempt to adopt out or euthanise. We will not continually bleed a rabbit that shows no effective antibody production.
If the animal stops producing antibodies of a suitable potency at any point in the study, we will remove the animal from the study and attempt to adopt out or euthanise.
No rabbit, will be kept for more than one year. At this point we will remove the animal from the study and attempt to adopt out or euthanise.

b. Detailed description of procedures (do not include details of in vitro procedures): Describe in narrative form all procedures to be carried out on living animals from initial contact to euthanasia.

Subcutaneous Injections (Sedation):
For a total volume 1 mL we will divide the dose over a minimum of 4 locations (250 uL/location) spaced far enough (>1 inch) apart in to the flank that any irritation will not coallesce. Injections will be made such that the animals will not be able to reach back and groom any immunisation site. Injections will all be made in the flank, but for boosters we will ensure that we rotate sites to ensure we are not using the same site.

Blood collection (Sedation):

The percutaneous central aural artery puncture will be performed on sedated animals. For rabbits, 5-10 ml of blood will be collected per rabbit at each time point (time points as described above) not to exceed 10% of circulating blood volume at any time point, with 2 weeks to recover between bleeds. A closed-chest cardiopuncture will be performed for terminal blood collection on terminally anesthetized rabbits. -

Euthanasia (Anaesthesia):

The rabbits will be euthanized by IV (marginal ear vein) or intracardiac injection of sodium pentobarbital at >150 mg/kg at the end of the study. Euthanasia via the ear vein will be accomplished by placing a 22-25 gauge catheter or over the needle catheter in to the ear vein of an anaesthetized animal before sodium pentobarbital infusion.

Disposal of animals:

Since the rabbits will not receive any infectious agent, they will be disposed in the regular necropsy room after being euthanized.

c. Indicate how you will identify animals

Cage card, individual animals by tattoo/permanent marking
d. Will the studies result in adverse effects on animal health or well being?
☒ Yes ☐ No If yes, please complete form H

Section VII. TERMINATION OF STUDY / EUTHANASIA

Unless specified otherwise by the Principal Investigator, Department of Animal Medicine personnel who are euthanizing animals on behalf of the Principal Investigator will do so using any method listed as acceptable for the species in the most recent edition of the euthanasia guidelines of the American Veterinary Medical Association. Neonatal and pregnant rats and mice will be euthanized using methods approved by the UMMS IACUC and posted on the IACUC intranet site.

a. Is death used as an endpoint in this study? Death as an endpoint means that the animal is permitted to die as a result of experimental manipulation, i.e. exclusive of planned euthanasia. If yes, explain why an earlier end point is not acceptable. (Studies using death as an endpoint are Category E)	☐Yes	☒ No

b. What criteria will be used to perform euthanasia earlier than planned?

Rabbits will be euthanized with any sign of distress or serious pain* or injury caused by events other than planned experimental procedures. We do not anticipate (based on published use of this adjuvant) any irritant reaction at the site of immunisation, however if we observe animals in pain we will euthanise.
*Head pressing; teeth grinding; may become more aggressive; increased vocalizations; excessive licking and scratching; reluctant to locomote
We are aware of the possibility of distress as a result of the inflammatory response. If we notice any of the above signs or weight loss (>10% which will be measured before each bleed- 10 days after each immunization) we will remove the animal from the study and attempt to adopt out or euthanise. If the animal does not appear to be in pain, but is losing weight/ in poor condition we will attempt to adopt out. If the animal is obviously in pain that we do not believe could be alleviated, we will euthanise.

c. Other Use – Will animals be available for further use by other investigators? Link to the Animal Medicine Exchange Board at http://i.umassmed.edu/ExchangeBoard/messagepage.aspx

No

d. Describe the method(s) of euthanasia for each species or procedure. For injectable drugs, give name, dose and route below. US Government policy requires the use of pharmaceutical grade drugs[1].
Must comply with the American Veterinary Medical Association (AVMA) Guidelines on Euthanasia.

Species	Method/Drug	Dose (mg/kg body wt.)	Route
Rabbit	Sodium Pentobarbital	>150 mg/mg	IV or intracardiac

e. Current rules require that after euthanasia, death be confirmed by using a second method. For example bilateral pneumothorax is done after euthanasia using CO_2. Indicate below how you will double kill your animals.

Bilateral thoracotomy with/without exsanguination.

Section VIII. DATABASE SEARCHES

In the space below, document that you have searched databases

1. To determine that you are not unnecessarily duplicating previous experiments,
2. To determine that alternatives to animal use are either not available or not appropriate,
3. To determine that procedures involving animals will avoid or minimize discomfort, distress and pain, AND
4. To determine that alternatives to procedures that may cause more than momentary or slight pain or distress to the animals have been considered

Sources of information regarding possible duplication and alternatives include:

- Medline (http://www.ncbi.nlm.nih.gov/pubmed)
- Animal Welfare Information Center (awic.nal.usda.gov/)
- UMMS veterinarians (508.856.3151)
- National Agricultural Library (www.nal.usda.gov/)
- WWW Virtual Library of Veterinary Medicine (http://netvet.wustl.edu/vetmed.htm)
- Altweb (http://www.jhsph.edu/~altweb)

a. Dates of Searches:

[1]A pharmaceutical grade compound is a drug, biologic, or reagent that is approved by the Food and Drug Administration (FDA) or for which a chemical purity standard has been established by the United States Pharmacopeia-National Formulary (USP-NF), or British Pharmacopeia (BP).

28-nov-2016
b. Name of the database(s) you searched:
PubMed
c. Years Covered by Searches:
1990-2016
d. Keywords Searched:
antibody antisense oligo, phosphorothioate antibody, ASO tracking, oligonucleotide tracking, oligonucleotide localization, alternative to animal use, computer simulation antibody production, rabbit antibody, rabbit ASO antibody, rabbit polyclonal antisense, rabbit polyclonal ASO
e. Other Sources of Information:
Personal Communication with several scientists and collaborators

Section IX. PARTICIPATING PERSONNEL (including PI)

a. Identify all personnel working with animals at the time of this application.

Name	UMMS Phone	Emer-gency Phone	What are this person's responsibilities with animals on this study?	Years of experience with the technique to be used	Years of experience with the technique in species to be used	[2]First time animal user at UMMS (Yes/NO)	[3]On-line training courses completed (Yes/No)	[4]Occupational health requirements completed (Yes/No)
Jonathan Watts	774-455-3784	774-243-7931	PI	0	0	No	Yes	No
Mike Moazami	774-455-3765	774-666-0180	All procedures	0	0	No	Yes	Yes
Maire Osborn		508-654-6215	All procedures	0	0	No	Yes	Yes

b. Briefly describe how the individuals with less than one year of experience with the technique(s) in species will be trained and/or supervised. Identify the individual(s) who would be responsible for training and/or supervising new or inexperienced personnel. Note that hands-on training can be given by an experienced personnel or designated Department of Animal Medicine staff. Those who perform survival surgery must receive aseptic Survival Surgery Training from the Department of Animal Medicine before approved as a surgeon. Please contact Van Gould (extension 66811) or Suzanne Wheeler (extension 62363) for training.

All personnel with limited experience will be trained by Van Gould in Animal Medicine

ADDITIONAL NOTES ON PARTICIPATING PERSONNEL:

IACUC training webpage IACUC new personnel webpage

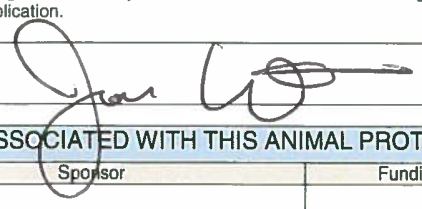
- Before other new personnel perform any procedures, a written minor amendment request must be submitted to and approved by the IACUC. All personnel new to UMMS or those who never worked with animals will have to complete all the Training and Occupational Health and Safety Requirements before added on an IACUC protocol.
- The principal investigator is responsible for ensuring that all personnel adhere to the conditions approved by the IACUC.

Section X. APPLICANT'S CERTIFICATION

IACUC is charged with carrying out the rules and regulations of the Federal Government's Animal Welfare Act governing the care and use of animals in research and instruction. The Act stipulates that (a) Principal Investigators must give written assurance that the activities do not unnecessarily duplicate previous experiments; (b) procedures involving animals must avoid or minimize discomfort, distress, and pain to the animals; (c) Principal Investigators must consider alternatives to procedures that cause more than momentary or slight pain or distress to the animals and give a written description of methods used to determine that alternatives are not available; and (d) paralytic agents cannot be used in unanesthetized animals. Accordingly, the Applicant, who must be a member of the faculty holding Principal Investigator status, is required to read and sign the following certification:

BY SIGNING BELOW, I CERTIFY THE FOLLOWING:

1. I am thoroughly familiar with the literature in the field of research proposed in this application, and I have determined that the research does not unnecessarily duplicate experiments, that appropriate non-animal models are not available, and that the research must be conducted on living animals.
2. I will abide by all UMMS policies and procedures regulating use of animals in instruction and research, by the provisions of the PHS/NIH Guide for the Care and Use of Laboratory Animals, and by all other applicable laws, policies, and regulations governing the use of animals in instruction and research.
3. I will supervise all experiments involving live animals. Furthermore, I will ensure that all listed participants are qualified or will be trained in proper procedures, including animal handling, anesthesia, surgery, post-procedural management, and euthanasia. Also, I will ensure that individuals not listed in the application will not have responsibility in experiments involving animals.
4. All listed personnel will read the IACUC-approved Application to Use Vertebrate Animals in Research or Instruction before undertaking any procedures on laboratory animals.
5. Survival surgery will be performed using standard aseptic procedures.
6. Animal Medicine clinical veterinary staff will be consulted as needed to ensure satisfactory veterinary care.
7. In the event of an animal health emergency, my staff or I will contact the Department of Animal Medicine. We will not attempt animal treatment by ourselves, unless it is a life-threatening emergency.
8. If I cannot be contacted, and animals in this project show evidence of illness or pain, emergency care, including euthanasia, may be administered at the discretion of the Animal Medicine veterinary staff.
9. Significant changes in study objectives and procedures require IACUC approval.
10. Unanticipated adverse events will be reported to the IACUC as required by UMMS IACUC policy
11. This application meets all animal use and care requirements of the funding agencies that have been asked to support the research.
12. By signing below, I certify that all animal studies described in grant proposals using this protocol are described in this animal use application.

PI's Signature:  Date: Dec 6, 2016

GRANTS ASSOCIATED WITH THIS ANIMAL PROTOCOL

Sponsor	Funding period	Grant ID #

FORM A TRANSPORTING ANIMALS FOR USE OUTSIDE OF OR TO ANOTHER AREA OF THE ANIMAL FACILITIES Animal Transfer Policies		
A1. Will animals be used in areas, e.g., laboratories, outside one of the general animal facilities? (A Level, BioTech II, BNRI 1st floor, LRB 1st floor, Rose Gordon, ASC Facility)	☐ Yes	☒ No
If "yes", list the building and room number(s) where animals will be housed or used outside the animal facility.		
A2. Indicate which of the following procedures will be used outside of the animal facility:		
☐ Fluid Collection/Tissue Harvesting ☐ Non-Surgical Procedure ☐ Non-Survival Surgery ☐ Survival Surgery ☐ Irradiation ☐ Core Service/Imaging ☐ Other:		
A3. Will animals be held, housed, and/or used in study areas outside of the animal facility for more than 12 hours for USDA regulated species or 24 hours for non-USDA regulated species? If "Yes", in the space below list the building and room number(s), and justify scientifically the need to hold animals.	☐ Yes	☒ No
A4. Use this space to describe how you will transport animals from the animal facility to a study area outside of the animal facility		
A5. Is a patient procedural area to be used for animal studies? If "Yes", use the space below to provide room number and/or location of patient area. If animals are to be transported to the patient area, you will require the approval of the Infectious Disease Committee (ICC). Please fill out the ICC Approval form and send to Mack, Deborah Ann (Infection Control) (UMMHC) DeborahAnn.Mack@umassmemorial.org. Contact Deborah Ann Mack at 508-856-5843, if you have questions.	☐ Yes	☒ No
A6. If "Yes", describe any special animal transport or facility procedures that will be followed to assure health and safety of both animals and patients.		
A7. Will animals for your use be transported to a different area of the animal facility or to a different UMMS animal facility? ☐ Yes. If yes see below.		
☐ I agree to use the Web Animal Ordering System for all transfers within the animal facility, except as noted below. ☐ Our work requires that we transport of animals to different areas of the animal facility for activities approved by the IACUC e.g. irradiator, biocontainment suite. Explain very briefly below.		

FORM B
BREEDING

Use this section if you are breeding animals for this project.

