UNIVERSITY OF SOUTHAMPTON

FACULTY OF ENGINEERING, SCIENCE & MATHMATICS

School of Chemistry

Novel approaches to dendrimer based radiopharmaceutical imaging agents and drug delivery systems

By

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Thesis for the degree of Doctor of Philosophy

May 2007

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF ENGINERRING, SCIENCE & MATHMATICS

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Doctor of Philosophy

NOVEL APPROACHES TO DENDRIMER BASED RADIOPHARMACEUTICAL IMAGING AGENTS AND DRUG DELIVERY SYSTEMS

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The work reported in this thesis details two novel approaches towards the use of poly(amidoamine) (PAMAM) dendrimers for medicinal applications. The introduction contains a brief overview on the synthesis and properties of dendrimers, focusing in particular on PAMAM dendrimers and the growing interest in their use as polymer therapeutics. The second part of the introduction contains an overview of the role of Tc^{99m} in nuclear medicine. The final part of the introduction gives an explanation of the genetic disorder, cystic fibrosis, concentrating on the degenerative lung disease associated with the disease and the role neutrophil elastase has in the degeneration of the lung. The next chapter then proceeds to describe the preparation of 4^{th} and 5^{th} generation PAMAM dendrimers with dioctylamine surface groups. These surface modified dendrimers are reported as potential $NaTc^{99m}O_4$ complexation systems for potential application as radiopharmaceutical agents for use in lung imaging. The next chapter describes the preparation of PAMAM dendrimers with β-lactam and saccharine-based inhibitors linked to the surface. These modified dendrimers are shown to inhibit intracellular neutrophil elastase in samples of human blood. The results indicate that the modified dendrimers could potentially be used to reduce the extent of lung damage in a cystic fibrosis patient and improve both their quality of life and life expectancy.

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Acknowledgements

Thank you to my girlfriend Keira for all her help, encouragement and support over the three years and for coming up with the Trojan horse analogy.

Thanks to my Mum, Dad and Sue for my initial years of sponsored inactivity at University and continued support over my PhD.

Thanks to those in my research group past and present for making the lab such a great place to work, Andy, Rojrit, George, Sophie, Daniel, James, Jon, Francesco, Alan, Alain, Michael, Polski and Chris.

Thanks to Martin for helping me and giving me such a great opportunity and allowing me to work with some great people.

Thanks to both my project students Tim Harridge and Rachael Robbins for all their hard work.

Thanks to Janis Shute and Sarah Clarke at Portsmouth University for their biological advice and sample testing.

Thanks for John and Julie of the Southampton Mass Spectrometry service and also Neil and Joan of the NMR service.

And thanks to the staff down in stores for keeping me in gloves and chemicals.

Abbreviations and Nomenclature

Solvent and reagent abbreviations

Acetonitrile	MeCN
Ammonia	NH_3
Ammonium chloride	NH_4Cl
tert-Butoxy carbonyl	Boc
Chloroform	$CHCl_3$
Deuterated Chloroform	$CDCl_3$
Deuterated dimethyl sulfoxide	d_6-DMSO
Deuterated methanol (d_1)	MeOD
Deuterated methanol (d_4)	CD_3OD
Dichloromethane	CH_2Cl_2
Diethyl ether	Et_2O
1, 8-Diazabicyclo [5.4.0] undec-7-ene	DBU
N, *N*-Dimethyl formamide	DMF
Dimethyl sulfoxide	DMSO
Ethanol	EtOH
Ethyl acetate	EtOAc
Ethylene diamine	EDA
Fluorescein isothiocyanate	FITC
Hydrochloric acid	HCl
Lithium chloride	LiCl
Magnesium Sulfate	$MgSO_4$
Methanol	MeOH
Nitrogen	N_2
Pertechnetate	TcO_4^-
Perrhenate	ReO_4^-
Poly(amidoamine)	PAMAM
Poly (ethylene glycol)	PEG

Poly (ethylene glycol) monomethyl ether	MPEG
Potassium carbonate	K_2CO_3
Potassium hydrogen carbonate	$KHCO_3$
Potassium hydroxide	KOH
Potassium perrhenate	$KReO_4$
Sodium carbonate	Na_2CO_3
Sodium hydrogen carbonate	$NaHCO_3$
Sodium hydroxide	NaOH
Sodium Iodide	NaI
Sodium pertechnetate	$NaTcO_4$
Sulfuric acid	H_2SO_4
Tetrahydrofuran	THF
Triethylamine	Et_3N
Trifluoroacetic acid	TFA
Trimethylsulfonium	TMS

Biological abbreviations

Cystic fibrosis	CF
Cystic fibrosis transmembrane conductance regulator	CFTR
Dry powder inhalers	DPIs
Neutrophil elastase	NE
Neutrophil elastase inhibitor	NEI
Magnetic Resonance Imaging	MRI
Positron Emission Tomography	PET
Pressurised metered dose inhalers	pMDIs
Secretory leukoprotease inhibitor	SLPI
Single photon emission computed tomography	SPECT

Miscellaneous abbreviations

Aryl	Ar
Analysis	Anal.
Boiling point	b.p.
Butyl	Bu
Calculated	Calcd.
Electrospray	ES
High resolution mass spectrometry	HRMS
Infra red	IR
Low resolution mass spectrometry	LRMS
Melting point	m.p.
Mass spectrometry	MS
Mass to charge ratio	m/z
Nuclear magnetic resonance	NMR
Parts per million	ppm
Propyl	Pr
Retention factor	R_f
Room temperature	R.T.
Singlet, doublet, triplet, quartet, multiplet	s, d, t, q, m
Strong, medium, weak, broad	s, m, w, br
Thin layer chromatography	TLC
Weight/volume	w/v

1. Introduction

1.1.1 Dendrimers

Dendrimers are macromolecules comprised of a series of branches extending outward from an inner core[1, 2]. The term dendrimer originates from the Greek *dendron*, meaning "tree". Dendrimers branch out in a controlled concentric "starburst" manner to produce highly branched three dimensional polymeric structures. Dendrimers are usually produced in a series of iterative steps, in which each additional iteration leads to a higher generation material. Dendrimers are comprised of three distinct parts: (i) a core, (ii) branching units and (iii) surface/terminal groups.

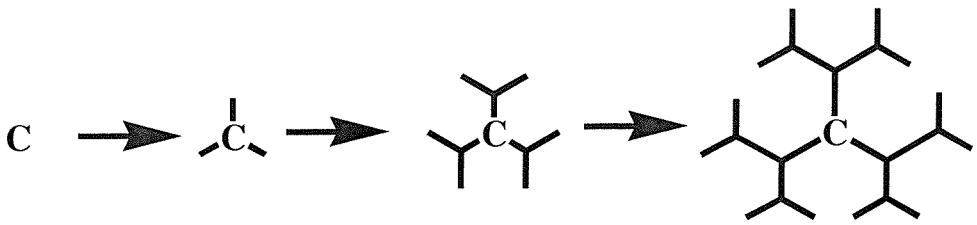


Figure 1. Stepwise dendrimer growth from an initiator core (**C**).

The controlled nature dendrimer growth allows for the size of the dendrimer molecules to be carefully regulated. It also allows for monodisperse populations to be prepared (>95 %)[3] rather than the great polydispersity of molecular weights normally associated with conventional chain polymerisation reactions.

1.1.2 Dendrimer development

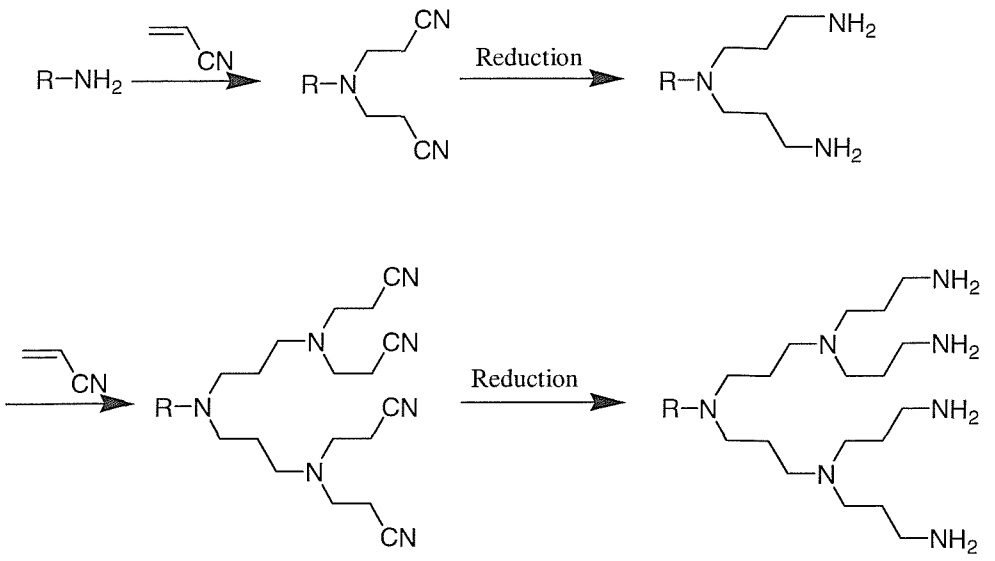
The progress towards the deliberate construction of macromolecules possessing branched architectures occurred in three periods[1]. The first occurred from the late 1940s to the early 1960s, when branched structures were considered responsible for the insolubility and intractability of materials formed in polymerisation reactions.

Synthetic control, mechanical separations and physical characterisation were primitive at that time meaning that isolation and proof of structure was not feasible.

In the second period (between the 1960s and 1970s) branched structures were considered primarily from a theoretical perspective. There were also initial attempts at their preparation using classical or single-pot polymerisation of functionally differentiated monomers.

The third period of development is considered to have started at the end of the 1970s and the beginning of the 1980s. There was preliminary success toward macromolecular assembly based on the iterative method. During this period, control of macroassembly construction was optimised. There were also advances in physical isolation and purification as well as spectroscopic characterisation.

It was in 1978 that Vögtle reported the first example of an iterative procedure applied to well defined branched structures, naming it "cascade"[4] synthesis, (Scheme 1). This involves Michael addition of an amine to acrylonitrile followed by reduction of the nitrile group to an amino group. Repetition of these steps leads to higher generation dendrimers.



Scheme 1. Vögtle's "cascade" synthesis.

1.1.3 Dendrimer synthesis

Dendrimer synthesis generally follows three steps. (i) An initiator, which can be converted into a dendrimer core in good yield. (ii) An iterative reaction in which the reactive initiator core is combined with the appropriate reagents or (partially protected) branched molecules leading to high yield conversions of branched assemblies with specific molecular surfaces. (iii) Reiteration of the previous steps or chain growth sequences to produce dendrimers possessing concentric generations of repeating units and branch junctions. A full layer (which may take more than one step of synthesis) is called a generation.

There are two different synthetic routes for the construction of high generation dendrimers, the *divergent* and *convergent* approaches. Both cases involve iterative repetitions of a sequence of reactions.

1.1.4 Divergent approach

This involves building the dendrimers outwards from a core in a stepwise fashion, (Figure 2). The main drawback from this approach comes from incomplete reactions at the developing chain termini of the dendrimer as this creates structural defects that accumulate with each new generation.

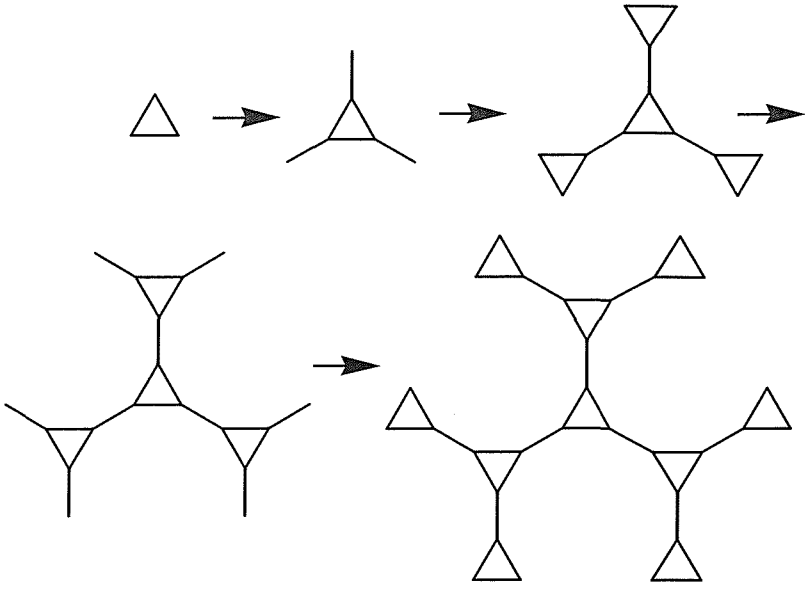


Figure 2. Divergent dendrimer synthesis.

As the by-products of the reaction often exhibit similar properties to the target molecule, separation of the desired dendrimer by, for example, chromatography is not always possible. It is to be expected that the higher generations of dendrimers constructed divergently will always contain a certain degree of structural imperfections[5]. Tomalia's Poly(amidoamine) (PAMAM) dendrimers[6-8], Newkome's "arborol" systems[9], (Figure 3) and Meijer's poly(propyleneimine)[10] dendrimers are all examples of dendrimers constructed by a divergent approach.

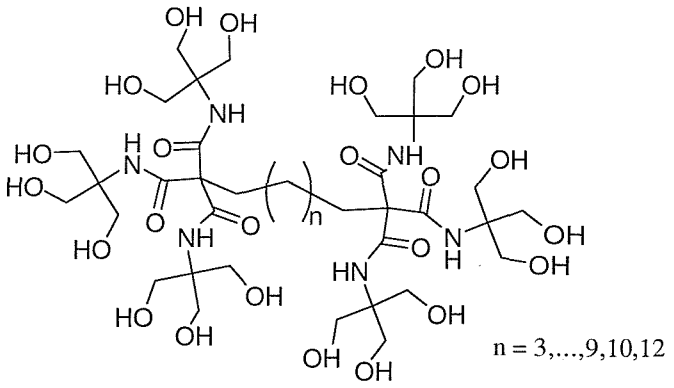


Figure 3. An example of Newkome's "Arborol" dendrimers[9].

1.1.5 Convergent approach

Convergent synthesis involves a number of preformed sections being added simultaneously to a central core molecule, (Figure 4).

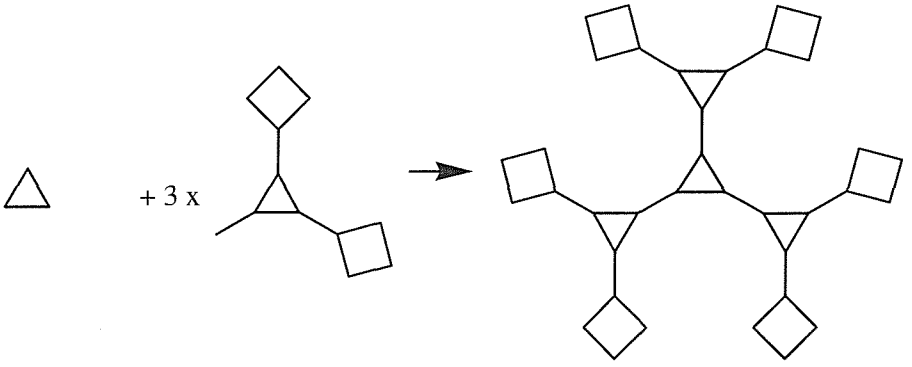
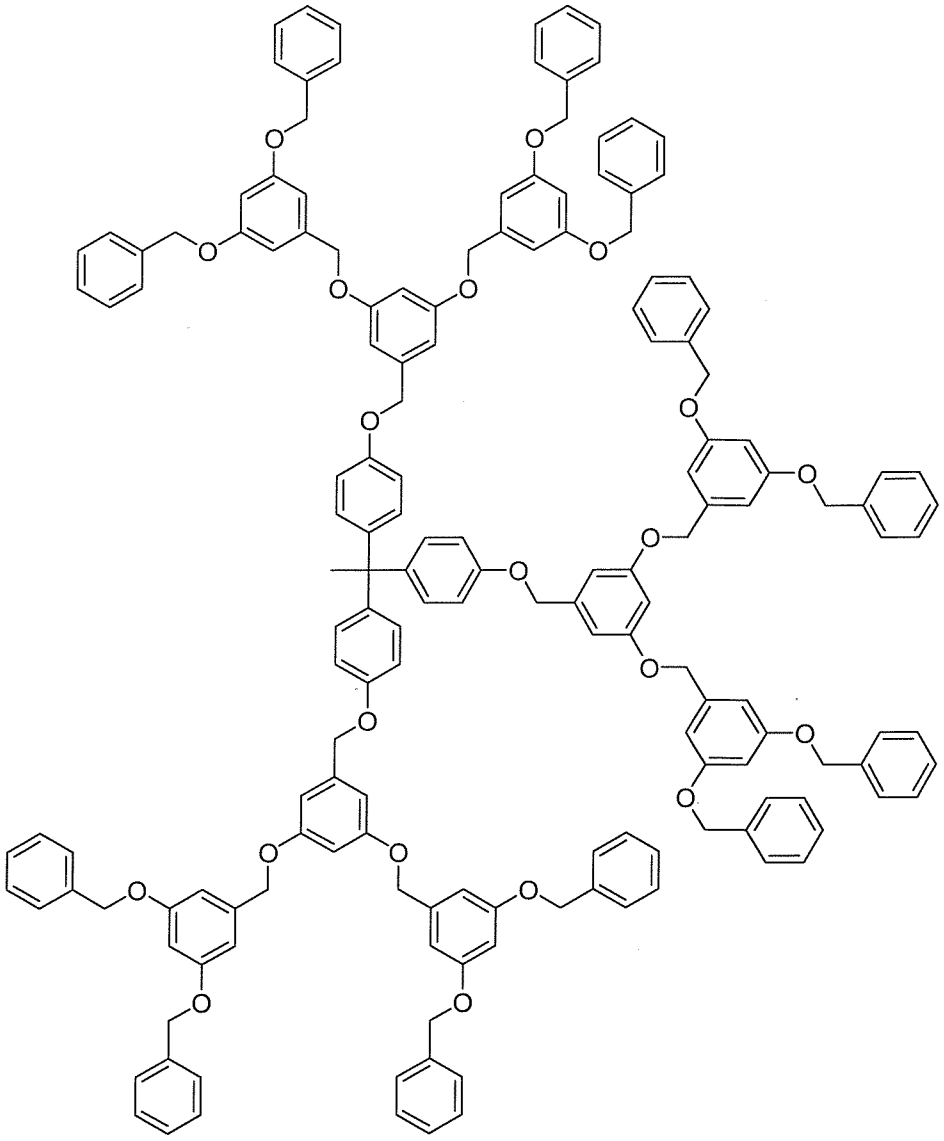


Figure 4. Convergent dendrimer synthesis.

Various different cores and wedges are available with different combinations providing unique sizes and shapes of dendrimers. Dendron wedges are prepared by the divergent route then attached to a multivalent anchoring. Examples of dendrimers synthesised by this approach are Moore's phenylacetylene dendrimers[11-14] and Fréchet's aromatic polyether dendrimers[15, 16] such as **1**, (Figure 5). In the latter case polyaryl wedges are made independently then coupled to a core unit.



1

Figure 5. An example of one of Fréchet's aromatic polyether dendrimers[15, 16].

1.1.6 Polymer therapeutics

One of the main interests in dendrimer development is for their use as drugs or multi-component conjugates in biological applications. Their use for this purpose defines them as being "polymer therapeutics"[17, 18].

Polymer therapeutics are complex, hybrid technologies often combining several components, e.g., polymer, drug, peptide, protein, glycoside or oligo-nucleotide. All use synthetic or natural polymers to create macromolecular drugs, polymer drugs and polymer-protein conjugates. This description reflects a regulatory authority perspective which sees these compounds as new chemical entities rather than conventional drug delivery systems or formulations which simply entrap, solubilise or control drug release without resorting to chemical conjugation[19].

Both successful and failed clinical trials involving polymer therapeutics have highlighted the need for careful choice of polymer for each intended application[19]. The most successful early constructs were synthesised using linear random coil polymers such as polyethylene glycol (PEG), *N*-(2-hydroxypropyl)methacrylamide (HMPA) copolymers, poly(glutamic acid) (PGA), poly(ethyleneimine) (PEI) and dextrin (α-1,4 polyglucose). Although these polymers have generated conjugates[20, 21] and polyplexes[20] which have been taken into clinical trials they present specific challenges for pharmaceutical development. A manufactured drug substance must be reproducible batch to batch, be composed of single, defined species whose identity, impurities and stability can be specified using validated techniques and whose pharmacokinetics and therapeutic index (activity and toxicity) can be precisely defined[19]. Many linear polymers and resulting conjugates are inherently heterogeneous and polydisperse. This variation in molecular weight can have a profound effect on biological activity in terms of toxicity and efficacy.

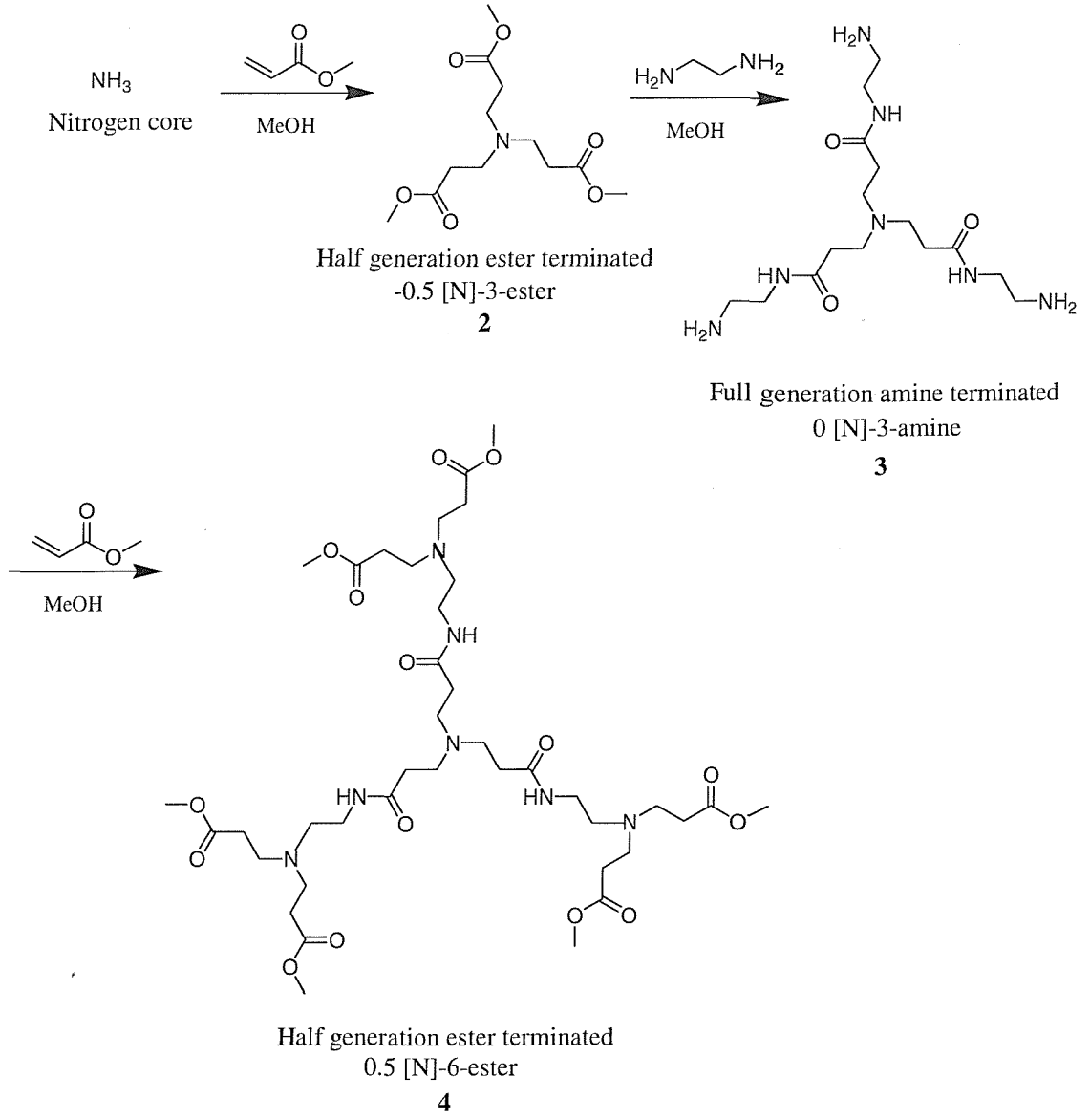
The monodisperse and defined chemical composition of dendrimers as well as a tailored multi-valency surface are particular reasons why dendrimers are attractive prospects for use as polymer therapeutics. However the complexity of many synthetic procedures presents different challenges in terms of cost-effectiveness of manufacture and challenging molecular characterisation[22].

The first dendrimer-based MRI imaging agent (SH L 634A; Gadomer-17)[23] entered clinical development in 2003. It was then followed in 2004 by a vaginal virucide

(Vivagel^{TM})[24]. Both examples are administered in different ways, SH L 634A; Gadomer-17 is given intravenously whereas Vivagel^{TM} is a topical cream in which the dendrimer enhances transdermal delivery.

1.1.7 PAMAM dendrimers

The most widely studied types of dendrimers for use as polymer therapeutics are polyamidoamine (PAMAM) type dendrimers. PAMAM dendrimers were the first complete dendrimer family to be synthesised, characterised and commercialised[26, 27]. These are AB_2 type condensation polymers that are constructed from tertiary amine and amide units. The first step in the synthesis of PAMAM dendrimers involves a Michael addition of a nucleophilic initiator core (e.g. ammonia or ethylenediamine (EDA)) with a multifunctional electrophilic reagent (e.g. methyl acrylate). The reaction of ammonia with methyl acrylate to form **2** can be seen in Scheme 2.



Half generation ester terminated
-0.5 [N]-3-ester
2

Full generation amine terminated
0 [N]-3-amine
3

Half generation ester terminated
0.5 [N]-6-ester
4

Scheme 2. A typical synthetic route to a PAMAM dendrimer.

Compound **2** is an example of a half-generation dendrimer. A full-generation dendrimer of which **3** is an example has expanded terminal multiplicity and any further growth to form **4** involves branching to give double the number of terminal chains. **3** is considered to be 0 generation and the ammonia core –1 generation. There is a general formula for the naming of PAMAM dendrimers, which is:

$$G\,[C]\,N_F$$

Where G is the generation number, C is the type of core, N is the number of terminal groups and F is the terminal group functionality. For example **2** is denoted –0.5 [N]-3-ester as it is –0.5 generation, has a nitrogen core and 3 terminal ester groups. (N.B. Older nomenclature has the initiator core being denoted as zero generation and the first full-generation to be 1st generation.)

The Michael addition to form **2** and other half-generation dendrimers using methyl acrylate is fast compared to the competing reaction which is nucleophilic attack by the amine on the ester and so **2** dominates as the major product.

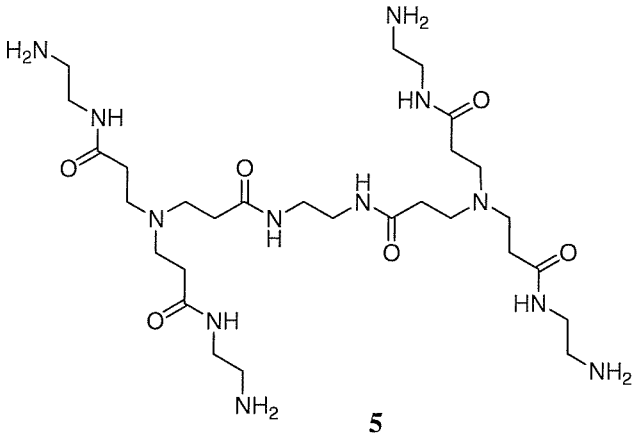
The second step is the reaction of **2** with a multifunctional nucleophile, (EDA) to produce **3**. This reaction involves nucleophilic attack by the EDA on the methyl ester groups to form an amide bond. The initiator core has an effect on the properties of the dendrimer; by using EDA as the core instead of ammonia there are 4, 8, 16 and 32 terminal groups for the 0, 1st, 2nd and 3rd full generations respectively compared to 3, 6, 12 and 24 terminal groups for ammonia cored full generations.

The use of ethanolamine in place of EDA leads to PAMAM dendrimers with hydroxy surface groups instead of primary amines. Further dendrimer growth using methyl acrylate to give higher generations is however no longer possible. Both amine and hydroxy terminated full generation dendrimers as well as ester terminated half-generation PAMAM dendrimers are commercially available.

1.1.8 Dendrimer defects and degradation

During dendrimer growth defects can occur, these defects then carry on through to the next step of production. When making a full-generation dendrimer, a large excess of EDA is needed to give a high dilution as this will prevent dimerisation. This occurs when EDA forms a bridge between two of the ester groups on different molecules, (Figure 6). **5** is an example of two half generation dendrimers becoming linked through an EDA bridge. Another unwanted side reaction is when EDA reacts

with two terminal ester groups of the same molecule of which **6** is an example. This effectively caps two branches of the dendrimer and produces defects in the dendrimer which prevent further growth at these terminal sites. Temperature also has unwanted effects, heating above 70 °C causes retro-Michael reactions which then lead to degradation of the dendrimer sample[3]. All of these unwanted reactions cause an increase in the polydispersity of the final dendrimer sample.



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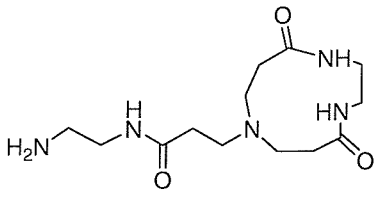


6

Figure 6. Examples of two of the main side products created in the formation of full-generation amine-terminated PAMAM dendrimers using the reaction of **2** with EDA as the example. i) Dimerisation of two molecules of **2** with an EDA bridge to give **5**. ii) Bridging of the two terminal ester groups of **2** with EDA to give **6**.