B1. Describe the species, strain, and source of each animal that you plan to breed

SPECIES / STRAIN	AGE	WEIGHT	VENDOR

B2. Specify what parameters you will assess to ensure that the animals are healthy. Check all boxes that apply.

Activity		Appearance		Appetite	
Excreta		Respiratory Pattern		Temperature	
Laboratory tests or other observations (specify)					

B3. In this space describe how many animals you anticipate breeding over the next 3 years. Briefly explain your calculations below.

B4. Anticipated health problems in the animals being bred. If there are known health or well being issues associated with the animals you are proposing to breed, or their offspring, please describe them in detail.

B5. Male:Female Ratio

☐ A ratio of 1:1 will be used. No continuous breeding

☐ A ratio of 1:1 with continuous breeding will be used. See below

☐ A ratio of 1:2 or 1:3 will be used (harem breeding). See below

☐ I have read the IACUC Mouse Breeding Policy that prohibits more than one litter per cage. I agree to separate pregnant females to other cages prior to their giving birth in harem breeding and to wean continuously bred litters at day 21 if the female is pregnant.

Mouse Breeding Policy

B6. If your animals have special needs, use the space below to list needs for special handling or housing.

B7: Describe how you will identify your animals (e.g. ear tags):

B8. Use this space to describe what will be the disposition of retired breeders or any excess animals.

B9. Will you require special caging? (If so it is your responsibility to provide it)

B10. How often will each male and female be bred?

B11. If you will be using inbred or outbred animals, describe how you will ensure that they remain in- or outbred:

B12. Describe any special handling that you anticipate:

B13. Will males be removed from cages before birthing?

☒ Yes ☐ No for continuous breeding
(if no, explain why not)

B14. What nesting materials will you use?
(For rodents, consider Nestlets or similar material)

B15. At what age will the young be weaned?
B16. Will the offspring be genotyped, and if so, at what age and by what method?
B17. Will mice be bred and transferred to other investigators? ☐YES ☐NO
If yes please check the attestation ☐ I agree that all transfers to another investigator whether within the Animal facility or directly to a laboratory must use electronic Web Animal Ordering system. I understand that failure to do so represents unapproved animal use under the donating investigator's (my) protocol and I will be responsible for all regulatory issues, violations, etc arising from such use. Use of animals not on the recipient's protocol also represents a protocol violation of his/her protocol.

FORM C
ANESTHESIA
Anesthesia and Analgesia

C1. PRE-ANESTHETIC AGENTS (e.g., tranquilizers, narcotics) and ANESTHETIC AGENTS: Describe how these agents will be used in your studies. If none will be used, enter "none" in the "Agents" column.

Frequency of administration	Species	Pre-anesthetic Agents & Anesthetic Agents	Dose	Route/Volume
prior to immunization & bleeding	Rabbit	Ketamine/Xylazine mix	15-30/3-6 mg/kg	IM
prior to immunization & bleeding	Rabbit	Ketamine/ Dexmedetomidine	15-30/0.05-0.1 mg/kg	IM
Terminal blood collection prior to euthanasia	Rabbit	Ketamine/Xylazine mix	30-50/ 6-10mg/kg	IM
Terminal blood collection prior to euthanasia	Rabbit	Ketamine/ Dexmedetomidine	30-50/0.05-0.1 mg/kg	IM

C2. MONITORING OF ANESTHESIA: Describe (a) what will be monitored (e.g., corneal reflex, heart rate, respiration, response to noxious stimulus) and (b) how frequently each of these variables will be monitored.

A form (suggested template) for recording Rodent Anesthesia and Analgesia can be obtained from the IACUC website: RODENT ANESTHESIA RECORD FORM

Heart rate and respiration throughout the immunization process. The animals will be monitored Q 15 min until ambulatory or able to maintain sternal recumbency.

C3. Use of Isofluorane: It is IACUC policy to ensure that animals do not contact liquid isofluorane. Please see the IACUC policy on isoflurane use.

C4. Describe the anesthetic gas scavenging system you will use to eliminate waste anesthetic gas. Follow the link for the policy on scavenging anesthetic gas.

C5. Are any of the anesthetic or pre-anesthetic agents listed in C1 non-pharmaceutical grade? A pharmaceutical grade compound is a drug, biologic, or reagent that is approved by the Food and Drug Administration (FDA) or for which a chemical purity standard has been established by the United States Pharmacopeia-National Formulary (USP-NF), or British Pharmacopeia (BP).

☐Yes ☒No If the answer is "yes," please explain

US government policy requires the use of pharmaceutical grade drugs especially for anesthetics or analgesics. Exceptions include unavailability of pharmaceutical grade drug and lack of a suitable alternative. Exceptions require scientific justification.

FORM D

SURVIVAL SURGERY INCLUDING LAPAROSCOPY, ENDOSCOPY, and any procedure that has a *reasonable* potential of causing a permanent physical or physiological handicap.

Complete this section if any animals will recover from local or general anesthesia after a surgical or other major operative procedure.

D1. In the space below, explain why it is necessary for the animals to recover from surgery/anesthesia.

D2. In the space below, describe pre-operative care (including physical examinations, lab tests, and any preconditioning apparatus). All anesthetic agents and pre-operative medications should be listed in Form C.

D3. Use the space below to describe in detail the surgical procedure(s)/other major procedure to be used.

D4. List all participating surgeons and assistants, and indicate the number of years of experience with the particular species and surgical or other major operative procedures to be used. Those who perform survival surgery must receive Surgery Training from the Department of Animal Medicine and submit individual form (download). Please contact Van Gould (extension 66811) or Suzanne Wheeler (extension 62363) for training.
Policy for Survival Surgery Training

Name	Role in surgery	Are you performing survival surgery?	Years of experience with the role

D5. Describe immediate postoperative care, and provide dosage, route, and frequency of administration of specified analgesics (pain relieving drugs) for the first 48 hours.

Note that "As needed" or "PRN" do not constitute an acceptable schedule for analgesia.

Species	Analgesic Agents	Dose	Route/Volume	Frequency of Administration

Describe the immediate postoperative care for the animal until it is able to maintain a sternal position (e.g., maintaining body temperature, fluid administration, bandaging, vital signs monitoring and monitoring frequency).	

D6. List the names of individual(s) who will check animals during recovery.

Name	AREA CODE/TELEPHONE#
MIke Moazami	
Jon Watts	
Andrew Coles	
Maire Osborn	

D7. In the space below describe any expected or potential postoperative complications and describe how you will handle them.

D8. Where will surgery/procedure be performed?	
D9. Where will animals be housed during recovery?	
D10. Where will animals be housed after recovery?	

D11. ARE MULTIPLE SURVIVAL SURGERIES PERFORMED ON THE SAME ANIMAL?

☐Yes ☐No

D11a: Justify the need for multiple survival surgeries

D11b: Give the species and number of animals that will have multiple survival surgeries.

D11c: Specify the time intervals between the surgical procedures

FORM E
ADMINISTRATION OF SUBSTANCES OTHER THAN ANESTHETICS
Non-Anesthetic Agents

List all 1) Therapeutic , 2) Cell lines or Cell/Tissue extracts and 3) Other Experimental/Study non-anesthetic agents that will be administered to the animals, including but not limited to: 1) drugs such as antibiotics, analgesics or local anesthetics used to minimize post-procedural pain, distress, or discomfort, 2) isolated cells, cell lines, cell or tissue/cell extracts and 3) drugs, infectious agents such as viruses or other substances under study. For drugs under study in the experimental component of your protocol, drug type or group (e.g., non-steroidal anti-inflammatory agents, α-adrenergic receptor blockers) will suffice; however specific drugs should be indicated if known.

E1. Therapeutic agents:

Species receiving the agent	Agent Name	Dose	Route	Volume	Frequency & Total Duration
Rabbit	Yohimbime	0.2 mg/kg	IM	<1 mL	For reversal of Xylazine anaesthesia
Rabbit	Atipamezole	0.5 mg/kg	IM	<1 mL	For reversal of dexmedetomidine sedation/anaesthesia
Rabbit	Flumazenil	0.1 mg/kg	IV/IM	<1 mL	For reversal of midazolam sedation/anaesthesia
Rabbit	Acepromazine	0.5-1mg/kg	IM/SC	<1 mL	Prior to injections/bleeds
Rabbit	Dexmedetomidine	0.05-0.1 mg/kg	IM	<1 mL	Prior to injections/bleeds
Rabbit	Midazolam	0.5-2 mg/kg	IM, SC	<1mL	Prior to injections/bleeds
Rabbit	Lidocaine cream	Dermal	Dermal	<1mL	Prior to bleeding

E2. Cells or Cell/Tissue Extracts (do not include purified cell products here) Rodent cell lines, serum and cell/tissue products can only be used in animals after confirming the absence of rodent infectious agents by MAP or PCR testing.
Please review the IACUC policy on cell line testing.

Species receiving the agent	Cells or Cell/Tissue extracts		Dose	Route	Volume	Frequency and Total Duration
	Name of cell line or tissue type	Species of origin				

E3. Experimental / Study Agents

Species receiving the agent	Agent/Substance Name	Dose Range	Route	Volume	Frequency & Total Duration
Rabbit	Therapeutic oligonucleotide (conjugated to protein)	1-2000 ug	SC- sites separated by at least an inch, no more than 250 uL per site.	0.5 mL	Every 2 weeks
Rabbit	Therapeutic oligonucleotide	1-2000 ug	SC- sites separated by at least an inch, no more than 250 uL per site.	0.5 mL	Every 2 weeks
Rabbit	Montanide ISA Adjuvant* Fodey, T.L., Delahaut, P., Charlier, C. and Elliott, C.T., 2008. Comparison of three adjuvants used to produce polyclonal antibodies to veterinary drugs. *Veterinary immunology and immunopathology*, *122*(1), pp.25-34.	Up to 0.5 mL diluted 1:1 with the test compound in sterile saline	SC- with the above	0.5 mL	Administered with either of the above, in the same syringe

E4. Are any of the therapeutic agents listed in E1 or drugs listed in E3 are non-pharmaceutical grade? A pharmaceutical grade compound is a drug, biologic, or reagent that is approved by the Food and Drug Administration (FDA) or for which a chemical purity standard has been established by the United States Pharmacopeia-National Formulary (USP-NF), or British Pharmacopeia (BP). More information for the use of non-pharmaceutical compounds.
☒ Yes ☐ No If the answer is "yes", please explain. US government policy requires the use of pharmaceutical grade drugs especially for anesthetics or analgesics. Exceptions include unavailability of pharmaceutical grade drug and lack of a suitable alternative. Exceptions require scientific justification. There is no pharmaceutical grade for these compounds. We will follow IACUC 3.01. Oligonucleotides are synthesized using standard solid phase synthesis and deprotection conditions, then purified using ion exchange chromatography before desalting on sephadex and analysis by LC-MS. Oligos are typically 90%+ pure. Preparation of KLH-conjugated oligos required preparation of a 3'-thiolated oligo which is subsequently coupled to pure KLH-maleimide and purified as per the product protocol.

FORM F

PROLONGED PHYSICAL RESTRAINT OR STRESS OF CONSCIOUS ANIMALS FASTING, FOOD RESTRICTION, ANIMALS UNDER THE CARE OF THE INVESTIGATORS STAFF RATHER THAN ANIMAL MEDICINE PERSONNEL

Complete this section if any unanesthetized animals will be restrained, except when the restraint is for a brief examination, sample collection, or injection. Also complete if noxious stimuli will be administered, if food or water will be withheld, etc.