1.1.9 Purification of dendrimers

Lower half-generation PAMAM dendrimers such as **2** and **4** are soluble in some organic solvents such as $CHCl_3$ and so can be purified after the reaction by column chromatography. However due to their size this is not practical for other half-generation ester terminated PAMAMs. Purification of dendrimers over 3000 kDa can be carried out by dialysis against deionised H_2O or MeOH.

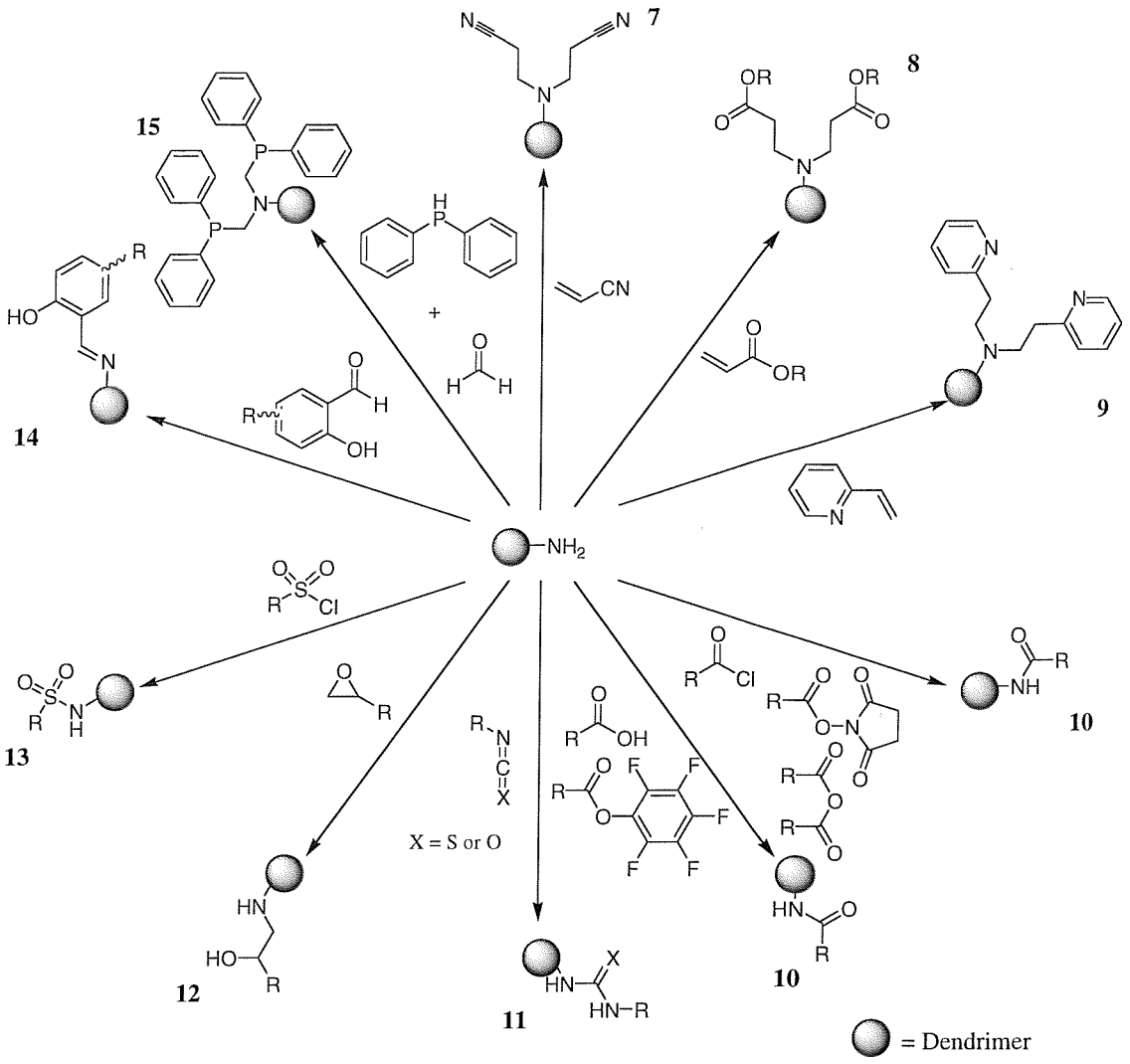
It is important to remove all excess EDA after the formation of full-generation amine-terminated dendrimers such as **3**. Purification of **3** by column chromatography is not feasible as the amine surface groups make the molecule too polar. Purification by dialysis is also not possible as the molecular weight is too low

to be contained by the dialysis tubing. Any remaining EDA will, in the next step act as a new dendrimer core and form -0.5 [EDA]-4-ester, which will be an unwanted impurity in the sample. Removal of EDA especially in the larger dendrimers is difficult and, although most can be removed by distillation, some remains in the internal cavities of the dendrimer. It can be removed by azeotropic distillation using toluene and MeOH in a 12:1 ratio. The MeOH helps dissolve the dendrimer and the toluene displaces any EDA held within the dendrimer cavities.

1.1.10 Functionalisation of PAMAM dendrimers

The peripheral amine groups of a PAMAM dendrimer can be fuctionalised in different ways. Owing to the high number of terminal groups per dendrimer only reagents which react selectively with the terminal groups and also have a high reactivity can be used. The reactions cannot be carried out in acidic conditions as this leads to protonation of the internal tertiary amines of the dendrimer which leads to degradation via reverse Michael addition reactions. The reactions cannot be heated above 70 °C as this also leads to dendrimer degradation.

These problems are the reason that very few reactions have been established for coupling functional groups to polyamino dendrimers. Many of the examples in Scheme 3 have only been carried out in individual cases[28].



Scheme 3. Reactions coupling functional groups to the surface of amine-terminated dendrimers.

For substrates which have a comparatively low boiling point, the reaction can be optimised by large substrate excess. Excess reagent can be removed by distillation. This strategy is applied in the skeleton-building reaction of polyamines and acrylonitrile to give the corresponding polynitrile[29-33] (**7**), as well as the functionalisation and skeleton constructing step using acrylic ester to yield polyesters[34] (**8**) and half generation PAMAM dendrimers[35, 36]. Vinyl pyridines have also been used to give polypyridines[37] (**9**). The conversion with activated carboxylic derivatives such as carboxylic acid chlorides[38-43], fluorides[44], acid anhydride[45] and *N*-hydroxysuccinimide esters[46-53] that lead to the corresponding polyamides (**10**) are

well established as well as broadly applicable. The separation of by-products such as chloride ions and *N*-hydroxysuccinimide succeeds thanks to the low solubility of the lipophilic dendrimers in water. Meijer *et al* have used pentafluorophenolic esters as coupling reagents which enabled the precipitation of dendritic products in non-polar sovents[48, 52]. As well as the active ester methods other approaches include the reaction with carboxylic acids in the presence of peptide coupling reagents to give amides[54] (**10**) and with isocyanates to urea derivatives[55, 56] (**11**). The coupling of isothiocyanates to give thiourea derivatves (**11**) has proved to be very useful in the synthesis of glycodendrimers[57-60].

Other reactions of polyamine dendrimers include reaction with epoxides to afford α-aminoalcohols[61] (**12**), with sulphonyl chlorides to sulphonamides[46] (**13**) and reaction with ortho-hydroxybenzaldehyde derivatives to give imines[62] (**14**). The double phosphinomethylation of the amine end groups via hydroxymethyldiphenyl phosphine formed *in situ* gives the corresponding bis(methyldiphenylphosphino)amines[63] (**15**).

1.1.11 Starburst limit

The starburst limit of a dendrimer is the limiting stage of uniformity in its construction. It is caused by surface crowding in the outer layer of the otherwise spherical dendrimers. When overcrowding of branches does occur, not all the groups at the edge of the dendrimer have sufficient room to continue to react and expand the molecule evenly. This does not stop the overall growth but results in some of the branches being unable to continue to grow, therefore control of the number of possible surface groups present and overall monodispersity is lost. The starburst limit in PAMAM dendrimers is reached at about the fifth generation[64], but could be controlled to some extent by the length of molecules used in constructing the inner layers of the dendrimer. This results in a size limit of approximately 1 to 50nm, which means that, amongst other applications, dendrimers are potentially useful in dealing with biological systems.

1.1.12 PAMAM dendrimers as polymer therapeutics

PAMAM dendrimers closely match the sizes and contours of many important proteins and bioassemblies[65]. For example, insulin (≈ 3 nm), cytochrome C (≈ 4 nm) and haemoglobin (≈ 5.5 nm) are approximately the same size and shape as generations 3, 4 and 5 respectively of ammonia-core PAMAM dendrimers. Generations 5 and 6 have diameters which are approximately equivalent to the ubiquitous lipid bi-layer membranes of biological cells[65]. PAMAM dendrimers have also been determined to be non-immunogenic and exhibit low mammalian toxicity, especially when their surface contains anionic or neutral groups such as carboxylic or hydroxylic functionalities[66].

These features have enabled PAMAM dendrimers to be developed for use in biological applications, such as carriers for chelating groups for MRI contrast agents[67-68]. These dendrimer-based MRI agents provide greatly enhanced images of the heart, blood vessels and a variety of other organs. Because of the high number of positive charges on their surfaces at physiological pH, PAMAM dendrimers can form stable electrostatic complexes with negatively charged nucleic acids. This has led to work in developing ^{111}In-labeled oligonucleotide-dendrimer complexes that target tumours and are detectable by diagnostic nuclear medicine[69]. PAMAM dendrimers also have the potential to be used as gene delivery agents[70], as they can mediate the high efficiency transfection of DNA into a variety of cultured mammalian cells, transfection being a function of both the dendrimer-DNA ratio and the diameter of the dendrimers. Other work has been carried out to conjugate biologically active molecules such as antibodies[71-72] and sugars[73, 74] to the surface of PAMAM dendrimers.

1.2.1 Nuclear medicine

The first application outlined in this thesis for PAMAM dendrimers as polymer therapeutics is as a diagnostic tool in the field of nuclear medicine.

Diagnostic nuclear medicine is a useful discipline wherein diagnostic and prognostic information is obtained by a non-invasive (i.e. non-surgical) procedure[75]. A radiopharmaceutical containing an emitting isotope is administered to the patient; the radiopharmaceutical is tailored to target a specific organ within the body. Subsequent visualisation of the organ with suitable imaging equipment provides information about both the structure and function of that organ.

Techniques such as ultrasound, X-ray and Magnetic Resonance Imaging (MRI) are invaluable tools for the diagnosis of disease. They provide detailed images of anatomical features but offer only limited information about biochemical and physiological function[76]. However such data may be obtained by diagnostic nuclear medicine which provides a unique way of assessing disease states and monitoring the effects of treatment.

1.2.2 Radionuclides in nuclear medicine

Two basic types of radionuclide are suitable for use in diagnostic nuclear medicine, β^+ and γ-emitters. When β^+ particles or positrons are emitted they have a high chance of impacting with an electron. When this happens a positron-electron annihilation takes place resulting in emission of collinear γ-rays each with an energy of 511 KeV. These emissions can be visualised by Positron Emission Tomography (PET)[77]. Examples of β^+ emitters include: ^{52}Fe, ^{64}Cu and ^{68}Ga.

γ-Rays are photons produced by internal nuclear transitions. They often, but not always accompany particle emissions. Although high in energy, the ionizing potential is very low and therefore they are ideally suited to radiodiagnosis[77]. Examples of γ-emitters include: ^{57}Co, ^{99m}Tc and ^{201}Tl. γ-Ray energies between 80-300 KeV are necessary and between 100-200 KeV is optimum for the instrumentation used for detection[76].

The ideal radionuclide should decay to a long-lived daughter isotope by γ or β^+-emission that is not accompanied by α or β^- particle emission. The length of the half-life should be between a few hours and a day as this will allow time for chemical preparation of the radiopharmaceutical, administration to the patient and the imaging procedure itself to be carried out whilst minimising the dose of radiation to the patient[76].

It would be convenient if the emitting radionuclide in the pharmaceutical were an isotope of carbon as ^{11}C ($t_{1/2}$ = 20.3 minutes) is a β^+ emitter, which gives it the right properties for potential use in a radiopharmaceutical. However the short half-life requires that; upon generation, the isotope must be quickly and efficiently incorporated into the potential drug. A cyclotron is also required for its generation. For these reasons this particular radionuclide has been limited to research applications[76].

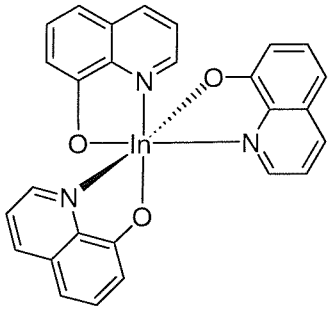
The radionuclides which have physical (or nuclear) properties for use in diagnostic or therapeutic nuclear medicine are predominantly metals[76]. A suitable metal can be incorporated into a radiopharmaceutical by complexation. By modifying the environment around the metal, specific *in vivo* targeting can be incorporated into the radiopharmaceutical. This type of radiopharmaceutical can be classified into two groups:

1.) Metal-essential, in which the biological distribution is determined by the properties of the coordination compound.

2.) Metal-tagged, where the properties of the carrier molecule determine the biological distribution. For example, by using a receptor-specific molecule or a monoclonal antibody. The targeting moiety is labelled with the radionuclide directly or with a bi-functional chelate. However, by attaching a separate chelating moiety the molecular weight is increased significantly, which may hinder the transport of the molecule across cell membranes[78].

1.2.3 Examples of radionuclides employed in nuclear medicine

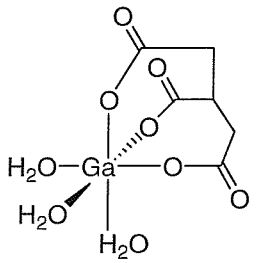
There are many examples of metal complexes that can be used in diagnostic nuclear medicine. For example ^{111}In-oxine (Indium 111 Oxyquinoline solution, Amersham International) **16** (Figure 7), is used to label leukocytes (white blood cells) and thus images sites of infection and inflammation. Once inside the cell ^{111}In becomes displaced and binds to other cytoplasmic components, therefore becoming trapped in the cell. Cells are labelled *in vitro* and are reintroduced to the patient's blood stream for imaging purposes[77].



16

Figure 7. ^{111}In-Oxine (**16**).

^{67}Ga-Citrate (Neoscan, mediphysics; Gallium Citrate Ga 67 Injection, Mallinckrodt; Gallium Citrate Ga 67 Injection, U.S.P., duPont-NEN), **17** (Figure 8), concentrates in certain viable primary and metastatic tumours as well as focal infection sites[77].



17

Figure 8. ^{67}Ga-Citrate (**17**).

1.2.4 Technetium

The metal which is employed most often in diagnostic nuclear medicine is technetium. One of its isotopes ^{99m}Tc is used in a high percentage of the scans annually performed[79]. Technetium was first detected in 1937 by C. Perrier and E. Segré[80] in a sample of molybdenum which had been bombarded with deuterons in a cyclotron. It was present in the form of ^{95m}Tc and ^{97m}Tc which have half lives of 61 and 90 days respectively. There are now twenty known isotopes of technetium (^{91}Tc - ^{110}Tc) and seven additional metastable nuclear isomers (excited states with a measurable lifetime). The only isotope which occurs naturally is ^{99}Tc which is formed by the spontaneous fission of ^{238}U and has been isolated in nanogram quantities from pitchblende[81]. The quantities available in the earth's crust are extremely low (of the order of 7 x 10^{-8}%, 0.0007 ppm)[82]. Technetium therefore has to be obtained from other sources such as from nuclear power stations where it makes up about 6% of uranium fission products[82].

For nuclear medicine the isotope of interest is ^{99m}Tc which is obtained from the decay of ^{99}Mo (Figure 9), which in turn is obtained from the fission products of natural or reactor uranium or from the neutron irradiation of ^{98}Mo. The radiophysical properties of ^{99m}Tc, which exists in a metastable nuclear excited state are nearly ideal for diagnostic nuclear medicine[83]. This is because as the 141 KeV γ-ray emitted from ^{99m}Tc falls within the optimum 100 - 200 KeV range for detection equipment and are sufficiently energetic to penetrate several layers of tissue, giving rise to a scintillation event on striking the NaI crystals used in the detection instrumentation. It has a short half life of 6.02 hours; this allows for the administration of high radiation counts which means that the biological dose is relatively small when integrated over the fairly short time it is emitted. There are no α or β^- particle emissions associated with its decay, these features allow for the repetition of diagnostic scans as needed[83]. Whilst some technetium may remain in body tissues for considerable periods, the daughter isotope ^{99}Tc emits only β-radiation with a maximum energy of 292 KeV and owing to its long half life of

2.1 x 10^5 years the additional radiation dose from the residual ^{99}Tc can be considered to be negligible[83].

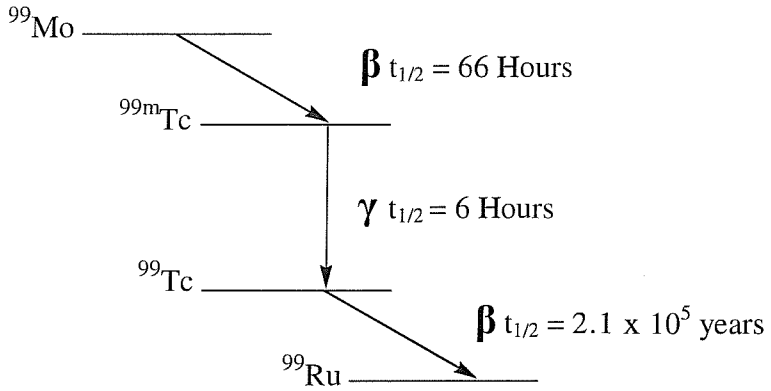


Figure 9. Schematic diagram of the decay of ^{99}Mo.

Two practical developments have led to the widespread use of technetium in radiopharmaceuticals[84]. In the 1950s and 60s Tucker and Greene developed the ^{99}Mo/^{99m}Tc generator; which allows production of technetium-based radiopharmaceuticals without having to be in close proximity to a cyclotron[85]. The technetium generator (Figure 10) contains molybdate $[^{99}MoO_4]^{2-}$ adsorbed at the top of a lead-shielded alumina ion-exchange column. The ^{99}Mo decays with a 66 hour half life by β^- emission to give pertechnetate $[^{99m}TcO_4]^-$. Due to the difference in charge of these ions the pertechnetate can be eluted from the column using physiological saline solution (0.15M NaCl). Since ^{99m}Tc undergoes decay to ^{99}Tc, the eluant contains both nuclides. The relative quantities of ^{99m}Tc and ^{99}Tc depend on the age of the column and the length of time since it was eluted. Total technetium quantities in the eluant are variable[86], but are usually in the range of 10^{-8} and 10^{-6} M. The adequate quantity of pure ^{99m}Tc necessary for satisfactory imaging is on the order of a few nanograms, so that even with several times the amount of ^{99}Tc present relative to ^{99m}Tc , a scan can still be performed and the chemical toxicity is negligible[83].

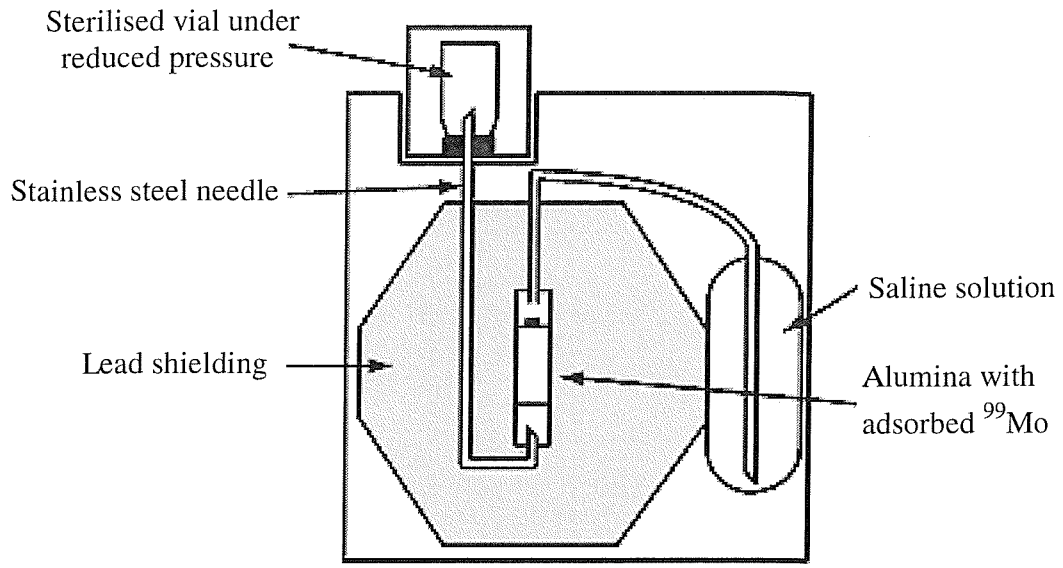


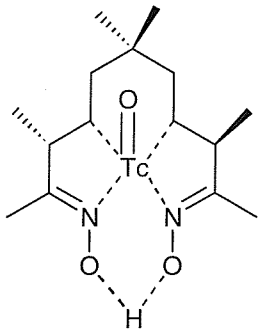
Figure 10. Structural arrangement of a $^{99m}TcO_4^-$ generator.

The second development that has led to the widespread use of technetium is the single-vial kit. This allows the rapid preparation of the radiopharmaceutical from the eluted pertechnetate solution[87]. The kit mainly consists of suitable reducing and chelating agents so that the desired radiopharmaceutical can be prepared quickly and in high radiochemical purity of > 90%[84]. In the absence of reducing agents the pertechnetate ion is quite stable in aqueous solutions and it usually only binds through electrostatic interactions. In general, co-ordination is achieved by reduction of the pertechnetate in the presence of a good complexing agent. Tin (II) compounds have been shown to be the most reliable reductants[88].

1.2.5 Technetium based imaging agents

There are many examples of technetium compounds that can be used in diagnostic nuclear medicine. Some of which include Ceretec™ (Nycomed Amersham) **18** (Figure 11), which is used in brain imaging for the evaluation of stroke. The ligand loses two amine protons and one oxime proton on coordination of the Tc(V) monooxo core giving a neutral complex. This is then able to pass through the blood

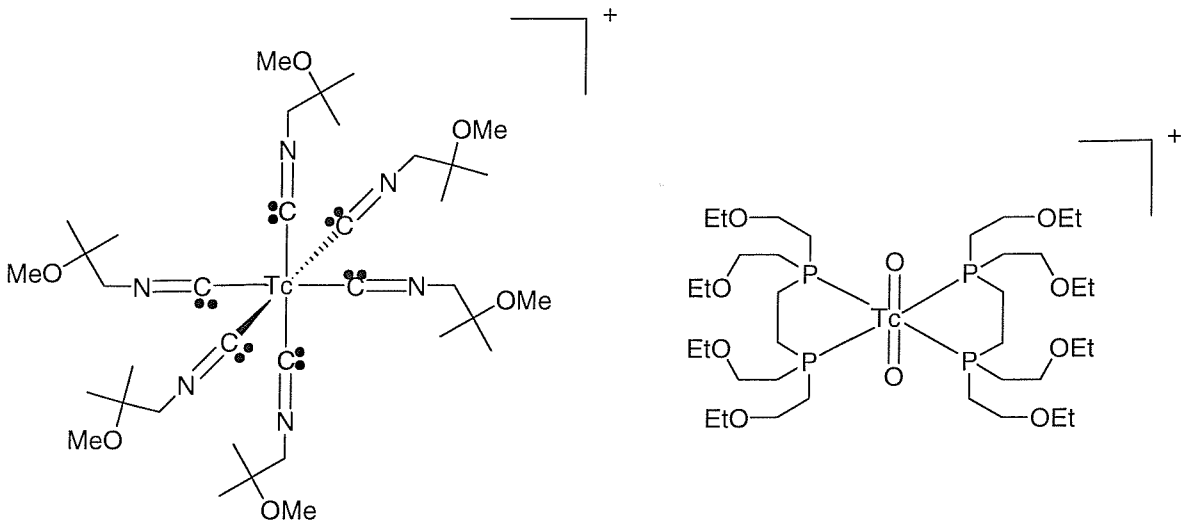
brain barrier where it is metabolised thereby blocking the diffusion back out of the brain; cerebral blood flow can then be imaged[89].



18

Figure 11. Ceretec (**18**).

Unipositively charged complexes concentrate in the muscle of the heart[90] (myocardium) in a similar way to K^+, Rb^+ and Tl^+. Cardiolite® (DuPont-Merck) **19** (Figure 12), features an octahedral array of isonitrile ligands surrounding the central Tc(I) ion. Myoview® (Nycomed Amersham) **20** (Figure 12), features a dioxotechnetium (V) core (TcO_2^+) bound by two neutral diphosphine ligands. Other radiopharmaceuticals have been developed for use in kidney, liver and skeletal imaging[91].



19 **20**

Figure 12. Cardiolite (**19**) and Myoview (**20**).

Because of the short half-life of ^{99m}Tc it is unsuitable for use in the characterisation and study of technetium complexes. Instead ^{99}Tc ($t_{1/2}$ = 2 x 10^5 years, E_β = 293 KeV) is used. This has a long half life and its radioactivity is comparable to ^{14}C (E_β = 156 KeV) and ^{32}P (E_β = 1703 KeV) which are both commonly handled in milligram amounts in laboratories where the appropriate safety precautions are employed[83]. The β-emission from ^{99}Tc is effectively stopped by solutions and laboratory glassware. The soft X-ray radiation (Bremsstrahlung) resulting from stopping the β-particle is not a significant problem[83].

1.2.6 Imaging techniques

There are two different techniques for detecting the resulting radiation, Positron Emission Tomography (PET) for β^+-emitters and Single Photon Emission Computed Tomography (SPECT) for γ-emitters such as ^{99m}Tc. SPECT works by detecting γ-rays which are emitted from radiopharmaceuticals within the body. The detector consists of Ti-doped NaI crystals which absorb a significant fraction of γ-radiation below 500 KeV. This absorption of radiation causes an ejection of a core electron in

the NaI which imparts its energy to the crystal matrix and results in an emission of a photon of light. This is known as a scintillation event; the emitted light photon is detected and turned into an electrical pulse by a photomultiplier or photodiode. This electrical pulse can then be turned into a graphical display. The emission of light photons is directly proportional to the number and energies of the incident γ-radiation[83]. Different camera systems can image different parts of the body and a three-dimensional image is obtained by mounting an Anger camera on a rotating gantry and recording images at different angles. The images are then combined to give a reconstruction of activity distribution[77]. Full body scans can be achieved using Anger cameras which have an array of Na(Ti) detectors each having a single hole collimator. Scans of smaller areas such as the thyroid can be imaged using a high resolution collimator focusing on a single wide crystal.

1.2.7 Application of nuclear medicine in lung imaging

Therapeutic aerosols such as dry powder inhalers (DPIs) and pressured metered dose inhalers (pMDIs) are the devices currently used for self administration of topical drugs to the lungs. They are routinely employed for the management of asthma and are capable of depositing approximately 10 to 30% of the nominally stated dose to the lungs[92, 93]. However little is known of the deposition of the drug whilst in the lungs, so the potential for targeting the drug to a specific site within the lungs for the improved management of airway diseases is therefore unknown. As well as current inhaled drugs, other therapeutic technologies are being developed, such as α-1 antitrypsin[94] for use against developing emphysema and gene therapy agents for the treatment of cystic fibrosis[95]. These drugs and others like them may be highly effective, but only if they can be administered directly to the disease site. It is therefore important to obtain information which details the regional deposition of the aerosols from these inhalers.

Such information can be achieved by inhalation of a radiolabelled aerosol from DPIs and pressurised metered dose inhalers pMDIs. Combined with imaging this can give

detailed information about the extent of aerosol deposition within the mouth, oropharynx, lungs and stomach. Sodium pertechnetate is absorbed onto the aerosol particles prior to inhalation, the deposition of these particles can then be visualised. As no chemical bonds are formed, the drug and radiolabel are released unchanged following dissolution of the particle on the lung surface. However because of pulmonary clearance mechanisms the $NaTcO_4$ has a short half-life in the lung of approximately 10 minutes[96]. This is adequate to obtain a two dimensional image of the lung by recording anterior and posterior images but this provides only limited information regarding the extent of lung penetration. Regions of interest overlap because the lungs are hemispherical in shape and when viewed in a single plane little differentiation can be made between large central airways, smaller bronchioles and the alveoli. Three-dimensional SPECT imaging provides a more sensitive measure of lung pentration[97-99]. However dual-head SPECT imaging requires a stable distribution of radiolabel within the body for approximately 15-30 minutes[98, 99]. This condition is not met with simple salts such as $NaTcO_4$, as less than 36% of the inhaled pertechnetate is remaining in the lungs at the end of the imaging period which leads to poor quality data.

1.2.8 Lung function and structure

The principal function of the lungs is to transport oxygen from the atmosphere into the blood stream, and to excrete carbon dioxide from the bloodstream back into the atmosphere. They are located inside the thoracic cavity and protected by the rib cage. The lungs are connected to the oesophagus via the trachea (windpipe). This divides into the left and right bronchus. The main right bronchus is wider, shorter and more vertical than the left bronchus. Each of the bronchi divides into two and three secondary bronchi that serve the left and right lungs, respectively. These then divide again several times into smaller bronchioles, these are the first airway branches that no longer contain cartilage and are smaller than 1 mm in diameter. These terminate into alveoli, each alveolus being composed of respiratory tissue. A network of fine capillaries transports blood over the surface of the alveoli. Oxygen

from the air inside the alveoli diffuses into the bloodstream across the thin alveolar membranes. Carbon dioxide moves from the blood to the alveoli by the same process.