F1. Explain rationale for use of restraint or induction of stress:

F2. Describe device, dimensions, etc.:

F3. Duration and frequency animal will be confined to device:

F4. Observation intervals during confinement:

F5. Qualified faculty or staff making observations:

Name	AREA CODE/TELEPHONE#

F6. Will pain or discomfort be induced? If yes, describe in detail using the space below.	☐Yes	☐No
F7. Will stimulation, including light and sound, be used to modify animal behavior? If yes, describe in detail.	☐Yes	☐No
F8. Will animals be fasted (food, approx. 24 hours and/or water, approx. 12 hours) or placed on a diet deficient in one or more nutrients? If yes, provide the details below. How long animals will be restricted? How will the general well-being of the animal be determined How often will the animal be weighed? ☐ I have read the IACUC policy on fasting and agree to label all cages with start dates, stop dates, and telephone contacts.	☐Yes	☐No
F9. Animal medicine staff will be restricted from caring for animals ☐ I have read the IACUC policy on investigators caring for their animals and agree to label all cages with start dates, stop dates, and telephone contacts. Please explain why the restrictions are required below:	☐Yes	☐No
F10. Will analgesics, sedatives, or tranquilizers be used to provide additional restraint? If yes, make sure that the agent(s) are listed in Form C.: ANESTHESIA	☐Yes	☒No

FORM G HAZARDOUS AGENT INFORMATION		
Protocol may require ancillary reviews by the Institutional Biosafety Committee (IBC), the Environmental Health and Safety (EH&S), the Radiation Safety Office prior to the IACUC approval. IACUC will notify you if ancillary reviews are required based on the information provided in the protocol. Omission of appropriate information may delay the approval process.		
G1. Will this project require the use of infectious biological agents? (pathogenic to man or animal) If Yes, IBC approval is required.	☐Yes	☒No
G2. Will this project require the use of recombinant DNA technology in live animals? If Yes, IBC approval is required.	☐Yes	☒No
G3. Will this project require creation of new transgenic animals in any UMMS laboratory or transgenic core facility? If yes, IBC approval is required	☐Yes	☒No
G4. Will this project require the breeding of transgenic animals that contain transgene encoding more that 50% of the genome of an exogenous eukaryotic virus from a single family or express transgene under the control of gammaretroviral long terminal repeat? If Yes, IBC approval is required.	☐Yes	☒No
G5. Will this project require the use of materials of human origin? If Yes, IBC approval is required.	☐Yes	☒No
G6. Will this project require working with live non-human primates or materials of non-human primate origin (tissues, cells, etc.)? If Yes, IBC approval is required.	☐Yes	☒No
G7. Will this project require the use of biotoxins? If Yes, IBC approval is required. Please see examples of biotoxins that require IBC approval:	☐Yes	☒No
G8. Will this project require IBC approval for the use of synthetic nucleic acids (e.g. siRNA, synthetic DNA)? Please see the footnote below for synthetic nucleic acids which require IBC approval[1].	☒Yes	☐No
G9. Will your research activities potentially expose humans, other than researchers and Animal Medicine personnel, to zoonotic infections (e.g., Q fever from sheep) [2]? If yes, IBC approval is required.	☐Yes	☒No
G10. Will this project require the use of ionizing radiation in live animals? If Yes, Radiation Safety approval is required	☐Yes	☒No
G11. Will this project require the use of hazardous chemicals in live animals? If Yes, Chemical Safety Review is required. Please see the footnote below for Occupational Health and Safety Administration (OSHA) definition of a hazardous chemical[3].	☐Yes	☒No
G12. If you will be using any of the above agents, use this space to describe briefly what you will be doing.		
IBC animal addendum has been prepared		

[1] Synthetic nucleic acids

If the answer to ANY of the following questions is "yes," IBC approval is required for synthetic nucleic acids:

1. Will synthetic nucleic acid molecules be modified, or used in a vehicle that is intended to enhance cell membrane penetration *in vivo* (e.g. bound to nanoparticles)?
2. Will synthetic nucleic acid molecules have ANY of the following characteristics?
   - *Able to express a transgene*
   - *Able to replicate in vivo*
   - *Able to integrate in cellular genome or modify genomic sequences*
   - *Prolonged (not transient) biologic effects if introduced into cells*

[2] The researchers and Animal Medicine personnel are required to be knowledgeable about the Occupational Health and Safety hazards of the animal species they will be working with. However, if there is the possibility of untrained individuals (e.g., if animals and patients are using the same imaging equipment) contracting serious zoonotic diseases by coming into contact with animals directly or indirectly , IBC has to review and approve procedures used for transporting, experimentation, decontamination, disposal of animal waste, etc.

[3] Chemical Safety Review

Purpose: Many chemicals used in conjunction with animals in research as part of the experimental test system have the potential to adversely affect the health and safety of researchers as well as animal care personnel. These 'hazardous chemicals' are broadly defined in the OSHA Hazard Communication Standard as chemicals that pose either a physical hazard or a health hazard to humans.

Definition: Among the chemicals that pose a 'physical hazard' are those that are combustible or flammable liquids, compressed gases, organic peroxides, oxidizers, and unstable or water-reactive. Chemicals that pose a 'health hazard' are those that may have acute or chronic health effects in exposed employees. Among these chemicals are carcinogens, toxic or highly toxic agents, reproductive toxins, sensitizers, hepatotoxins, nephrotoxins, neurotoxins, agents which act on the hematopoietic system, and agents which damage the lungs, skin, eyes, or mucous membranes. See 29 CFR 1900.1200(c) for the complete definition.

Process: Chemicals used with animals that are considered to be potentially 'hazardous' according to OSHA need to be listed in the IACUC Animal Study Protocol application, and their use reviewed by EH&S. Material Safety Data Sheets may be consulted for guidance regarding whether or not a given chemical may pose a chemical or physical hazard to researchers and/or animal care personnel. You may contact EH&S for additional information.

G13. If you have IBC approval for the studies described in the protocol, please check the box below and fill in the requested information	
☒ I (PI of this IACUC protocol) have reviewed the IBC protocol and verified that all the hazardous biological agents used in live animals under this protocol have IBC approval. There is an IBC approved animal addendum for use of animals at animal biosafety level >ABSL-1.	IBC protocol number I-656-16 PI of the IBC protocol Jonathan Watts

FORM H
ADVERSE EFFECTS OF PROCEDURES AND EXPERIMENTS/MONITORING AND MANAGEMENT

H1. What will be monitored to assess the presence of pain, discomfort, or other potential adverse effects caused by your studies? NOTE: This period includes the time from initiation of experiments until the animals are removed from the study; for surgically operated animals, this includes the time after anesthesia recovery until animals are removed from the study. Check all that apply.

Activity	X	Appearance	X	Appetite		Behavior	
Excreta		Grooming		Guarding		Heart rate	
Licking, biting		Posture	X	Respiratory rate	X	Temperature	X
Vocalizing		Weight loss		Wound site	x	Other	
Laboratory tests or other evaluation (specify)							

H2. Indicate the frequency with which you will monitor your animals during and after all procedures.
Please indicate both monitoring interval and total length of time.

Continuously during procedure and daily for 5 days post injection to assess skin.

H3. Describe the conditions and complications that would lead to removal of an animal from the study and how this will be accomplished (e.g., stopping treatment and/or euthanasia).

If any sign of distress or serious pain or injury caused by events other than planned experimental procedures, animals will be removed from the study by euthanasia.
Ketamine reaction in rabbits may occasionally occur when Ketamine injection is at the leg muscles (this approach will be used minimally when lumbar muscles are used for injections of compound or multiple SC injections needed on the back). If the minor Ketamine reaction happens, the rabbits will be treated by the veterinary staff. If any severe Ketamine reaction occurs, the rabbit will be euthanized.
*Head pressing; teeth grinding; may become more aggressive; increased vocalizations; excessive licking and scratching; reluctant to locomote
We will mark injection sites using a pen and check on the rabbits each day for 5 days post injection for any redness/swelling/abscess formation/bleeding. If we notice serious skin issues, such as abscess formation the animals will be treated by veterinary staff and removed from the study.

FORM I
PROCEDURES DONE IN UMMS CORE FACILITIES

For use a UMMS core facility for this study, you need to get the approval for those animals used in the core facility under this protocol. However, you do not need to list details of the specific procedures performed in the core facility by its staff if the procedures are part of an IACUC approved core protocol.

I1. List below the UMMS core facility services you will be using for this protocol.

Name of the core facility	Name of the PI of the core facility	Protocol # (if applicable)	Briefly describe the procedures/services provided by the core facility

I2. Is any of the procedure(s) performed by personnel other than the core facility personnel?	☐ Yes ☐ No
If the answer is "Yes" please name the individuals performing the procedures under this protocol in the core facility and identify the procedure(s) by core facility SOP# or by other means.	

I3. If any of the procedure(s) used in the core facility is different from the IACUC approved core facility protocol, please describe below the procedure deviations.

I4. Use this space to describe how you will transport animals between the animal facility and the study area

FORM J
ANIMAL WORK DONE OUTSIDE UMMS

Please fill out this form, if a UMMS investigator is going to perform animal studies in collaboration with a non-UMMS collaborator or commercial vendor. This includes production of antibodies by an outside company. According to Federal Regulations, UMMS, being the grantee institution, is responsible to verify that all animal work done outside UMMS has been reviewed and approved by a PHS assured institution, and approved by an IACUC.

J1. List below the details of animal work done outside UMMS

Name of institution or company	Name of the PI or contact (if a company), & contact information (phone # or email address)	IACUC Protocol number, if applicable	Animal Species used in the study

J2. Provide a brief description of the animal work done at the non-UMMS site below:

J3. List below the supporting documents provided with this application.

UMMS IACUC may require one or more of the following documents to attached to this application:

☐ Evidence of collaboration or service (letter from the collaborator or company indicating willingness for doing the animal work)
☐ Copy of the IACUC approval from the non-UMMS institution to indicate that this work has been reviewed and approved by an IACUC. If this approval letter is in a foreign language, please provide an English translation along with a copy of the original Approval Letter.
☐ Evidence for PHS Assurance of the non-UMMS institution (A copy of the Assurance Letter from NIH to the institution or company will be sufficient). This is not required if the Assurance number and period of validity is indicated in the IACUC Approval Letter)
☐ A copy of the protocol (translation into English, if in another language), if the collaborating institution or company is from outside United States, or non-human primates are used in the protocol.
☐ Memorandum of Understanding (requested by the IACUC)

FORM K

IF ANIMALS ARE USED FOR TRAINING COURSES (TO TRAIN STUDENTS, RESIDENTS, FELLOWS AND OTHERS)

Please fill out this form, if this application is designed for training courses. The trainees should be under the supervision of a trained UMMS investigator during any contact with live animals. Please note that the trainees are not permitted to work independently with live animals; to do so, they have to be listed as personnel in the protocol (please see section IX). According to Federal Regulations, the IACUC is to ensure the occupational health and safety of personnel working with animals, and that all individuals working with animals receive appropriate training. Because the training participants are not known at the time of protocol submission, IACUC recommends the following options to meet the Occupational Health and Safety and Training requirements, and requests that a list of participants submitted to the IACUC Office before the training session(s).

K1. Occupational Health Evaluation of the trainees; please check all the choices that you are planning to recommend the training participants. Each participant has to full fill this requirement by full filling one the choices below.

a. Are the participants UMMS student, resident, employee or volunteer? Please submit an Initial Health Evaluation form to the Employee Health Service for obtaining Health Clearance.	☐Yes ☐No
b. Are the participants going to receive clearance for working with animals from a qualified physician? Health Clearance from a qualified physician is acceptable, if the participant is not affiliated with UMMS. The physician must review and accept the criteria established by the UMMS Occupational Health and Safety Policy. The UMMS Occupational Health and Safety Policy and Health Clearance Forms are available from the IACUC office.	☐Yes ☐No
c. Are the participants going to sign the "Informed Consent Form?" (Participants who are not affiliated with UMMS) In lieu of obtaining health clearance from a qualified health care practitioner participant may submit a signed Informed Consent form indicating that they are aware of and understand the risks of working with animals . and participate at his/her own risk. This option is only allowed if the trainees are only attending one or two training session(s).	☐Yes ☐No

K2. Training options: please check the option(s) below for participant training

a. Are you planning to provide an instructional lecture on working with animals? The PI or his designee can provide training in a lecture format (the lecture should include an overview of Federal Regulations, ethical guidelines for using animals, hazards and risks associated with the use of animals, species-specific information, humane techniques for animal procedures, and other appropriate information relevant to the experimental procedures being used). IACUC could provide the PIs with PowerPoint slides on the UMMS IACUC Animal Care and Use Program upon request. Please note that these slides do not cover Federal Regulations, Occupational Health Hazards and Species Specific information.	☐Yes ☐No
b. Are the participants UMMS students, residents, staff or volunteers? The trainees may fulfill this requirement by completing the online Training courses available to the New Animals Users at UMMS to meet all the training requirements.	☐Yes ☐No
K3. Are the trainees going to perform SURVIVAL SURGERY? If the trainees are going to perform survival surgery, they are required to complete Survival Surgery Training provided by the Dept of Animal Medicine.	☐Yes ☐No

The PIs are required to provide a list of trainees on or before the day of training to the IACUC office. Please see next page for a template for listing trainees. The list should include the names, affiliation and contact information, and evidence for completion of the above requirements (K1 to K3). Failure to submit a list of participants prior to the training session may be considered a protocol violation.