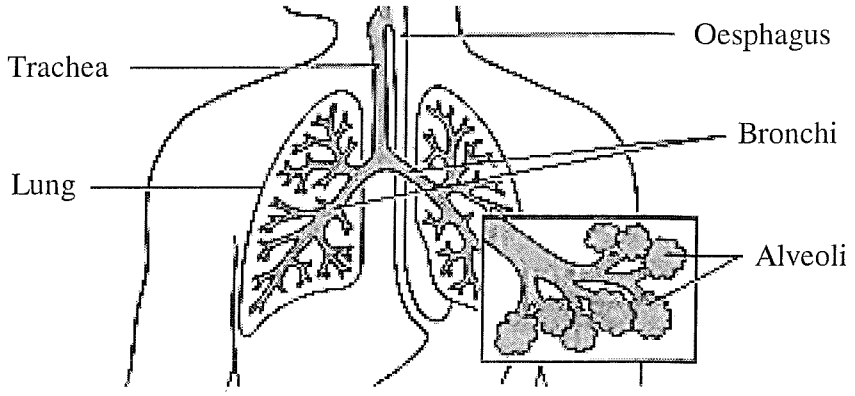


Figure 13. Diagram of the lungs.

1.2.9 Pulmonary clearance of $NaTcO_4$

It is understood that a significant contribution to pulmonary clearance of the $NaTcO_4$ may be through a large population of "water filled pore-like structures" in the epithelium[100]. Pulmonary epithelial transport studies have been conducted to correlate molecular size, weight, lipophilicity and charge to pulmonary clearance rate[101-106]. The evidence suggests that size is the predominant criterion that determines the precise clearance mechanism employed and therefore the clearance rate. The binding of $NaTcO_4$ to a macromolecule of sufficient size such as a PAMAM dendrimer would afford a complex that is too large to be removed from the lung through the epithelium and clearance would then be left to other slower processes. This would increase the half-life of the $NaTcO_4$ in the lung allowing for a better quality three-dimensional SPECT scan. A high generation PAMAM dendrimer is of sufficient size so that it is too large to be removed from the lung through the epithelium and clearance would then be left to slower processes such as epithelial transcytosis, phagocystosis or mucocilliary clearance.

Binding $NaTcO_4$ to dendrimers of different sizes may also affect distribution of the radiopharmaceutical within the lung allowing specific areas to be targeted for imaging.

1.3.1 Cystic fibrosis

The second application explored in this thesis for PAMAM dendrimers as polymer therapeutics is to use them as drug delivery platforms which could potentially be employed as a treatment for the genetic disorder known as cystic fibrosis.

Cystic fibrosis (CF) is the most common genetically inherited disease in the western world, 1 in 2500 children being born with two of the 1000+ different mutations in the CF transmembrane conductance gene[107]. This gene codes for the cystic fibrosis transmembrane conductance regulator (CFTR) a c-AMP regulated chloride channel which normally resides at the apical surface of many epithelial cell types. This leads to the production of a defective CFTR protein that disrupts chloride ion transport resulting in impaired water fluxea across the epithelial layer. This causes sticky mucus secretions that obstruct the secretory glands of the lungs, digestive tract, pancreas, liver, sweat glands and reproductive tract[108].

In the lungs the CFTR channel is found in surface airway epithelial cells and the cells on the submucosal glands[109]. In a non-CF airway islands of mucus float on top of beating cilia. One of its roles is to trap and dispose of inhaled bacteria, the mucus is cleared from the lungs by being pushed upwards by the cilia toward the oesophagus and the stomach by the mucociliary escalator, (Figure 14).

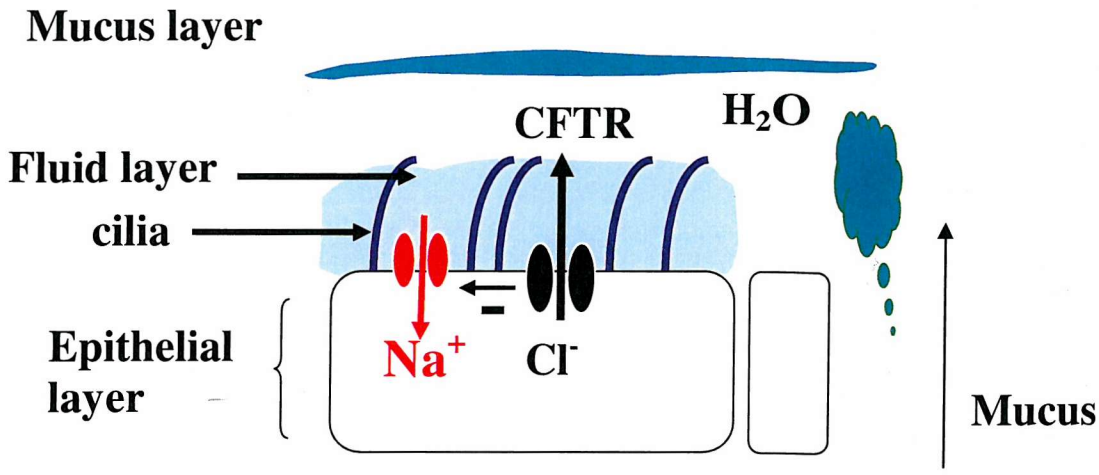


Figure 14. CFTR in a healthy airway.

In a CF airway the chloride transport is disrupted. This leads to mucus secretions that are poorly hydrated and hypoxic. The abnormal mucus is plastered down on the cilia and so inhibits lung clearance via the mucocilliary escalator. This causes airway obstruction and the stagnant mucus invites chronic infection from a range of organisms such as *H. influenzae, S. aureus, B. cepacia* and *P. aeruginosa*[110, 111]. *H. influenzae* and *S. aureus* may predominate in early life but 80% of young adult CF patients are chronically infected with *P. aeruginosa*[110-112]. Some strains of *P. aeruginosa* have the ability to mutate rapidly in the lungs of patients with CF and these hypermutatable strains demonstrate an increased ability to resist antibiotics[109]. *P. aeruginosa* also has the ability to organise into a biofilm within the lungs. A biofilm is where the cells are irreversibly attached to a substratum or interface with each other. They embed themselves in matrix of polymeric saccharide which they have produced[113]. Biofilm formation protects the bacteria from changes in environmental conditions, antibiotic and host defences. This allows the bacteria to persist in the airways of CF patients, (Figure 15).

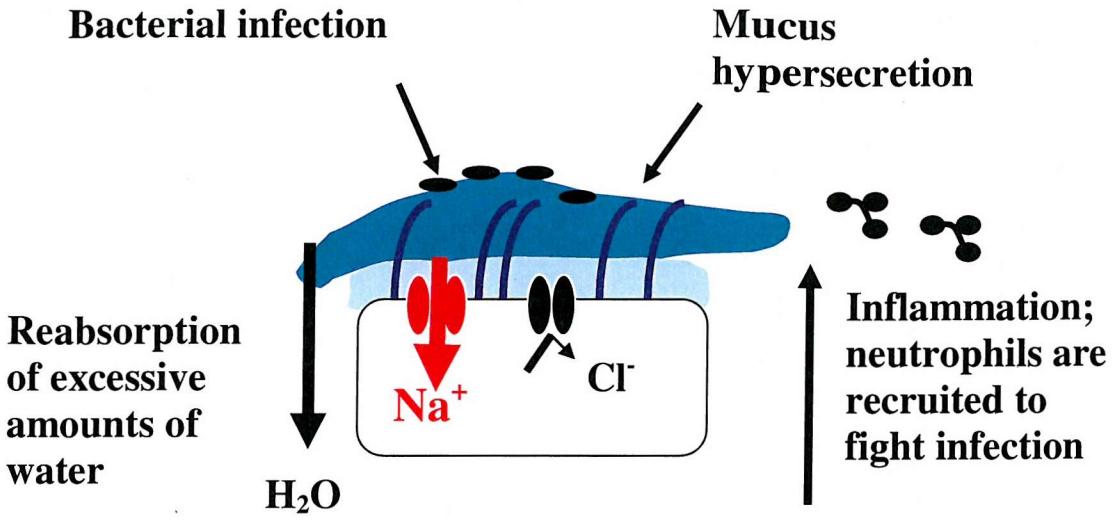


Figure 15. CFTR in a CF airway.

CF patients respond to infection with a vigorous inflammatory response. A class of macrophages known as neutrophils are recruited to the inflammatory site.

1.3.2 Neutrophils

Neutrophils are the body's first line of defence against bacterial and fungal infections. They are the most abundant white blood cells in the blood, accounting for 40-65% of white blood cells. They are found at concentrations usually in the range of 3-6 x 10^6 cells/mL of blood. This number can increase dramatically (up to ten times) in cases of infection. They have a half-life in blood circulation of about 8-20 hours, but this may extend to several days if the cells leave the circulation and enter tissues. In an average adult human there are about 2 x 10^{10} neutrophils in circulation which are replaced two or three times per day, meaning that around 5 x 10^{10} cells are generated every day.[114]

Neutrophils are produced in the bone marrow and circulate in the blood. They are about 10μm in diameter and whilst in the blood they are spherical in shape and are described as being in a non-activated state. When an infection occurs in the body,

chemotactic factors are released from bacteria and damaged host cells. These are 'sensed' via receptors on the surface of the neutrophils whilst they are passing through capillaries. The neutrophils then become activated and their morphology changes, they flatten to assume an amoeboid shape and become polarised so that they assume a front and rear end. They then pass through gaps between the endothelial cells and into the body tissue where they migrate up the chemotactic gradient by the process of chemotaxis until they reach the invading organism[114].

Neutrophils kill the target pathogens by the process of phagocytosis, but first the neutrophil needs to recognise the pathogen as being 'foreign'. Sometimes the surface properties of the pathogen are so unusual (compared with host tissues) that recognition is achieved without the involvement of any other factors. However this recognition is usually achieved by the coating of the pathogen with opsonins, such as antibodies, complement fragments, acute-phase proteins and fibronectin. The neutrophils possess receptors for portions of the opsonin molecules so that any particle coated with opsonins is labelled as a target for phagocytosis[114].

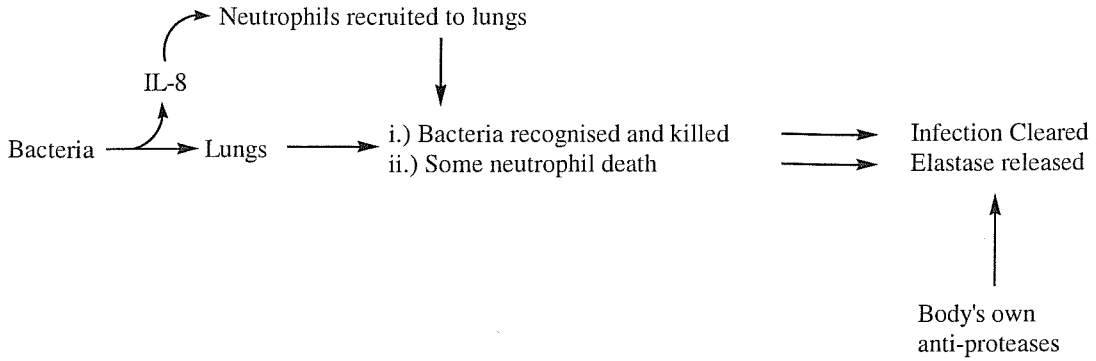
Once the organism is enclosed within the phagocytic vesicle the neutrophil activates its microbicidal arsenal. Anti-microbial proteins are packaged within azurophilic granules within the neutrophils, sometimes in a latent form. They are only active when released from the granule. This prevents damage to host tissue or the neutrophil itself. These proteins include proteases, hydrolytic enzymes and a number of highly specific proteins which affect the permeability of microbial targets. The granular components may be released extracellularly if the target organism is too large for phagocystosis. This is called frustrated phagocytosis[114].

Once the infection is cleared the neutrophil function is down regulated and the cells die by apoptosis, the killed bacteria and any remaining enzymes or components which may damage host tissue being sealed inside. The dead neutrophils are then cleared by phagocytosis via tissue macrophages[114].

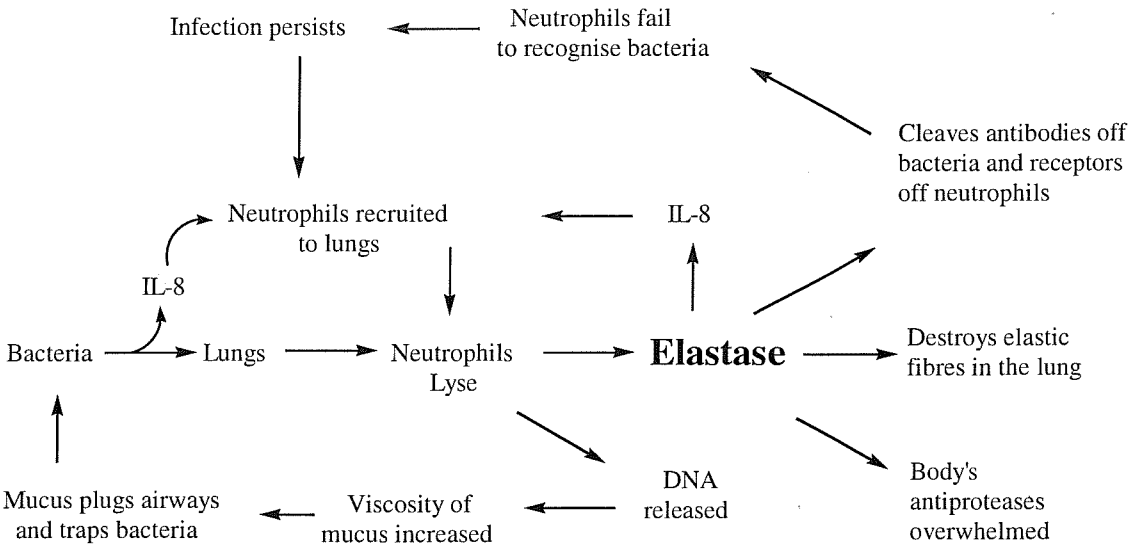
1.3.3 Neutrophils in a CF airway

One of the main proteolytic enzymes used to catabolise phagocystosed protein is an enzyme known as neutrophil elastase (NE) which is stored in the azurophilic granules within the neutrophils. In a normal lung, any NE released by neutrophils is controlled by the body's own anti-proteases such as α1-antitrypsin. In a CF lung the neutrophil numbers may be a million times higher than a normal lung and the concentrations of NE a thousand times higher. However concentrations of α1-antitrypsin are only increased by 4-5 times[115] so the natural anti-protease capacity is exceeded and there is a large amount of elastase-induced tissue damage. When the neutrophil dies, instead of apoptosis, necrosis occurs and the neutrophil breaks open releasing both NE and DNA. The NE is released into the extracellular matrix and cleaves elastin, collagen and proteoglyccin, which contribute to tissue damage in CF[116]. The DNA released mixes with the mucus making it even more viscous which leads to trapping of more bacteria. In a CF airway the NE stimulates the synthesis of the neutrophil chemo-attractant IL-8 by the airway epithelial cells[117]. This proteolytically inactivates the elastase inhibitor α1-antitrypsin and causes the release of more neutrophils thus perpetuating a self-amplifying role shown[115] in Scheme 4. Pseudomonas elastase, the protease produced by *P. aeruginosa* is more potent than NE on a per mg basis with respect to elastin degradation and may contribute to lung pathology, however it is NE which is the predominate elastase in CF sputum[118].

a.) Healthy Lung



b) Cystic Fibrosis Lung



Scheme 4. The mechanism by which the body copes with infection in a.) a healthy airway and b.) a CF airway.

Following tissue damage, growth factors that mediate tissue repair are released from binding sites in the extracellular matrix. It is the repeated cycle of tissue destruction and repair which leads to remodelling of lung architecture and eventual loss of lung function.

Vulnerability to infection in CF occurs only in the airways, and not at other sites such as skin or urinary tract, so there is no systemic immune defect in CF. However, excess inflammation does occur at other sites. The prevalence of inflammatory

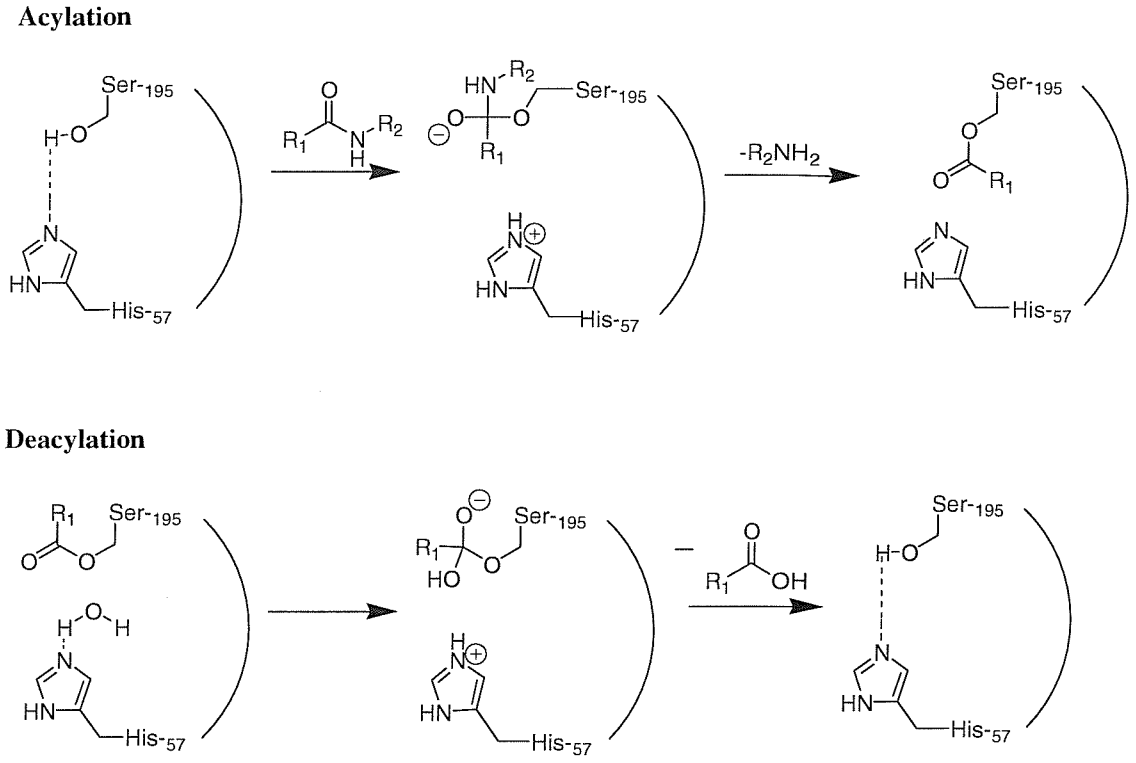
bowel disease and pancreatitis is markedly increased[119, 120]. There have been considerable advances in the treatment of the intestinal symptoms, for example by the oral administration of digestive enzymes. But it is lung disease that is responsible for 95% of the morbidity and mortality of CF patients.

1.3.4 Neutrophil elastase

NE is a 218 amino acid glycoprotein that is normally constrained within the azurophillic granules of the neutrophil[121]. Synthesis of NE begins early in the neutrophil's development whilst it's still in the bone marrow[122]. Once in circulation, mature neutrophils contain their full complement of NE and lose their capacity for further synthesis[109]. A mature neutrophil contains approximately 400 NE granules per cell with approximately 67,000 – 100,000 NE molecules each. This means that each cell contains approximately 1-2 picograms of NE[123, 124].

NE belongs to a class of enzymes known as serine proteases, so called as its active site contains the amino acid triad of histidine (His 57), aspartic acid (Asp 102) and serine (Ser 195)[121]. In addition to this catalytic triad, the enzyme possesses an oxyanion binding site which is made from the backbone amide NH groups of Ser 195 and glycine (Gly 193). After non-covalent binding of the substrate to the enzyme active site region, the OH group of Ser 195 attacks the carbonyl carbon of the scissile bond via general base catalysis by His 57. This action leads to formation of the tetrahedral intermediate. The structure of the tetrahedral intermediate is stabilised through the newly formed oxyanion component by forming hydrogen bonds to the backbone NH groups of Ser 195 and Gly 193 residues. His 57, acting now as a general acid, transfers a proton to the amine of the tetrahedral intermediate; this subsequently breaks down, a product amine leaves and the covalent acyl enzyme intermediate is formed. The acyl enzyme complex is attacked by a water molecule to generate a new tetrahedral intermediate. This intermediate collapses, assisted by general acid catalysis from His 57, resulting in the formation of product acid and the

regenerated Ser 195 OH leaving group. Scheme 5 shows the mechanism of peptide hydrolysis by NE[125].



Scheme 5. Mechanism of peptide hydrolysis by neutrophil elastase.

1.3.5 Enzyme and substrate interactions

The active site of the enzyme also contains an extended binding domain where non-covalent interactions occur between the enzyme and the amino acid residues of the substrate extending from either side of the scissile bond. It is these binding sites that impart specificity of action to a protease with regards to its substrate, thus enabling the diversity of the physiological function that exists within the protease class[126]. The most important interaction between the enzyme and its substrate occurs between the S_1 subsite of the enzyme and the P_1 residue of the substrate[¶]. This interaction is the primary determinant of substrate specificity and consequently defines the point of cleavage. NE has a shallow hydrophobic S_1 subsite which preferentially binds

medium sized aliphatic amino acids: isoleucine, valine and alanine. Since the S_1 subsite is quite small, enzyme substrate recognition is also influenced by interactions between secondary binding sites S_2 and S_2'.

1.3.6 Therapeutic benefit of NE inhibition in CF

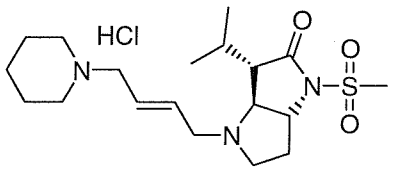
The ability to inhibit both the free extracellular and intracellular NE would provide great therapeutic benefit to a CF patient. There are two potential avenues for drug delivery of an effective inhibitor: either systemic administration or through inhalation of aerosolised solutions. It would be preferable to inhibit intracellular NE prior to lung recruitment as this would offer the greatest possibility of near maximal enzyme inhibition[127]. Regardless, in CF the mode of drug administration is not as important as the necessity to achieve and maintain high levels of sustained and uniformly distributed NE inhibition because the massive excess of NE in this disease causes continued acute inflammation[128]. Reduction of this inflammation could improve bacteriocidal and clearance mechanisms and possibly reduce sputum bacterial density and the incidence and severity of pulmonary exacerbations. It would also reduce the ongoing proteolytic lung destruction which would in the long term preserve pulmonary function and reduce the rate of exaggerated decline in forced expiratory volume[127]. All of these factors will have positive impacts on a patient's morbidity and possibly mortality[129].

1.3.7 Neutrophil Elastase Inhibitors (NEIs)

There have been experiments involving the use of recombinant proteins as potential neutrophil elastase inhibitors (NEIs). In animal studies inhaled and intravenous recombinant secretory leukoprotease inhibitor (SLPI) enhanced the protective screen of the pulmonary epithelium[130, 131] and inhaled prolastin (α1-antitrypsin isolated from human blood) suppressed lung inflammation and bacterial density in rats with chronic *Pseudomonus aeruginosa* infection[132]. It has also been shown that inhaled administration of peptide inhibitor EPI-HNE-4 led to a reduction of NE mediated

lung damage whilst intravenous administration reduced neutrophil influx in rat lungs[133]. However trials of SLPI in CF patients have been hampered by a short half-life in the lung and poor accessibility of recombinant protein to diseased areas[134].

Since the 1980's there has been extensive research carried out into developing low molecular weight mechanism-based NEIs[135]. A mechanism-based inhibitor[136, 137] is an inherently unreactive molecule that, following binding to the active site of an enzyme is processed by the catalytic machinery of the enzyme, to yield a highly reactive electrophilic species. Subsequent reaction with an active site nucleophilic residue leads to irreversible inactivation of the enzyme. Since the latent reactivity in this type of inhibitor is unmasked following catalytic processing of the inhibitor by the target enzyme only, high enzyme specificity could potentially be attained. In some instances, the latent electrophilic species is unmasked following an enzyme-induced rearrangement or fragmentation. Pyrrolidine *trans*-lactams[138, 139] and lactones such as **21** (Figure 16) are an example of small molecular weight NEIs. Its mode of action is to enter the enzymes active site and acylate the Ser 195 causing enzyme deactivation.



21

Figure 16. Example of a lactam-based NEI.

1.3.8 Dendrimers as drug delivery platforms

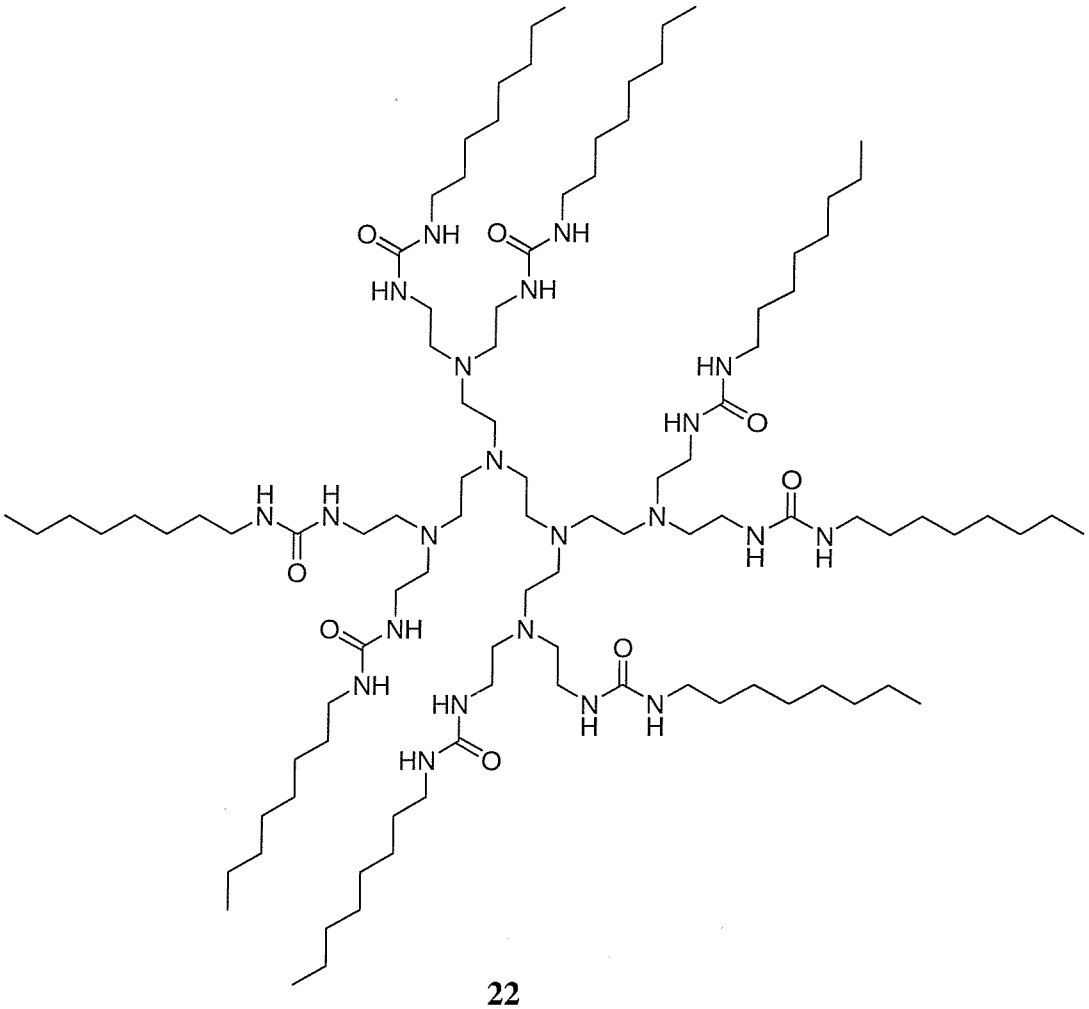
One of the aims of this project is to attach low molecular weight NEIs to a dendrimer for delivery to the lung by inhalation. It is hoped that the size of the dendrimer-NEI conjugate would significantly increase the half-life of the NEIs in the lung and therefore lead to a significant reduction of active NE present.

2. Results and Discussion

2.1.1 Technetium binding

One of the aims of this project was to bind $NaTcO_4$ to a macromolecule such as a PAMAM dendrimer so that the half life of $NaTcO_4$ in the lung would be increased as this would allow for a better quality 3D SPECT scan. Binding $NaTcO_4$ to dendrimers of different sizes may also affect the distribution of radiopharmaceutical within the lung, allowing specific areas to be targeted for imaging.

Previous work by Vögtle *et al*[140] used lipophilic dendrimers to bind oxyanions such as TcO_4^-. These were created by taking 2nd and 3rd generation poly(propylenimine) dendrimers and reacting the primary amine surface groups with octyl and dodecyl isocyanate. **22**, is a 2nd generation poly(propylenimine) dendrimer functionalised with octyl alkyl surface groups.



22

At physiological pH (7.4) more than 50% of the amino groups in these dendrimers are protonated, resulting in a polycation[141]. Vögtle *et al*[140] showed that these polycations are capable of binding anionic guests such as TcO_4^- and transferring them from an aqueous solution to an organic phase. ReO_4^- is often used as an analogue for TcO_4^- in extraction experiments[142] as it is similar in size and other properties but is less expensive and more widely available. It was shown with respect to these lipophilic dendrimers the same trend on influence on extraction[140] is observed for ReO_4^- as for TcO_4^-.

It was thought that functionalisation the surface of 4th and 5th generation amine terminated PAMAM dendrimers with long alkyl chains would create a species which could potentially bind $NaTcO_4$ by extraction from an aqueous solution. The functional group chosen to attach to the dendrimer surface was ethyl di-*n*-octylamine. This was selected as tertiary alkyl amines have been shown provide a high degree of extraction of metal anions[143-145] such as TcO_4^- from an aqueous acidic solution[146] into an organic solvent, (eqn. 1).