TRAINING PARTICIPANT LIST (template; make additional copies if necessary)

Note: Do not have to fill this out at the time of protocol submission; to be submitted to the IACUC Office (hardcopy or pdf) on or before the day of training

PI Name:	
Docket Number:	
Name of personnel conducting the training session if other than PI:	
Date of training:	

TRAINING PARTICIPANTS								
Name	Indicate if affiliated to UMMS, OR provide the name and address of the non-UMMS affiliation	Contact information (Email and phone number)	Occupational Health Evaluation and clearance to work with animals, OR Informed Consent singed (check applicable box below)			Training (check applicable box below)		
			By the Employee Health Service	By a qualified physician[1]	Informed Consent Form[2]	Online training courses	Classroom instruction	Surgery Training (if performing survival surgery)

[1] Please submit copies of Heath Clearance certificate to the IACUC Office
[2] Please submit copies of the Informed Consent Form to the IACUC Office

WORKSHEET 1 (Optional)

Animal number worksheet (addendum to section IV)-*add on more rows as needed*
Use this worksheet to show the details of animal numbers to be used for 3 years

Species:---------- (please use one sheet/species)

A	B	C	D	E	F	G	H			I			
#	Experiment or procedure[1]	Number of animals per group[2]	Number of groups, including replicates	Total number of animals per experi-ment or procedure	Estimated number of additional animals to compen-sate for possible death or protocol failure	Total number of animals requested (Sum of E and F)	Source of animals			Pain level			
							To be bred	To be purchased	To be transferred from another protocol	B	C	D	E
Grand total													

[1] The specific experiment(s) and procedure(s) listed here should be referenced in the Experimental Design (section IV)
[2] Should be based on justification (power analysis, previous publications, etc.) for the number of animals as described in section IV

WORKSHEET 2 (Optional)

Breeding worksheet (add more rows if needed)

Species:---------- (please use one sheet/species)

A	B		C	D	E	F	G	H	I	J
#	Animal strain, genotype, phenotype, etc.		Number of animals from outside source used in mating [1]	Number of matings	Expected litter size	Total number of pups expected	Number of pups expected to be used in experiments	Number of pups which may not be used in experiments	Number of pups to be used for continued breeding	Total number of animals (sum of columns C and F)
	Offspring	Parents								
Grand total				Not applicable						

[1] Animals purchased or obtained from other investigators used in breeding, do not use animals bred in house used for breeding in this column

C.3 IBC Protocol 656-16

University of Massachusetts Medical School
UMASS

Institutional Biosafety Committee
Office of Research, Rm. S1-859
University of Massachusetts Medical School
55 Lake Avenue North
Worcester, MA 01655-0002 USA
508.856.2563 (office) 508.856.5004 (fax)

Institutional Biosafety Committee

Report on Biosafety Committee Action

APPROVAL LETTER – AMENDMENT

Date: 03-16-2017

IBC Docket No: I-656-16

Investigator: Jonathan Watts, Ph.D.

Title: Development of Oligonucleotide Therapeutics

Amendment: To add: human materials (human blood) and flow sorting of mouse cells

Review Type: Administrative

Amendment Approved: BSL-2; BSL-2 for Flow Sorting Mouse Cells

Highest biocontainment for entire project: BSL-2/ABSL-1+BBP; BSL-2 enhanced for flow sorting

This is to certify that the Institutional Biosafety Committee (IBC) of the University of Massachusetts Medical School has reviewed and approved the research as described in your Amendment to your Biological Agents Registration. A copy of the reviewer's comments is enclosed.

This committee has considered compliance with applicable policies and guidelines including, but not limited to: "NIH Guidelines for Research Involving Recombinant DNA Molecules" and "Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th Edition". Any major changes in the study in the future must be reported to the IBC before implementation.

Institutional Approval Authorization:

Thomas Greenough, M.D., Chair, Institutional Biosafety Committee

Policy Notes to the Principal Investigator:

- The Principal Investigator is responsible for ensuring that all personnel working under this IBC registration are appropriately trained and familiar with the procedures, biosafety precautions and accidental exposure plans.
- The Principal Investigator is responsible for immediate reporting of significant problems, violations of the NIH Guidelines, or research-related accidents involving recombinant DNA, infectious agents, or biological toxins. Illnesses or adverse effects potentially resulting from exposures to recombinant DNA, infectious agents or biological toxins must also be reported immediately. Reporting instructions link: http://inside.umassmed.edu/content.aspx?id=48294
- This approval is restricted to the biological hazards (recombinant DNA, infectious agents, or biological toxins), personnel, bio-containment and locations specified in the IBC registration. An amendment must be submitted to the IBC if there are any changes in these features of the registration.
- For studies involving animals, IBC approval alone does not grant permission to initiate work. Formal IACUC approval is always required before initiating activities with animals.

- All personnel who will be working with human and/or non-human primate samples or cell lines are required to complete Blood Borne Pathogen training. They are also expected to undergo evaluation and counseling by the Employee Health Service regarding Hepatitis B immunization.

University of Massachusetts Medical School
UMASS Medical School

Office of Research
University of Massachusetts Medical School
55 Lake Avenue North
Worcester, MA 01655-0002
508-856-5416 (office) 508-856-5004 (fax)

INSTITUTIONAL BIOSAFETY COMMITTEE
BIOLOGICAL AGENTS REGISTRATION

PRINCIPAL INVESTIGATOR ASSURANCES

PROJECT TITLE:	**Development of Oligonucleotide therapeutics**

PRINCIPAL INVESTIGATOR NAME	Jonathan Watts

Docket No (IBC-Assigned)	I- 656-16

Registration Type (Check One):

	New
x	Amendment
	Renewal

Statement of Principal Investigator
(Initial each statement below, then sign and date at the bottom)

JW **I attest** that the information provided in the attached registration and supporting documents is accurate and complete to the best of my knowledge. I acknowledge that this assurance statement applies to all documents, including any revisions made as a condition for formal IBC approval.

JW **I agree** to abide by the current *NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules* (*NIH Guidelines*), the NIH/CDC, USDA and/or APHIS Biocontainment and Biosecurity Guidelines, and the State and Local Biocontainment and Biosecurity Guidelines pertaining to the proposed research.

JW **I agree** to conduct this project in accordance with Institutional policies developed by the UMMS IBC.

JW **I agree** to comply with the requirements pertaining to shipment and transfer of biohazardous materials and / or recombinant and synthetic nucleic acid molecules (r/s NA). These include Department of Transportation (DOT)/International Air Transport Association (IATA) regulations, and Export Control and Trade Sanctions laws and regulations.

JW **I accept responsibility** for ensuring that all personnel working under this IBC registration are appropriately trained and familiar with the procedures, biosafety precautions and accidental exposure

plans before they start experimentation.

JW **I understand** that I am responsible for ensuring that personnel who may be at risk are offered appropriate medical surveillance.

JW **I accept responsibility for immediate reporting** of significant problems, violations of the *NIH Guidelines*, or research-related accidents involving recombinant or synthetic NA, infectious agents, or biological toxins. I **also understand** that illnesses or adverse effects potentially resulting from exposures to recombinant or synthetic NA, infectious agents or biological toxins must also be reported immediately.

JW **I understand** that an amendment or a new registration must be submitted to the IBC if there are changes in personnel, Biosafety levels, or that introduce new r/s NA or biological hazards for any proposed research.

JW **I understand** that I am not to continue experimentation beyond the expiration date of the IBC approval for this protocol. **I accept the responsibility to submit a renewal application and receive IBC approval** before the expiration date, if continued experimentation is planned.

JW **I will only** carry out the work described in the attached application if formal approval by the UMMS IBC has been granted, and all requirements have been met.

Signatures:

Principal Investigator _____ Date Feb 28, 2017

Additional Investigator _____ Date _____

For IBC Use Only:

Administrative approval: X Yes _____ No

IBC meeting date documenting discussion or administrative approval:

Reviewed and Approved _____ Date 3/20/17

IBC Chair

Approved 041714

UNIVERSITY OF MASSACHUSETTS MEDICAL SCHOOL

INSTITUTIONAL BIOSAFETY COMMITTEE REVIEW FORM

Study Title: Development of Oligonucleotide Therapeutics

Investigator: Watts, Jonathan

IBC Docket Number: I- 656-16

Contact person: Michael Moazami Phone # 774-455-3874

IBC Registration (check box) ☐New ☐5-Year ☒Amendment

Reason for IBC Review (check all that apply)

☒r/s NA ☐Infectious Agents ☒Human/Primate Samples ☐Biotoxins

Biosecurity checklist (check all that apply)

☐ Yes ☒ No Involves SAT

☐ Yes ☒ No Enhances the harmful consequences of an agent or toxin

☐ Yes ☒ No Disrupts immunity or the effectiveness of an immunization against an agent or toxin without clinical and/or agricultural justification

☐ Yes ☒ No Confers to an agent or toxin resistance to clinically and/or agriculturally useful prophylactic or therapeutic interventions against that agent or toxin or facilitates their ability to evade detection methodologies

☐ Yes ☒ No Increases the stability, transmissibility, or the ability to disseminate an agent or toxin

☐ Yes ☒ No Alters the host range or tropism of an agent or toxin

☐ Yes ☒ No Enhances the susceptibility of a host population to an agent or toxin

☐ Yes ☒ No Generates or reconstitutes an eradicated or extinct agent or toxin

Recommendations (check all that apply)

NIH Guidelines Sections: ☐III-A ☐III-B ☐III-C ☐III-D ☐III-E ☒III-F ☐NA

Containment Levels (in vitro): ☐BSL1 ☒BSL2 ☐BSL2+ ☐BSL3

☒BSL-2 flow sorting ☐BSL-2 enhanced flow sorting ☐BSL-3 flow sorting

☐Good Large Scale ☐BL-1 Large Scale ☐BL-2 Large Scale

Containment Level (in vivo): ☐ABSL1 ☐ABSL1+BBP ☐ABSL2 ☐ABSL3

Additional Precautions

Exempt Activity: ☒ Yes ☐ No

Approval: ☒ Yes ☐ No

Review by full committee in a convened meeting: ☐ Yes ☒ No

Discussion by full committee about developing SOP with employee health: ☐ Yes ☒ No

Signature: Samuel Varghese **Date:** 3/16/17

Reviewer Name: Samuel Varghese

Do you have any objection to revealing your name to the PI? ☐ Yes ☒ No

If this registration is approved with action items, should the revised version be directed to you as primary reviewer for a final review and approval? ☒ Yes ☐ No

UNIVERSITY OF MASSACHUSETTS MEDICAL SCHOOL

INSTITUTIONAL BIOSAFETY COMMITTEE REVIEW FORM

Study Title: Development of Oligonucleotide Therapeutics

Investigator: Watts, Jonathan

IBC Docket Number: I- 656-16

BRIEF SUMMARY (3-5 sentences)

Overview and Objectives: The goal of this laboratory is to develop oligonucleotide based treatment for human diseases. This amendment is: (1) to add working with human blood and (2) to perform flow sorting of mouse cells with synthetic oligonucleotides.

Experimental Approach: Blood collection, analysis of oligonucleotide bound serum proteins and flow sorting of mouse cells from animals subjected to administration of oligonucleotids.

ACTION ITEMS

For PI:

Section A1: Mention the intent to do flow sorting of mouse cells harvested from animals administered with oligonucleotides

A2: Briefly describe flow sorting

A7: Check YES for flow core

A8: Any additional details on flow sorting

H5: Mention about following the UMMS BloodBorne Pathogen Exposure Control Plan

J4, J5 and J6: Provide more specific responses to address the saftey related questions

Flow Sorting addendum; A3: Check BSL-2

Flow Sorting Que. 3: Fill in missing information

For IBC: None

FULL REVIEW

Researchers plan to collect blood samples from volunteers, prepare serum and incubate serum with oligonuceotides for subsequent analysis of oligonucleotide binding by size exclusion chromatograpy and electrophoresis. They have prior IBC approval to work with synthetic oligonucleotides with complex chemical modifications and human samples. However, the protocol will need to include some details about precautions for human material handling, waste disposal and management of accidental exposure. BSL-2 biocontainment will be appropriate for working with the human samples.

The researchers had been approved to adminster oligonucleotides into to mice. This amendment proposes flow sorting of homogenized tissues harvested from the animals. These cells may harbor synthetic oligonucletides that have been designed to modulate expression of various target genes. In case of accidental exposure, effect on human genes will depend on homolgoy between the mouse and human genes. However, it is felt that the handling of these samples at BSL-2 will be adequate. Consistent with the International Society for Advancement of Cytometry guidelines, the mouse cells can be sorted at BSL-2 containment.

After addressing the minor action items, this amendment can be approved at BSL-2 for working with human materials and BSL-2 for flow sorting of mouse cells containing sythetic oligonucleotides.

Information from past reviews:

The original protocol was approved on 2/05/16 for testing chemically modified single-stranded or double-stranded oligonucleotides in cells and rodents for enhanced uptake and efficacy. Approval granted at BSL-2/ABSL-1 housing.

The following amendments were approved by IBC after initial approval on 2/5/16:

Approval Date: 12/13/2016

This is an amendment to add: (1) human cell lines (MRC-5, MCF7, HEK293, HeLa, and glioblastoma cell lines U87, GBM6 and GBM8)(2) vectors for CRISPR/Cas9 based genome editing system for human cell lines, (3) non-pathognic E coli

Approval Date: 01/13/2017

Updated their most recent amendment, discussed in Oct. 2016 IBC meeting, by submitting two animal addenda: 1) administration of human cells and then oligonucleotides into mice, 2) chemically modified oligonucleotides into mice and rats.

Approval Date: 02/01/2017

This is an amendment to inject rabbits with chemically modified oligonucleotides. They intend to raise antibodies against antisense oligonucleotides by immunizing rabbits subcutaneously and drawing blood.

University of Massachusetts Medical School
UMASS

Office of Research
University of Massachusetts Medical School
55 Lake Avenue North
Worcester, MA 01655-0002
508-856-5416 (office) 508-856-5004 (fax)

INSTITUTIONAL BIOSAFETY COMMITTEE

AMENDMENT TO BIOLOGICAL AGENTS REGISTRATION

Sections that must be completed in full: A, H, I, J, K, and L.

Please add rows to tables as needed by unprotecting the document (under tools menu), then copying and pasting an empty row, then re-protecting the document as a form.

Submit an electronic copy (preferably a Word file) of the registration with the required attachments at least 20 days prior to the next scheduled IBC meeting date to IBC@umassmed.edu.

Meeting dates and submission due dates are posted: http://inside.umassmed.edu/Biosafety/Institutional-Biosafety-Committee/IBC-Registration-and-Approval/

This form can be used for amendments only if no changes in the IBC approved management of biohazards, plans for accidental exposures, biocontainment and biosafety precautions are expected. For registration changes that do not meet these criteria, please update your original IBC registration form and highlight the changes in red.

Date: 9-Feb-2017

Project Title: Development of Oligonucleotide Therapeutics ☐ Check box if this is a different title from the original registration

Principal Investigator[1] Last Name	**Watts**
Principal Investigator First Name	**Jonathan**
☐ Check box if there is a change in Principal Investigator	
Co-Investigator Name (Last, First)	
Alternate Contact Name (Last, First)	**Moazami, Michael**
Campus (University, Memorial)	**UMass Worcester (University Campus)**
Department	**RTI**
Office Address (include room #)	**368 Plantation Street, Worcester, MA, 01605**
Mailing Address (if different)	
Telephone Number	**774-455-3874**
Fax Number	
Email Address	**jonathan.watts@umassmed.edu, michael.moazami@umassmed.edu**
Docket No (IBC-Assigned)	**I-656-16**
Biosafety Levels (IBC assigned)	**BSL-2; BSL-2 for Flow Sorting Mouse Cells**

Applicable Sections of *NIH Guidelines* (IBC assigned)

☐IIIA ☐IIIB ☐IIIC ☐IIID ☐IIIE ☒IIIF ☐NA

[1] Principal Investigator must have a UMMS faculty appointment to be eligible

IMPORTANT MESSAGE AND RESOURCES

IT IS THE RESPONSIBILITY OF THE PRINCIPAL INVESTIGATOR to be familiar with relevant regulations and guidelines from federal, state and local agencies. Links are provided below:

- **NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules (*NIH Guidelines*)**
  http://oba.od.nih.gov/rdna/nih_guidelines_oba.html

- **CDC/NIH Biosafety in Microbiological and Biomedical Laboratories**
  CDC/NIH BMBL 5th Edition Link: http://www.cdc.gov/biosafety/publications/

- **INFECTIOUS AGENTS (Centers for Disease Control, US Dept of Agriculture/Animal and Plant Health Inspection Service, NIH, WHO)**
  CDC General Information: http://www.cdc.gov/
  CDC Biosafety – Permits, etc.: http://www.cdc.gov/biosafety/
  APHIS General: http://www.aphis.usda.gov/
  APHIS Permits: http://www.aphis.usda.gov/permits/
  World Health Organization: http://www.who.int/en/index.html
  World Organization for Animal Health (OIE): http://www.oie.int/

- **CDC SELECT AGENTS**
  http://www.selectagents.gov/

- **MASSACHUSETTS DEPARTMENT OF PUBLIC HEALTH REGULATIONS**
  http://www.mass.gov/eohhs/gov/laws-regs/dph/
  http://www.mass.gov/eohhs/docs/dph/regs/105cmr480.pdf

- **WORCESTER DEPARTMENT OF PUBLIC HEALTH**
  http://www.worcesterma.gov/ocm/public-health
  http://www.worcesterma.gov/uploads/28/ec/28ecbc3974edbaca6b6e006c2f31c1b5/revised-ordinances-2008-1.pdf

- **DUAL USE AGENTS AND BIOSECURITY**
  http://oba.od.nih.gov/biosecurity/about_nsabb.html

- **OSHA**
  http://www.osha.gov/pls/oshaweb/owasrch.search_form?p_doc_type=STANDARDS&p_toc_level=1&p_keyvalue=1910

- **EXPORT CONTROL AND TRADE SANCTIONS**
  http://www.bis.doc.gov/
  http://www.pmddtc.state.gov/
  http://www.treas.gov/offices/enforcement/ofac/
  http://www.treas.gov/offices/enforcement/ofac/programs/index.shtml
  http://www.access.gpo.gov/bis/ear/ear_data.html#ccl

- **INTERNATIONAL AIR TRANSPORT ASSOCIATION AND DEPARTMENT OF TRANSPORTATION**
- http://www.iata.org/whatwedo/cargo/dgr/Pages/infectious_substances.aspx
- http://www.phmsa.dot.gov/hazmat

Section A: Summary of Changes to Project

A1. Describe proposed changes to the registration that may impact biosafety requirements (including new procedures with previously registered agents), and list ALL AGENTS ADDED:

Addition of Human Blood. Addition of Flow Sorting of Mouse Cells

Provide goals/objectives of project related to AGENTS ADDED:
To assess the serum binding profile of the oligonucleotides and dendrimers that we are interested in and to determine serum halflife of the same.

A2. Provide a brief summary of experiments or procedures involving AGENTS ADDED (with sufficient detail to enable risk assessment):
Fresh blood is drawn and serum collected. (labelled) Oligonucletide is added to the serum and incubated at room temp for up to 24 h, then analysed by size exclusion column.

Alternatively, oligo is inculbated in serum for a period of time (5 mins to 1 week) and analysed by PAGE.

Tissue from mice both treated and untreated with therapeutic oligonucleotides (approved a priori) is dissociated to a single cel suspension and antibody stained before sorting with flow cytometry.

A3. Type of AGENTS ADDED in this project; indicate ALL applicable:

Select Yes or No for ALL items listed; both *in vitro* and *in vivo*

	IN VITRO	*IN VIVO*
Recombinant nucleic acids (r NA) using vectors/plasmids For any YES response: **sections A4 and B must be completed**	☐ Yes ☒ No	☐ Yes ☒ No
Synthetic nucleic acids (s NA) NOT in vectors/plasmids For any YES response: **section C must be completed**	☐ Yes ☒ No	☐ Yes ☒ No
Human/non-human primate materials For any YES response: **section D must be completed**	☒ Yes ☐ No	☐ Yes ☒ No
Infectious agents, excluding select agents For any YES response: **section E must be completed**	☐ Yes ☒ No	☐ Yes ☒ No
Biological toxins For any YES response: **section F must be completed**	☐ Yes ☒ No	☐ Yes ☒ No
Select agents For any YES response: **section G must be completed**	☐ Yes ☒ No	☐ Yes ☒ No

A4. If AGENTS ADDED include new recombinant or synthetic nucleic acid (r/s NA) molecules, declare the Section(s) of the *NIH Guidelines* that apply (Check all that apply. See reference table):

Section	Does your research involve the following:
Examples	
III-A ☐ **Yes** ☒ **No**	**Experiments that Require IBC Approval, RAC Review, and NIH Director Approval Before Initiation**. http://oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.htm - _Toc351276229 e.g. Deliberate transfer of a drug resistance trait to microorganisms that are not known to acquire the trait naturally, if such acquisition could compromise the ability to control disease agents in humans, veterinary medicine, or agriculture
III-B	**Experiments That Require NIH/OBA and IBC Approval Before Initiation.** http://oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.htm - _Toc351276231

☐ **Yes** ☒ **No**	e.g. Cloning of toxin molecules with LD_{50} of less than 100 ng/kg body weight
III-C ☐ **Yes** ☒ **No**	**Experiments that Require IBC and IRB Approvals and RAC Review Before Research Participant Enrollment.** http://oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.htm - _Toc351276234 e.g. Deliberate transfer of r/s NA molecules, or DNA or RNA derived from r/s NA molecules, into one or more human research participants
III-D ☐ **Yes** ☒ **No**	**Experiments that Require IBC Approval Before Initiation.** http://oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.htm - _Toc351276236 Example: Introduction of r/s NA molecules into Risk Group 2, 3 or 4 agents e.g. Experiments in which DNA from Risk Group 2, 3 or 4 agents is transferred into nonpathogenic prokaryotes or lower eukaryotes e.g. Experiments involving the use of infectious or defective Risk Group 2, 3 or 4 viruses in the presence of helper virus e.g. Experiments involving whole animals in which the animal's genome has been altered by stable introduction of r/s NA molecules, or nucleic acids derived therefrom, into the germ-line (transgenic animals) e.g. Experiments involving viable r/s NA molecule-modified microorganisms tested on whole animals e.g. Experiments to genetically engineer plants by r/s NA molecule methods e.g. Experiments involving more than 10 Liters of culture of cells containing r/s NA molecules e.g. Experiments with influenza viruses generated by recombinant or synthetic methods
III-E ☐ **Yes** ☒ **No**	**Experiments that Require IBC Notice Simultaneous with Initiation.** http://oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.htm - _Toc351276244 e.g. Experiments that are appropriately conducted at BSL-1 involving r/s NA molecules that are not categorized in other sections of the *NIH Guidelines* e.g. BSL-1 experiments involving r/s NA molecules containing no more than two-thirds of the genome of any eukaryotic virus, propagated in the absence of helper virus e.g. BSL-1 experiments involving rodents in which the animal's genome has been altered by stable introduction of r/s NA molecules into the germ-line
III-F ☒ **Yes** ☐ **No**	**Exempt Experiments (Note: UMMS Institutional Policy requires that ALL research involving r/s NA must be registered).** http://oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.htm - _Toc351276248 e.g. Experiments with r/s NA that: (1) can neither replicate nor generate nucleic acids that can replicate in any living cell (e.g., oligonucleotides or other synthetic nucleic acids that do not contain an origin of replication or contain elements known to interact with either DNA or RNA polymerase), and (2) are not designed to integrate into DNA, and (3) do not produce a toxin that is lethal for vertebrates at an LD50 of less than 100 ng/kg body weight e.g. Experiments with r/s NA that consist entirely of nucleic acids from a prokaryotic host, including its indigenous plasmids or viruses when propagated only in that host (or a closely related strain of the same species), or when transferred to another host by well-established physiological means e.g. Experiments that do not present significant threat to health or environment (*E. coli* K-12 host vector system, recombinant or synthetic nucleic acids containing less than one-half of any viral genome maintained or propagated in cells in culture)

A5. Are animals that require IACUC approval used for research involving AGENTS ADDED:

☐ **Yes** ☒ **No** **If YES, provide IACUC Docket Number: A -**

If checked "YES", an animal addendum should be provided

NOTE: Animal addendum is required for use of agents in vertebrate animals in the Animal Medicine Facility. This is an important source of information for animal care personnel as they care for animals.