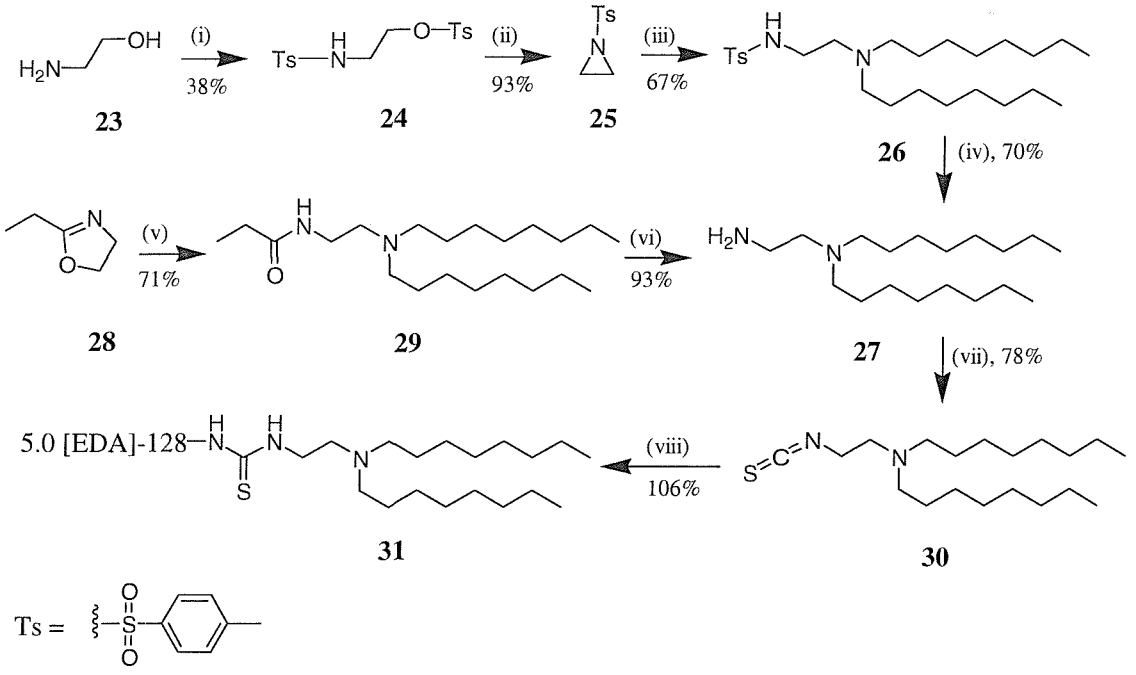
$$(R_3NHHSO_4)_{(Org)} + TcO_4^-{}_{(Aq)} \rightleftharpoons (R_3NH)TcO_{4(Org)} + HSO_4^-{}_{(Aq)} \qquad \textbf{Eqn. 1}$$

The metal anions exchange for the inorganic acid and the protonated amine and the metal anion form a donor-acceptor complex. As the amines go from primary to secondary to tertiary to quaternary the electron density on the nitrogen atom increases due to inductive effects which then increases the extractability of the metal ions[147]. Effects such as steric hindrance and the competitive effect of mineral acids reduce the extent to which metal ions such as pertechnetate are extracted[148].

Functionalising the surface of a PAMAM dendrimer with a tertiary alkyl amine such as ethyl di-*n*-octylamine should enable the dendrimer to bind pertechnetate.

2.1.2 Synthesis of lipophilic dendrimer

The alkyl amine functional group can be attached to an amine-surfaced PAMAM dendrimer via a thiourea bond. Scheme 6 shows two different routes for making a suitable functional group (2-isothiocyanatoethyl)-di-*n*-octylamine **30**.



Scheme 6. (i) TsCl, Pyridine. (ii), $KOH_{(aq)}$, PhMe. (iii) Di-*n*-octylamine, MeCN, reflux. (iv) Li/NH_3, Et_2O. (v) Di-*n*-octylamine, Cat. $Zn(OAc)_2.H_2O$, reflux. (vi) Conc. HCl/*n*-BuOH, reflux. (vii) Thiophosgene, Na_2CO_3, CH_2Cl_2, 0 °C, overnight (viii) 5.0 [EDA]-128-amine, DMSO/$CHCl_3$ (1:1), 40 °C.

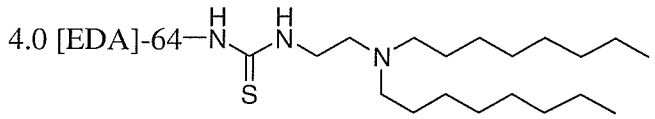
The first route involves five steps as outlined in Scheme 6. The first of these involves reaction of *p*-toluene sulphonyl chloride with ethanolamine **23** to give di-tosylate **24**. This then undergoes a 3-exo-tet cyclisation in the presence of aqueous KOH to form *N*-tosylaziridine **25**. The aziridine ring is opened (step iii) by nucleophilic attack by di-*n*-octylamine under reflux conditions in MeCN to form **26**. The tosyl group can then be removed using a Birch reduction[149] to form 2(amino) ethyl di-*n*-octylamine **27.** The primary amine can be converted to an isothiocyanate using thiophosgene and Na_2CO_3 in CH_2Cl_2 to give **30** (step viii).

The second route however involves only three steps, the first being a ring opening of 2-ethyloxazoline (Scheme 6, step v). Under reflux conditions zinc acetate acts as a Lewis acid by coordinating with the oxygen of 2-ethyl oxazoline (**28**), this activates the adjacent carbon towards nucleophilic attack by di-*n*-octylamine which leads to

ring opening to give **29.** The amide bond in **29** can be hydrolysed in conc. HCl to give **27** which, as in the first route can then be converted to the isothiocyanate **30.**

Both routes proved successful by affording satisfactory yields of the desired product **30**, but the second route is preferred as it involves two fewer steps.

The surface functionalised dendrimer **31** is formed by heating 5.0 [EDA]-128-amine with over 128 equivalents of isothiocyanate **30** overnight at 40 °C in a 1:1 mixture of DMSO and $CHCl_3$. Removal of excess **30** and DMSO proved possible by dialysis in $CHCl_3$. The yield for this reaction was 106%, the additional weight is most likely due to residual $CHCl_3$ trapped within the lipophilic internal cavities of the dendrimer. 4.0 [EDA]-64-amine was used to create **32** by the same methodology.



32

It was not possible to definitively determine that there was complete surface coverage of the dendrimer as it was not possible to obtain an accurate measurement of the mass by MALDI TOF spectroscopy. The ^{1}H and ^{13}C NMR spectra show the presence of ethyl di-*n*-octylamine however it is not possible to determine the extent of surface coverage by these techniques. The percentage of sulphur found by elemental analysis of **31** was reasonably close to the theoretical value so it was assumed that most if not all of the surface dendrimer sites were filled ethyl di-*n*-octylamine groups.

It was found that the addition of alkyl groups onto the dendrimers surface had an effect on the solubility. Amine-terminated PAMAM dendrimers have low solubility in organic solvents such as $CHCl_3$ and CH_2Cl_2. The attachment of alkyl chains to the surface of the dendrimer mean that this is no longer true for both **31** and **32.**

2.1.3 Extraction experiments

A set of experiments were carried out to determine the complexation behaviour of **31** and **32** with metal anions via liquid-liquid extraction. These experiments used a known concentration of $KReO_4$ (1×10^{-5} mol) in deionised H_2O at pH 7 to which had been added a negligible concentration of $NaTc^{99m}O_4$ which acted as a radioactive tracer. $KReO_4$ was used in these experiments as a substitute for $NaTcO_4$. 5 mL of $KReO_4$ solution was then shaken for one minute with 5 mL of differing concentrations of **31** and **32** in $CHCl_3$ with 1 mL of conc. HCl. The addition of conc. HCl ensured a high level of protonation of the tertiary amine sites both on the surface groups and within the dendrimer. HCl was used as in similar experiments it has been shown to not have a negative effect on extraction efficiency[146] as Cl^- does not compete for binding sites. After shaking, the mixture was passed through a phase-separating filter paper, the gamma activity in both the aqueous and organic phase was then measured. Four control experiments were also carried out, the first measured the extent of extraction when no dendrimer was present in the organic phase, the second measured the extent of extraction when no dendrimer was present in the organic phase without addition of conc. HCl. The final two control experiments measured the extent of extraction at the highest concentrations of **31** and **32** with no addition of conc. HCl. Each experiment was repeated twice and an average of the three results recorded. Control experiments to measure the extent of extraction using non-surface functionalised amine terminated dendrimers were not possible due to their high degree of insolubility in both CH_2Cl_2 and $CHCl_3$.

2.1.4 Results of extraction experiments

By measuring the γ activity in both the aqueous and organic phases the percentage of $KReO_4$ extracted into the organic phase can be calculated, (eqn. 2).

$$\% \text{ of } KReO_4 \text{ extracted} = (\text{Activity}_{(\text{organic})} / (\text{Activity}_{(\text{aqueous})} + \text{Activity}_{(\text{organic})})) \times 100, \quad \textbf{Eqn. 2}$$

Figure 17 shows the percentage of ReO_4^- extracted into the organic phase against different concentrations of both **31** and **32**. Both sets of control experiments without dendrimer in the organic phase showed negligible extraction which resulted in a value of zero.

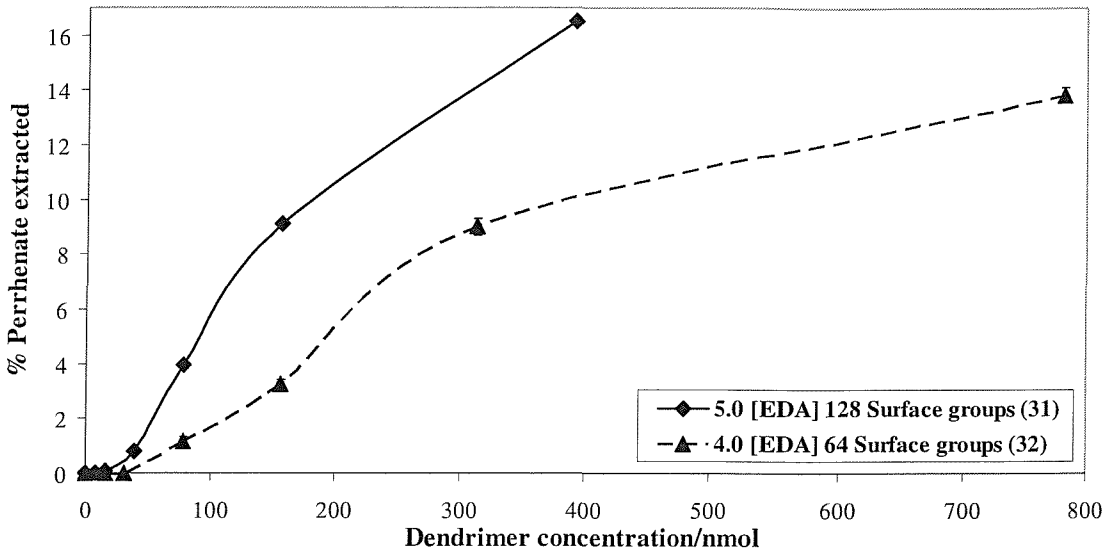


Figure 17. Plot showing the percentage of perrhenate extracted in to the organic phase against dendrimer concentration.

It can be seen that the percentage of ReO_4^- extracted with **31** is higher at lower concentrations than for **32**. However **31** has 128 functional groups on the surface of the dendrimer whilst **32** has 64. It is possible to adjust the x axis of Figure 17 so that it is expressed a ratio of the concentration of dendrimer surface groups to the concentration of $KReO_4$ (S), (Figure 18).

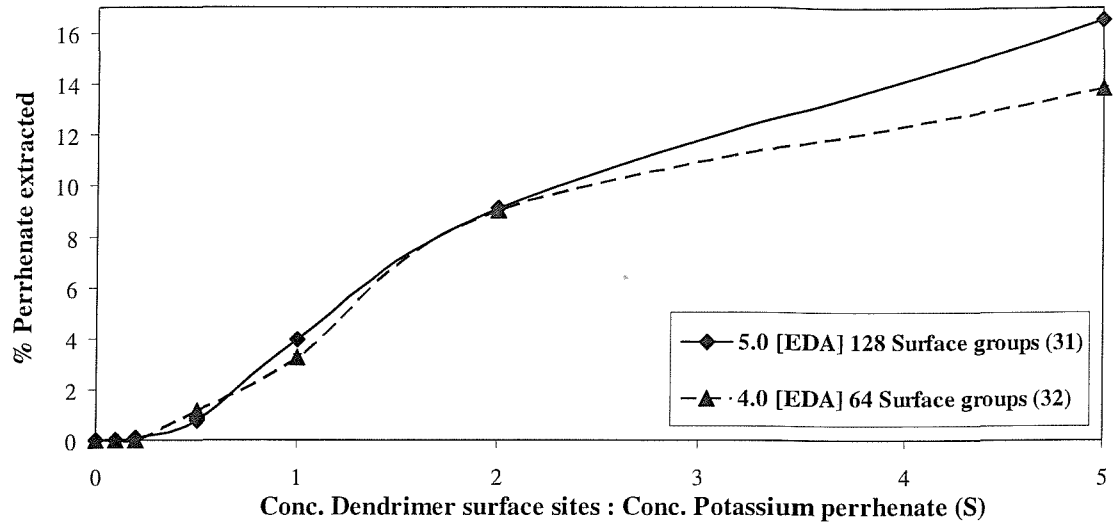


Figure 18. Plot showing the percentage of perrhenate extracted into the organic phase against the ratio of the concentration of dendrimer surface groups to the concentration of potassium perrhenate (S).

Up until S = 2 both **31** and **32** show similar trends of extraction; at a 1:1 ratio of surface groups **31** extracts 3.9% of the ReO_4^- whilst **32** extracts 3.2%, whilst at S = 2 both extract approximately 9% of the ReO_4^- into the organic phase. As S is increased to 5 then a difference begins to appear as **31** extracts approximately 16.5% of the ReO_4^- whilst **32** extracts only 13.8%.

A greater number of results need to be obtained using higher values of S to give a better understanding of the method of complexation of ReO_4^- by the dendrimer. The value of S needs to be increased so that the percentage of ReO_4^- either reaches 100% or else plateaus. Although the ethyl di-*n*-octylamine surface groups provide the most accessible binding sites, there are many tertiary amines within the hydrophobic core of the dendrimer where binding may also take place. Furthermore, once the dendrimer is protonated to form a polycation the extent, if any, of transfer of dendrimers **31** and **32** from the organic to the aqueous phase remains unknown.

The final two control experiments measured the extent of extraction of **31** and **32** when S = 5 with no addition of conc. HCl before shaking and phase separation.

These can then be compared to the results obtained when conc. HCl was added, (Figure 19).