A6. Declare the HIGHEST biosafety level(s) required for experiments involving the AGENTS ADDED: (check appropriate responses)

in vitro

BSL-1 ☐ Yes ☒ No
BSL-2 ☒ Yes ☐ No
BSL-2+ ☐ Yes ☒ No **(this is BSL-2 with BSL-3 practices; Lab SOP required)**
BSL-3 ☐ Yes ☒ No **(Lab SOP required)**

Good Large Scale Practices (for culture volumes > 10 liters) ☐ Yes ☒ No **(Lab SOP required)**
BL1-Large Scale (for culture volumes > 10 liters) ☐ Yes ☒ No **(Lab SOP required)**
BL2-Large Scale (for culture volumes > 10 liters) ☐ Yes ☒ No **(Lab SOP required)**

in vivo

ABSL-1 ☐ Yes ☒ No
ABSL-1+BBP ☐ Yes ☒ No **(this is for human or nonhuman primate cell xenotransplants)**
ABSL-2 ☐ Yes ☒ No
ABSL-3 ☐ Yes ☒ No **(Lab SOP required)**

A7. Indicate if any experiments ADDED involve utilization of UMMS Core Facilities as described:

Select YES or NO	UMMS Core Facility	Description of Core Activity/Involvement
☐ Yes ☒ No	Transgenic animals	• Generation of new transgenic animals (note that this includes gene insertions, mutations, modifications and knock outs)
☐ Yes ☒ No	Viral vector	• Generation of recombinant viral vectors with new gene inserts
☐ Yes ☒ No	RNAi	• Utilization of vectors available through the Core
☐ Yes ☒ No	Mutagenesis	• Utilization of TALEN and CRISPr technologies available through the Core
☒ Yes ☐ No	Flow Core	• Sorting of unfixed human or non-human primate cells / cell lines • Sorting of any unfixed cells containing r/s NA • Sorting of other potentially infectious material **If checked "Yes," fill out the Flow Core Addendum form:** http://inside.umassmed.edu/Global/Institutional%20Biosafety%20Committee/Addendum_Flow_Sort%20questions_080614.docx

☐ **Yes** ☒ **No**	**Electron Microscopy**	• **Manipulation of unfixed human or non-human primate cells/cell lines** • **Manipulation of any unfixed cells containing r/s NA** • **Manipulation of other potentially infectious material**
☐ **Yes** ☒ **No**	**Light Microscopy**	• **Manipulation of unfixed human or non-human primate cells/cell lines** • **Manipulation of any unfixed cells containing r/s NA** • **Manipulation of other potentially infectious material**
☐ **Yes** ☒ **No**	**Proteomics**	• **Manipulation of unfixed human or non-human primate cells/cell lines** • **Manipulation of any unfixed cells containing r/s NA** • **Manipulation of other potentially infectious material**
☐ **Yes** ☒ **No**	**Animal imaging**	• **Imaging animals using instruments also used for human studies** • **Imaging animals that are experimentally infected** • **Imaging animals that are treated with agents containing r/s NA**

A8. If the response to any of the above items in A7 is "YES", briefly describe the experiments here (or in Section A2 above). Be sure to indicate the biocontainment level required for the Core activities as pertains to your research.

BSL-2 required. Animals used will be healthy and will not be reasonably anticipated to require a higher biosafety level.

Examples:

Flow core sorting of unfixed, untested human PBMC to isolate resting CD4 T cells; sorting at BSL-2 enhanced.

Transgenic Core generation of mice over-expressing Insulin-like Growth Factor, using replication defective lentivirus vector transduced ES cells; lentivirus transduction at BSL-2 and animal housing at ABSL-1.

If TALEN/CRISPr technologies are used, indicate the genes targeted and how the mutant cells or animals are used as part of the description.

A9. Potential dual use research of concern (DURC). Indicate if CHANGES to your research involve any experiments that (check all that apply):

☐ Yes ☒ No Enhances the harmful consequences of an agent or toxin

☐ Yes ☒ No Disrupts immunity or the effectiveness of an immunization against an agent or toxin without clinical and/or agricultural justification

☐ Yes ☒ No Confers to an agent or toxin resistance to clinically and/or agriculturally useful prophylactic or therapeutic interventions against that agent or toxin or facilitates their ability to evade detection methodologies

☐ Yes ☒ No Increases the stability, transmissibility, or the ability to disseminate an agent or toxin

☐ Yes ☒ No Alters the host range or tropism of an agent or toxin

☐ Yes ☒ No Enhances the susceptibility of a host population to an agent or toxin

☐ Yes ☒ No Generates or reconstitutes an eradicated or extinct agent or toxin

A10. List AGENTS REMOVED from registration

Section B: ADDING or REMOVING Recombinant or Synthetic NA Molecules (using vectors/plasmids)

See Training Slides on IBC website:
http://inside.umassmed.edu/uploadedFiles/IBC_UMMS_Researcher%20Training_rsNA_V4_Approved_2013-11-21.pdf

Is this section applicable? ☐ **Yes** ☒ **No**

If NOT applicable, check "No" and move to next section

B1. Describe recombinant or synthetic genetic element(s) to be ADDED, including mutated forms. Indicate if the gene is an oncogene, immunomodulator or toxin (please add rows to table, if needed. See instructions on first page)**:**

Select Yes or No.

Gene Name	Origin (species)	Biological function	Oncogene	Immuno-modulator	Toxin
			☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
			☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
			☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
			☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
			☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
			☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
			☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
			☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
			☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
			☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No

B2. Types of ADDED vector(s) that will be used (check ALL appropriate responses):

Select (X)	
☐	**Bacterial** (plasmids for genetic manipulation, prokaryotic or eukaryotic expression)
☐	**Yeast** (expression vectors)
☐	**Baculoviral** (expression vectors)
☐	**Retroviral** (expression vector designed to be packaged in virus particle)
☐	**Lentiviral** (expression vector designed to be packaged in virus particle)

☐	**Adenoviral** (expression vector designed to be packaged in virus particle)
☐	**Adeno-associated viral** (expression vector designed to be packaged in virus particle)
☐	**Poxviral** (recombinant virus)
☐	**Herpesviral (Amplicon)** (including bacterial artificial chromosomes)
☐	**Herpesviral** (recombinant virus)
☐	**Other virus (specify)** (e.g. Sendai virus, Influenza virus, bacteriophage)

B3. List and briefly describe ADDED vectors/plasmids below (please add rows to table, if needed. See instructions on first page)

Refer to the **vector policy** at the UMMS IBC website to be sure that required information is complete: http://inside.umassmed.edu/uploadedfiles/UMMS_IBC_Vector_Policy.pdf

a. Vector name As listed in vendor catalog or vector database (e.g. Addgene), or in a publication	**b. Source** Company name and catalog #, or Colleague's name and literature reference	**c. Proposed *in vitro* biosafety level** (BSL-1, BSL-2, BSL-2+, BSL-3, GLSP, BL1-Large Scale, or BL2-Large Scale)	**d. Proposed *in vivo* biosafety level** (ABSL-1, ABSL-1+BBP, ABSL-2, or ABSL-3)

B4. Provide additional information about the ADDED vector/plasmid constructs (e.g. any custom modification) that would impact biosafety level:

List all ADDED vectors that contain ≥ 50% genome of a eukaryotic virus:

B5. Experimentation with ADDED vectors/plasmids in prokaryotes (e.g. for genetic manipulations, plasmid propagation, protein expression) (please add rows to table, if needed. See instructions on first page)

Species	**Strain** (for *E. coli*, indicate if it is NOT derived from the K-12 strain)

B6. Describe cells and systems used for ADDED viral vector production (please add rows to table, if needed. See instructions on first page):

Cell lines and strains	**List plasmids/vectors**	**Reference or Vendor Manual**

B7. List VECTOR(S) REMOVED from registration

B8. New Transgenic Animals ADDED:

Provide ALL requested information if animals are TO BE GENERATED at UMMS by the PI or transgenic animal core.

Provide selected information if animals are ALREADY GENERATED (by the PI, transgenic animal core, collaborator or vendor)

	TO BE GENERATED	ALREADY GENERATED
Animal species		
Specify vector(s) and methods used to generate Tg animals (e.g. lentiviral transduction; plasmid transfection)		NA
List gene(s) expressed or modified		NA
Is any gene a biological toxin? If answer is "yes," describe briefly.		
Is there incorporation of more than one-half of the genome of an exogenous eukaryotic virus from a single family of viruses? If answer is "yes," describe briefly.		
Is there incorporation of a transgene that is under the control of a gammaretroviral long terminal repeat (LTR)? If answer is "yes," describe briefly.		

B8. Genetically modified plants:

Plant species	
Specify vector(s) and methods used to generate GMO plant	
List gene(s) expressed or modified	
Is any gene a biological toxin? If answer is "yes," describe briefly.	
Is there incorporation of more than one-half of the genome of an exogenous virus from a single family of viruses? If answer is "yes," describe briefly.	

Section C: ADDING or REMOVING Synthetic NA molecules (NOT in vectors) that are chemically, or by other means, synthesized or amplified

See Training Slides on IBC website:
http://inside.umassmed.edu/uploadedFiles/IBC_UMMS_Researcher%20Training_rsNA_V4_Approved_2013-11-21.pdf

Is this section applicable? ☐ **Yes** ☒ **No**

If NOT applicable, check "No" and move to next section

C1. Will ADDED synthetic nucleic acid molecules be administered deliberately to human subjects?

☐ **Yes** ☐ **No**

C2. Will ADDED synthetic nucleic acid molecules be modified, or used with a vehicle that is intended to enhance bioavailability and activity in animals or humans (e.g. bound to nanoparticles)?

NB: This does NOT apply to most siRNA used for experimental manipulation *in vitro*.

☐ **Yes** ☐ **No**

C3. Will ADDED synthetic nucleic acid molecules have ANY of the following characteristics:

Able to express a transgene
Able to replicate in vivo
Able to integrate in cellular genome or modify genomic sequences
Prolonged (not transient) biologic effects if introduced into cells

☐ **Yes** ☐ **No**

C4. If you answered "YES" to ANY question (in C1, C2 or C3) above describe the ADDED synthetic nucleic acid molecule(s):

Agent type (e.g. shRNA, synthetic DNA)	
Name of the agent if applicable	
Commercial source of the agent	
Expected biological function and effects with exposure (deliberate or accidental)	
Describe briefly how the synthetic nucleic acid is modified (if applicable)	
Describe the vehicle used (if applicable)	
Proposed biosafety level for *in vitro* use	
Proposed animal biosafety level for *in vivo* use	

C5. List synthetic nucleic acids REMOVED from registration

Section D: ADDING or REMOVING Material Of Human And Non-Human Primates (NHP) Origin

Biosafety level when working with materials of human origin: ≥ BSL-2. The investigator should use universal precautions described in the OSHA-bloodborne pathogens Standard, when handling any material of human origin.

Plans for immunization against Hepatitis B virus must be included in Section H6 of this registration if there is potential exposure to bloodborne pathogens.

Is this section applicable? ☒ **Yes** ☐ **No**

If NOT applicable, check "No" and move to next section

D1. Indicate if your research involves the ADDED use of any of the following material(s) from human and/or non-human primates (NHP) origin, living or dead (check all appropriate responses):

Check	Type of material to be used (BSL-2 biosafety level required for *in vitro* work)	Proposed *in vivo* biosafety level, if used in animals
☐	**Unfixed cells**	
☐	**Organs**	
☐	**Tissues**	
☒	**Blood**	
☐	**Blood products**	
☐	**Other body fluids (urine, feces, saliva, etc)**	

Human or non-human primates cell lines (please add rows to table, if needed. See instructions on first page)	Proposed *in vivo* biosafety level, if used in animals

D2. If human or non-human primate materials are used in animals, identify the specific ADDED agent(s) and animal species (please add rows to table, if needed. See instructions on first page)**.**

Agent (e.g. NHP cells, Human cell lines)	Animal Species

D3. List human/non-human primate cell line(s), human/non-human primate research material (tissues, body fluids, etc.) REMOVED from registration

Section E: ADDING or REMOVING Infectious Agents (Excluding Select Agents)

Is this section applicable? ☐ **Yes** ☒ **No**

If NOT applicable, check "No" and move to next section

E1. Name, risk group, and source of ADDED infectious agent(s) (please add rows to table, if needed. See instructions on first page)**:**
(http://www.absa.org/riskgroups/index.html OR http://www.cdc.gov/biosafety/publications/bmbl5/index.htm OR http://osp.od.nih.gov/sites/default/files/NIH_Guidelines.html#_APPENDIX_B._CLASSIFICATION)

Agent name and strain	Risk Group	Source (Vendor, collaborator)	Proposed *in vitro* biosafety level	Proposed *in vivo* biosafety level

E2. For ADDED infectious agents that are ≥ risk group 2, provide relevant antimicrobial susceptibility (please add rows to table, if needed. See instructions on first page)**:**

Agent name and strain	Antibiotic, antifungal or antiviral susceptibility profile (for the specific infectious agent/strain being used)

E3. List INFECTIOUS AGENT(S) REMOVED from registration

Section F: ADDING or REMOVING Biological Toxins*

*Poisonous substances that are specific products of the metabolic activities of living organisms (plant, animal, fungus, bacteria). Contact EH&S at x6-3985 with questions about safe storage, handling or disposal.

Is this section applicable? ☐ **Yes** ☒ **No**

If NOT applicable, check "No" and move to next section

F1. Name and source of the ADDED toxin(s) (please add rows to table, if needed. See instructions on first page)**:**

Name	Source (Vendor, collaborator)	Proposed *in vitro* biosafety level	Proposed *in vivo* biosafety level

F2. LD_{50} for the ADDED biological toxin(s) – if available (please add rows to table, if needed. See instructions on first page):

Toxin	LD_{50}

F3. List biological toxins REMOVED from registration

Section G: Select Agents (CDC/USDA list) MAY NOT BE ADDED USING THIS FORM

Section H: Management of Biohazards of AGENTS ADDED (Complete ALL parts of this section)

H1. Describe the maximum anticipated titer/concentration of the preparations of each potentially biohazardous agent ADDED (e.g. virus stocks) (please add rows to table, if needed. See instructions on first page)**:**

Agent or vector	Maximum anticipated titer/concentration
Human Blood	50 mL

H2. Describe the maximum anticipated volume of the preparations of each potentially biohazardous agent ADDED (e.g. bacterial cultures) (please add rows to table, if needed. See instructions on first page)**:**

Agent or vector	Maximum anticipated volume
Human Blood	50 mL

H3. Briefly describe the biohazard potential of each potentially biohazardous agent ADDED (What might happen to a human with significant exposure to the agent? What disease process might result?) (please add rows to table, if needed. See instructions on first page)**:**

Agent or vector	Biohazard potential
Human Blood	Potential for transmission of disease

Appendix D

Miscellaneous Data

D.1 Biodistribution Images

D.1.1 Liver

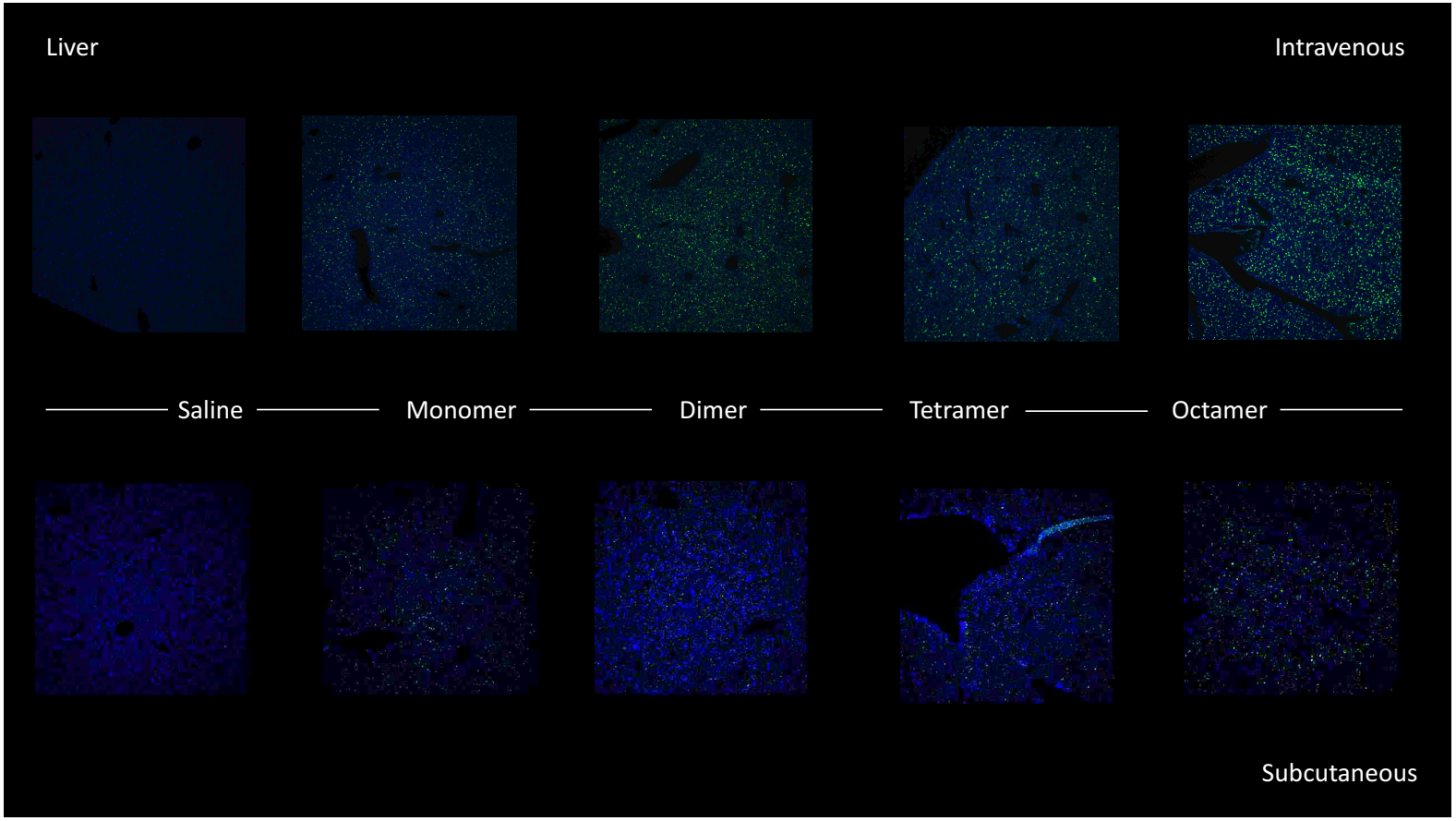


Figure D.1: Biodistribution of oligonucleotide dendrimers following injection of $10\,\mathrm{mg\,kg^{-1}}$
Blue: DAPI, Green: Anti-ASO pAb

D.1.2 Kidney Cortex

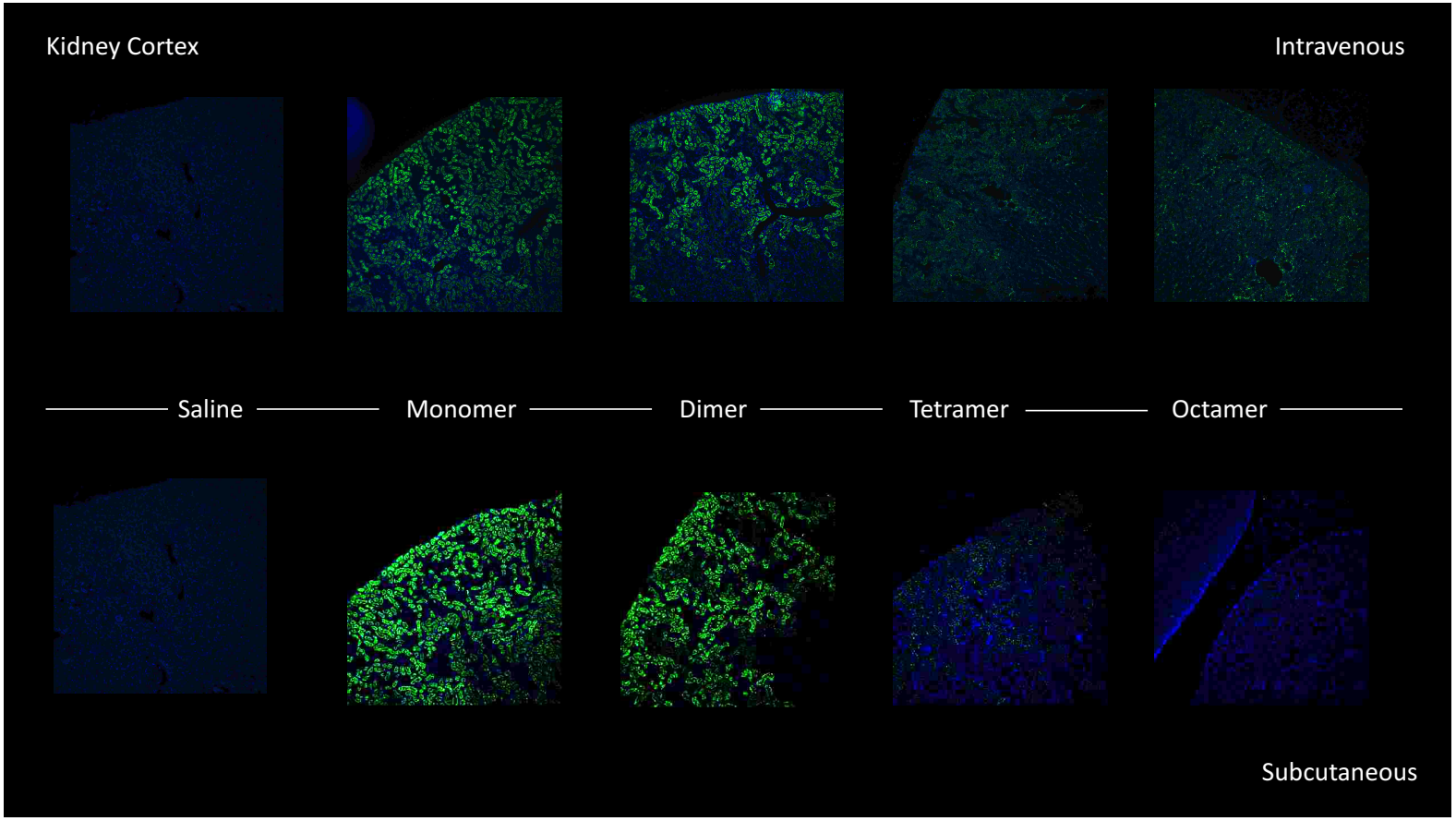


Figure D.2: Biodistribution of oligonucleotide dendrimers following injection of $10\,\mathrm{mg\,kg^{-1}}$
Blue: DAPI, Green: Anti-ASO pAb

D.1.3 Kidney Medulla

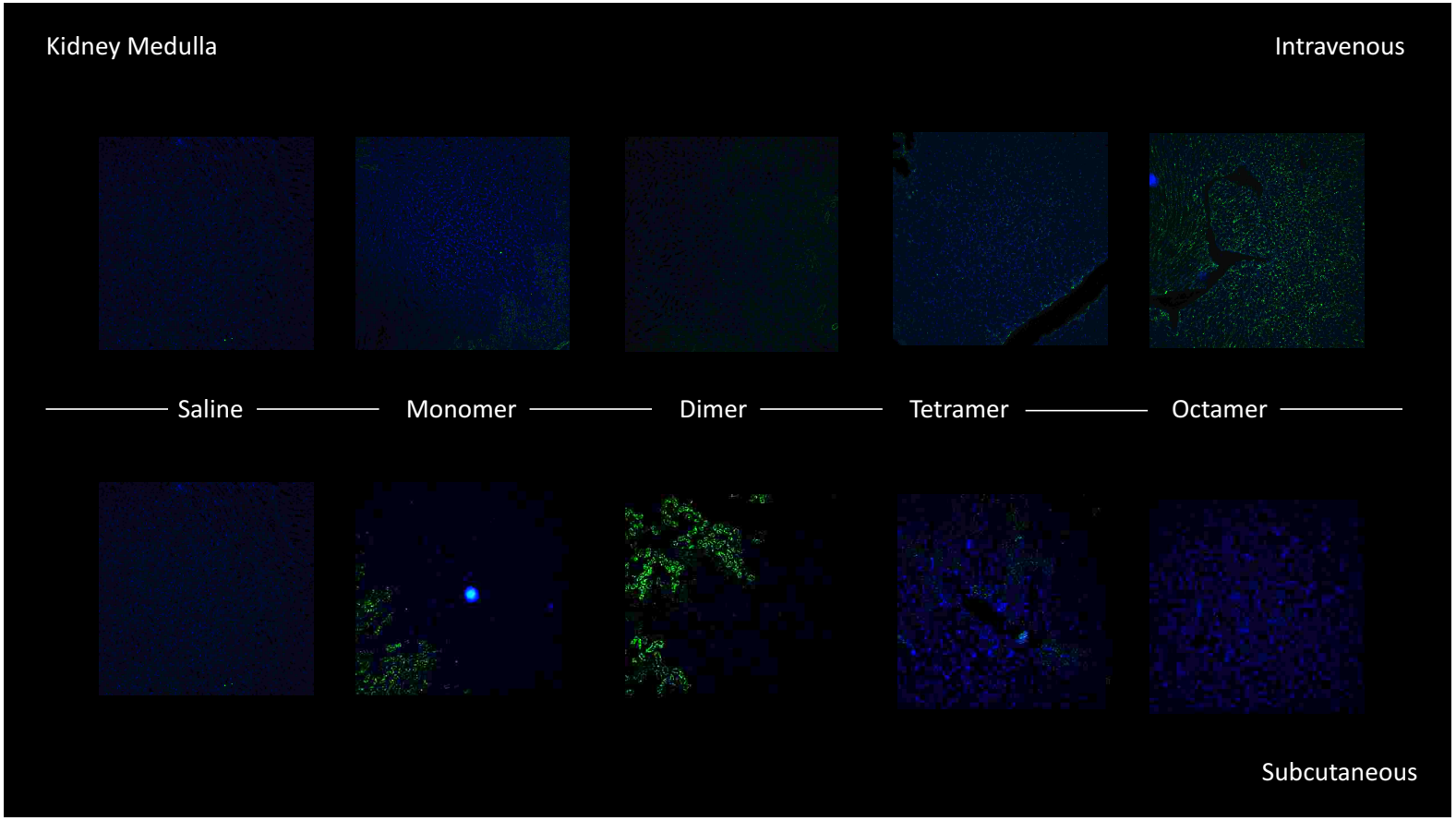


Figure D.3: Biodistribution of oligonucleotide dendrimers following injection of $10\,\mathrm{mg\,kg^{-1}}$
Blue: DAPI, Green: Anti-ASO pAb

D.1.4 Lung

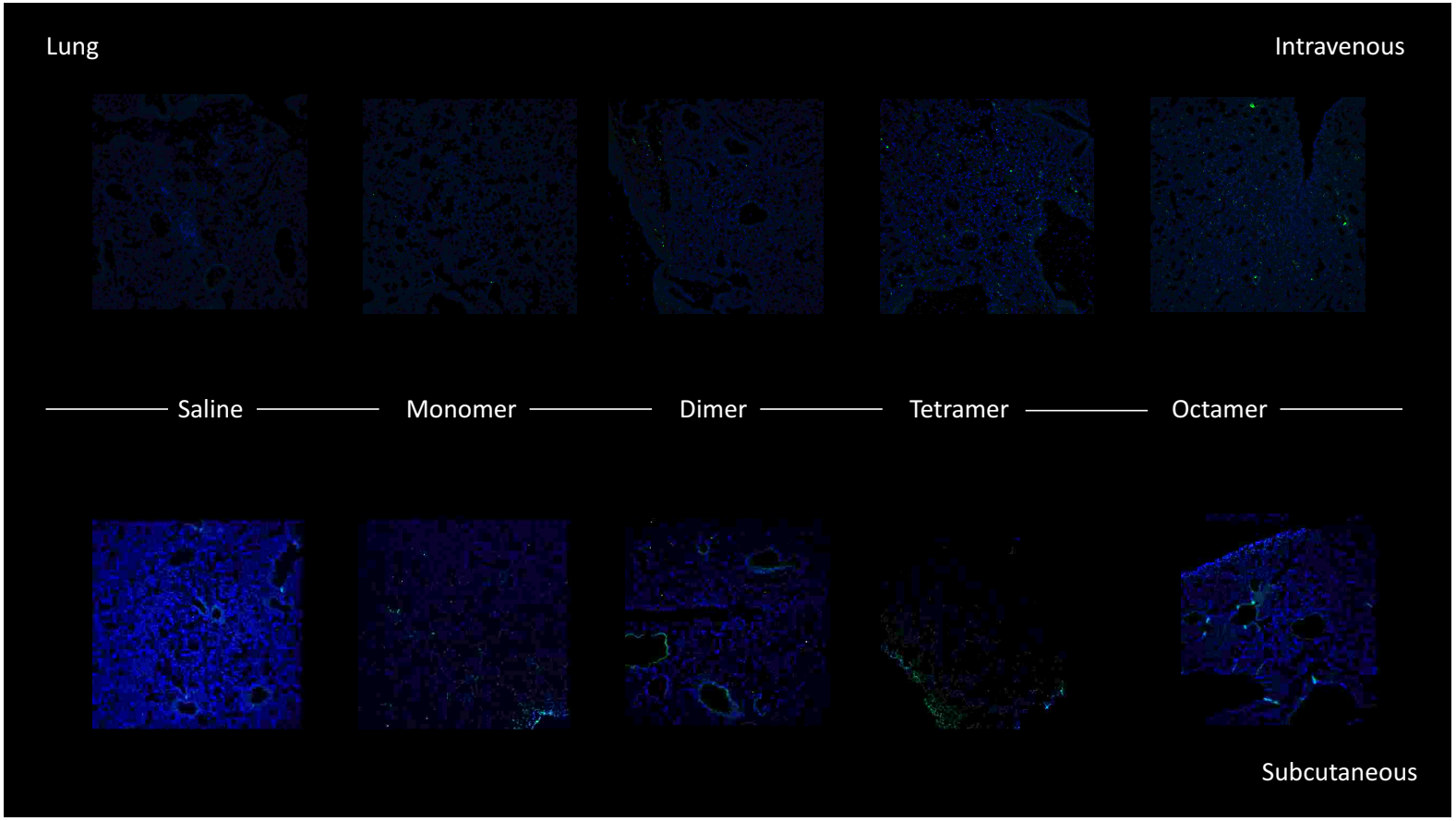


Figure D.4: Biodistribution of oligonucleotide dendrimers following injection of $10\,\mathrm{mg\,kg^{-1}}$
Blue: DAPI, Green: Anti-ASO pAb

D.1.5 Heart

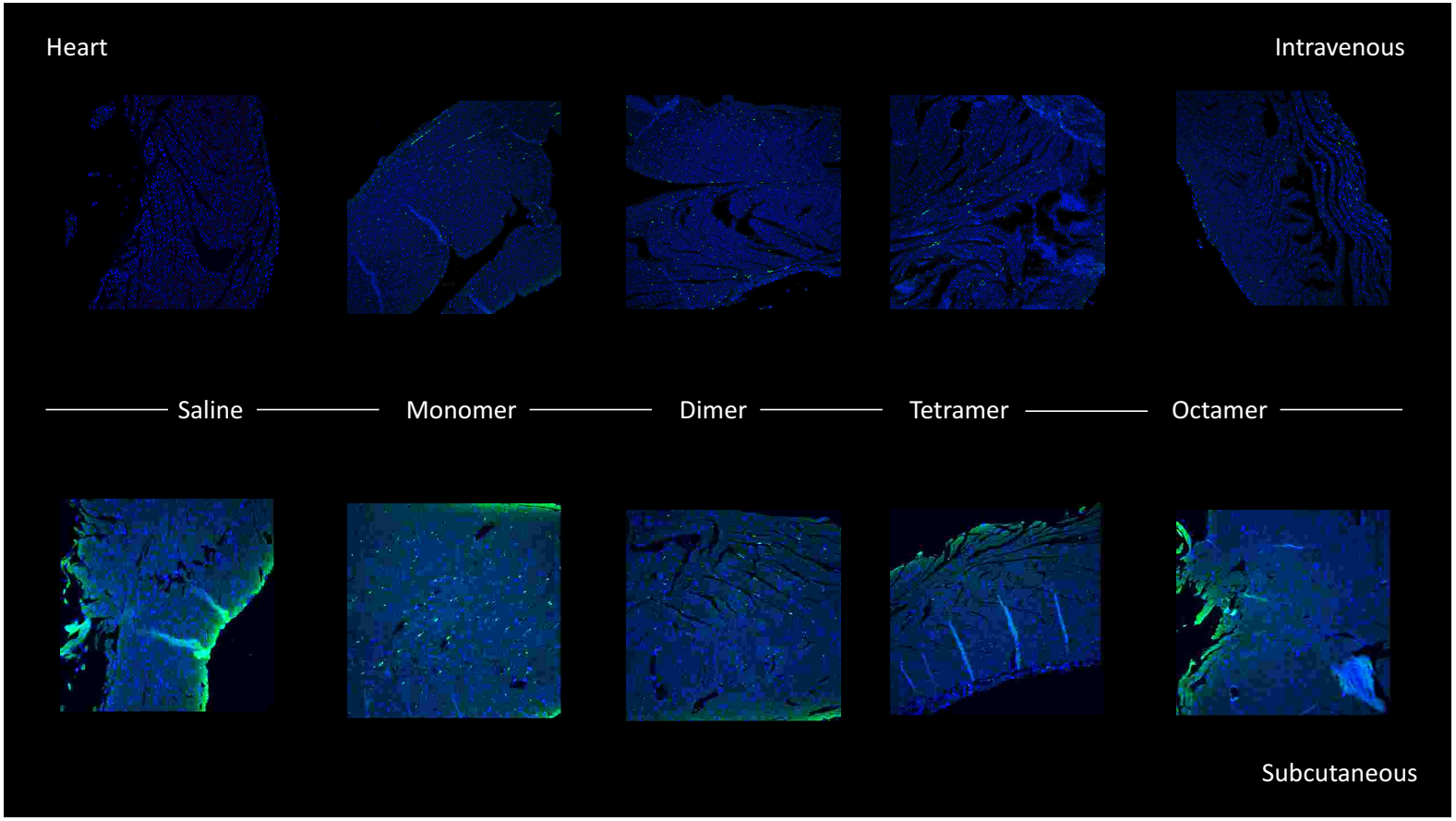


Figure D.5: Biodistribution of oligonucleotide dendrimers following injection of $10\,\mathrm{mg\,kg^{-1}}$
Blue: DAPI, Green: Anti-ASO pAb

D.1.6 Intestine

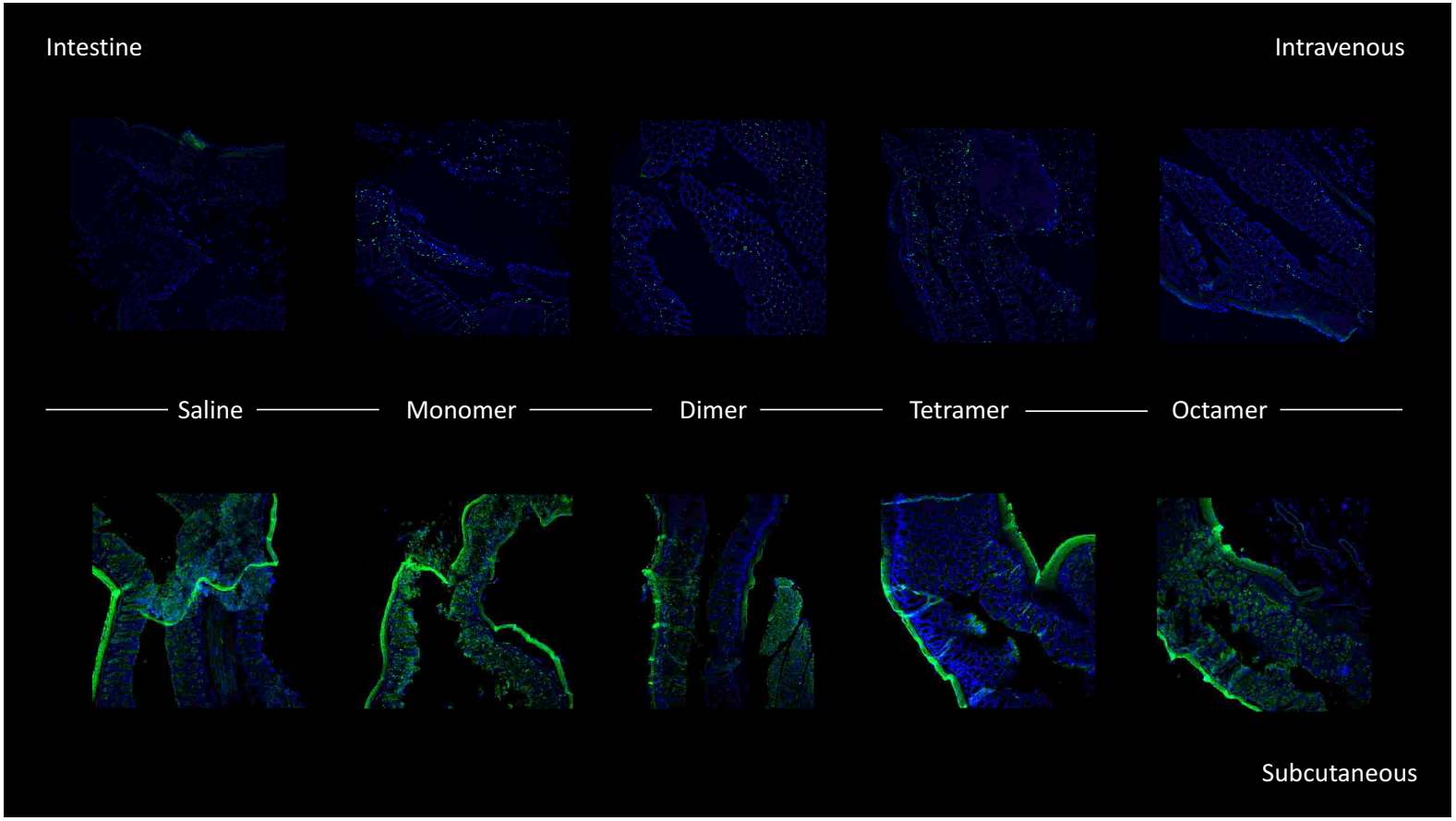


Figure D.6: Biodistribution of oligonucleotide dendrimers following injection of $10\,\mathrm{mg\,kg^{-1}}$
Blue: DAPI, Green: Anti-ASO pAb