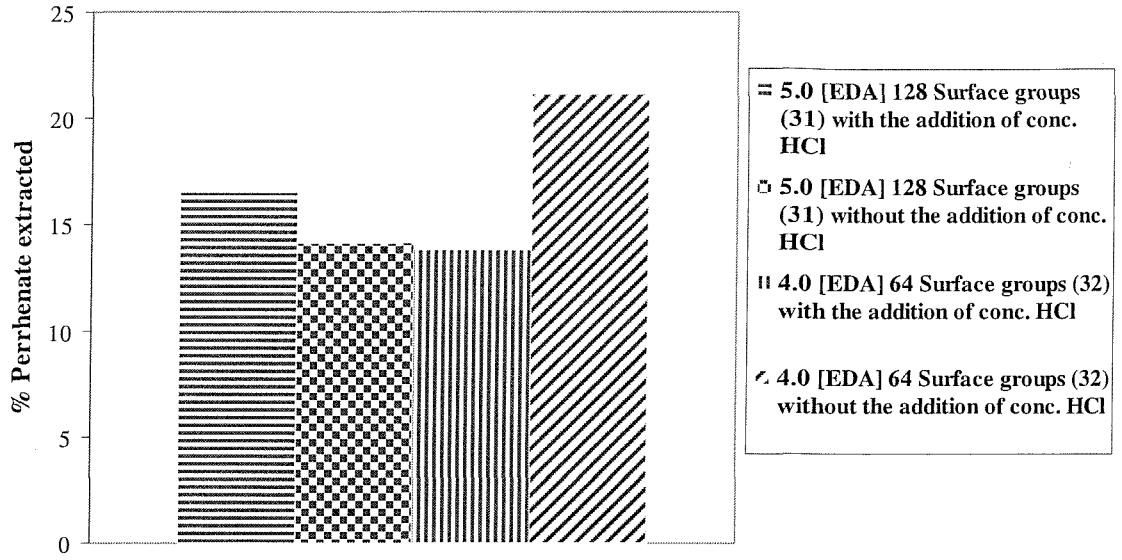


Figure 19. Graph showing the percentage of perrhenate extracted into the organic phase when S = 5 for both **31** and **32** with and without the addition of conc. HCl before shaking.

It can be seen from these results that both **31** and **32** still extract ReO_4^- without the addition of conc. HCl. This is most likely because, at pH 7, a significant proportion of the tertiary amines present on the surface groups and within the dendrimer are protonated and so capable of binding ReO_4^-. The percentage of ReO_4^- that **31** extracts is greater with the addition of conc. HCl than without at 16.5% and 14.2% respectively. This is not true for **32** as the percentage of ReO_4^- that is extracted is less with the addition of conc. HCl than without at 13.2% and 21.1% respectively. This may be because the dendrimer is also capable of extracting Cl^- as well as ReO_4^- and there is competition for binding sites, however, a greater number of experiments would need to be carried out in order to define any trend so as to present a hypothesis.

2.1.4 Conclusion

These experiments have shown that both dendrimers **31** and **32** are capable of extracting ReO_4^- from an acidic or non-acidic aqueous solution into $CHCl_3$. The results obtained in the present study have shown that 7.8 x 10^{-7} Mol of **32** was capable of binding 2.1 x 10^{-6} Mol of ReO_4^-. It can be reasonably assumed that both **31** and **32** will extract $NaTcO_4$ to a similar extent. Since the quantity of Tc^{99m} necessary for satisfactory imaging is on the order of a few nanograms[83] even though the percentage of extraction is low, it is still enough to justify further development for their potential use as radiopharmaceuticals. However in order to act as a radiopharmaceutical the solvent would need to be removed from organic phase and the dendrimer/anion complex would have to be adsorbed onto a powder for inhalation via a dry powder or pressured dose inhaler. Once *in vivo* the complex would be in an aqueous environment and further investigation needs to be undertaken to observe the rate at which reverse binding of the anion occurs. If it occurs too quickly then the half-life of Tc^{99m} in the lung will not be significantly improved enough to obtain a better quality 3D SPECT scan than simply by using $NaTc^{99m}O_4$.

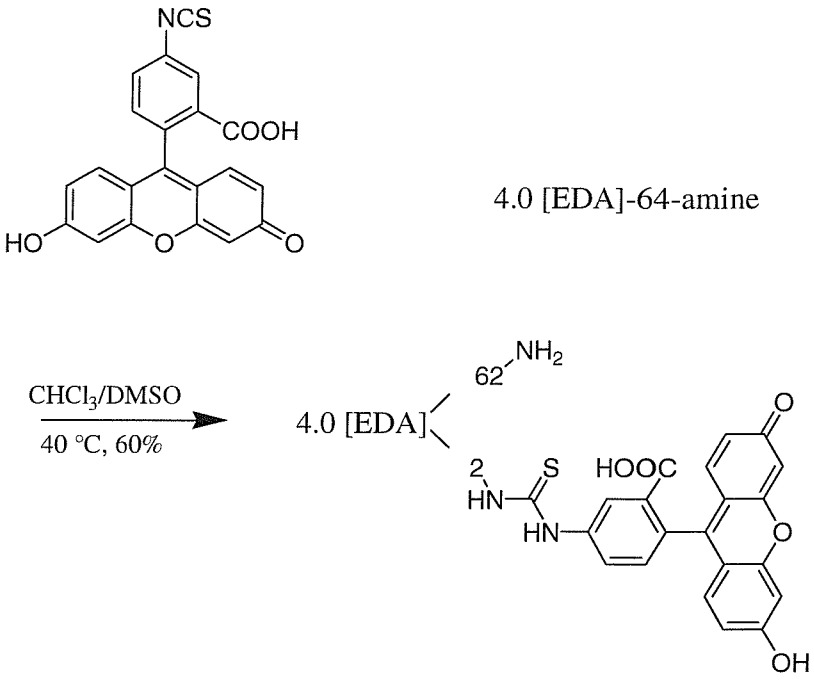
2.2.1 Dendrimers as drug delivery platforms

The second application outlined in this thesis for PAMAM dendrimers as polymer therapeutics is to use them as drug delivery platforms which could potentially be employed as a treatment for cystic fibrosis (CF).

It was originally conceived that a dendrimer-NEI conjugate could be delivered *in vivo* by way of inhalation. This approach could potentially solve several of the problems which reduced the effectiveness of inhibitors previously mentioned in the literature such as poor solubility and biodistribution. Solubilising groups could be added to the dendrimer's surface along with neutrophil elastase inhibitors (NEIs) to reduce overall lipophilicity. The macromolecular size of the dendrimer-NEI

conjugate would increase the half life of the pharmaceutical in the lung which should in turn increase the amount of extracellular elastase inhibited.

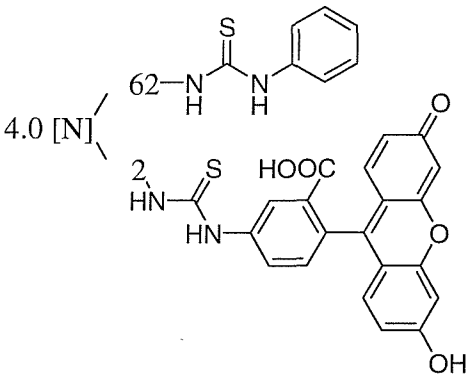
A short investigation was carried out to see how PAMAM dendrimers behave in whole blood. By introducing fluorescein to a small number of surface sites on 4.0 [N]-64-amine to produce **33** it was possible to visualise the dendrimer after being mixed with a blood sample using confocal microscopy. The fluorescein was attached to the dendrimer via a thiourea bond by the addition of a solution of fluorescein isothiocyanate (FITC) in $CHCl_3$ to 4.0 [N]-64-amine dissolved in an equal amount of DMSO and stirring the reaction mixture overnight at 40 °C. By using double the concentration of FITC with respect to concentration of dendrimer it was possible to fill on average approximately 2 surface sites per molecule of dendrimer with fluoroscein whilst leaving the remaining 62 surface sites as primary amines to give **33** in a 60% yield, (Scheme 7).



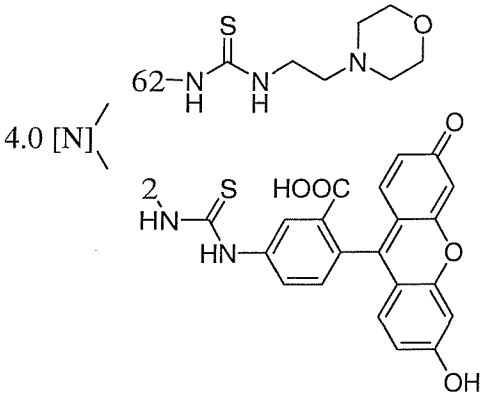
33

Scheme 7.

Two other fluorescein tagged 4th generation nitrogen core PAMAM dendrimers with different surface sites were also prepared: **34** in a yield of 64% which has phenyl surface groups; and **35** in a yield of 71% which has ethyl morpholine surface groups.



34



35

Figure 20 is a gallery of images taken at vertical cross sections of a sample of whole blood to which **33** has been added. The green areas of the image are caused by the FITC tagged dendrimer and are clearly seen within the neutrophil. The neutrophil uptake of dendrimers **34** and **35** was shown to be similar.

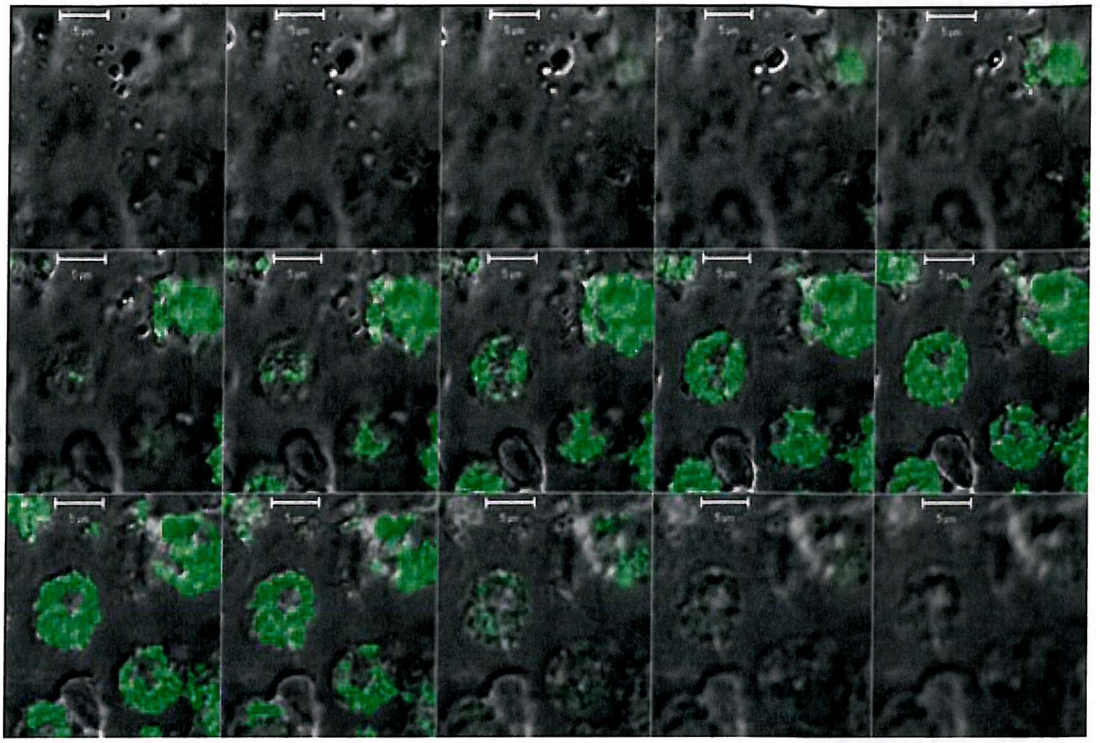


Figure 20. Vertical cross-section of images taken by fluorescent confocal microscopy showing neutrophils in a sample of white blood cells having absorbed FITC-tagged dendrimer **33**.

For all three compounds **33-35**, independent of the type of surface group the neutrophils absorbed the dendrimer by an unknown mechanism. The majority of the intercellular dendrimers congregated around the azurophilic granules. It was also observed that none of the other monocytes present in the blood absorbed the dendrimers.

This changed the method of proposed drug delivery. Instead of an inhaled drug which would inhibit extracellular elastase, it was now envisaged that the drug could be delivered systemically. As the dendrimers are absorbed inside the neutrophils whilst still in the blood it could potentially be possible inhibit intracellular elastase before the neutrophils are recruited to the lungs.

2.2.2 Strategies for forming a dendrimer-inhibitor complex

Two strategies were proposed for using PAMAM dendrimers to deliver NEIs into the neutrophil.

1.) The dendritic box approach. Previous work in the literature has shown that small molecules can be captured inside a dendrimer during dendrimer synthesis[46]. The first approach would be to trap the NEIs inside the hydrophobic interior of the dendrimer. It is assumed that as the neutrophil selectively absorbs the dendrimer, it will then try to break the dendrimer down. As it is broken down then the NEIs will be released inside the neutrophil.

2.) The second approach would be to attach the NEIs directly onto the surface sites of the dendrimer.

It was decided to pursue the second approach as the mechanism of enzyme inactivation of several of the small molecule NEIs found in the literature contained a leaving group. It was decided to link the NEI to a dendrimer via the leaving group so that each inhibitor molecule can be removed sequentially from the dendrimer surface by the NE, (Figure 21).

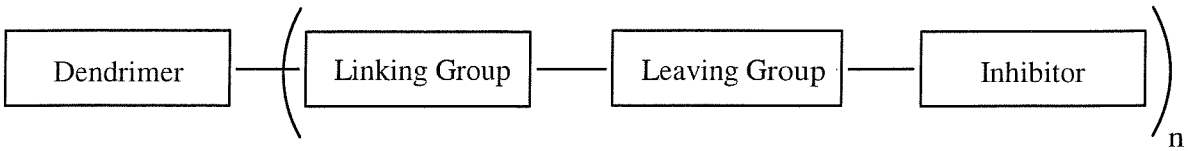


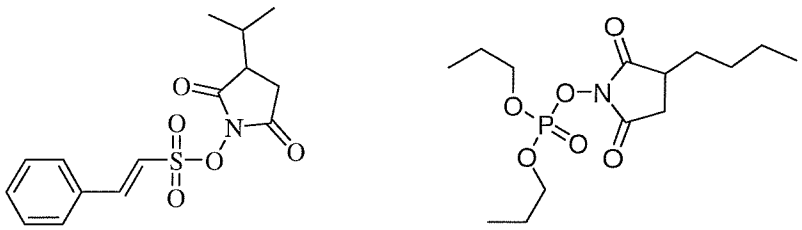
Figure 21. Schematic showing that the inhibitor would be linked to the dendrimer via a leaving group. n = number of surface groups.

Amine and hydroxy-terminated full generation dendrimers as well as ester-terminated half generation PAMAM dendrimers are commercially available and their synthesis and characterisation is well documented. However it was decided that the best strategy would be to design the attachment of NEIs to the surface of an

amine-terminated PAMAM dendrimer as there are more documented reactions for the functionalisation of such surfaces[29-63].

2.3.1 Succinimide-based NEIs

The types of NEIs initially identified as suitable candidates for attachment to a PAMAM dendrimer surface were *N*-sulphonyl succinimide[149] and *N*-succinimydyl phosphate[150] derivatives, for example **36** and **37**, Figure 22.



36 **37**

Figure 22. Example succinimide-based NEIs.

2.3.2 *N*-sulphonyl succinimide-based NEIs

R_1 = Isopropyl, isobutyl
R_2 = Leaving Group
* = Chiral center

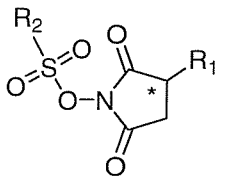
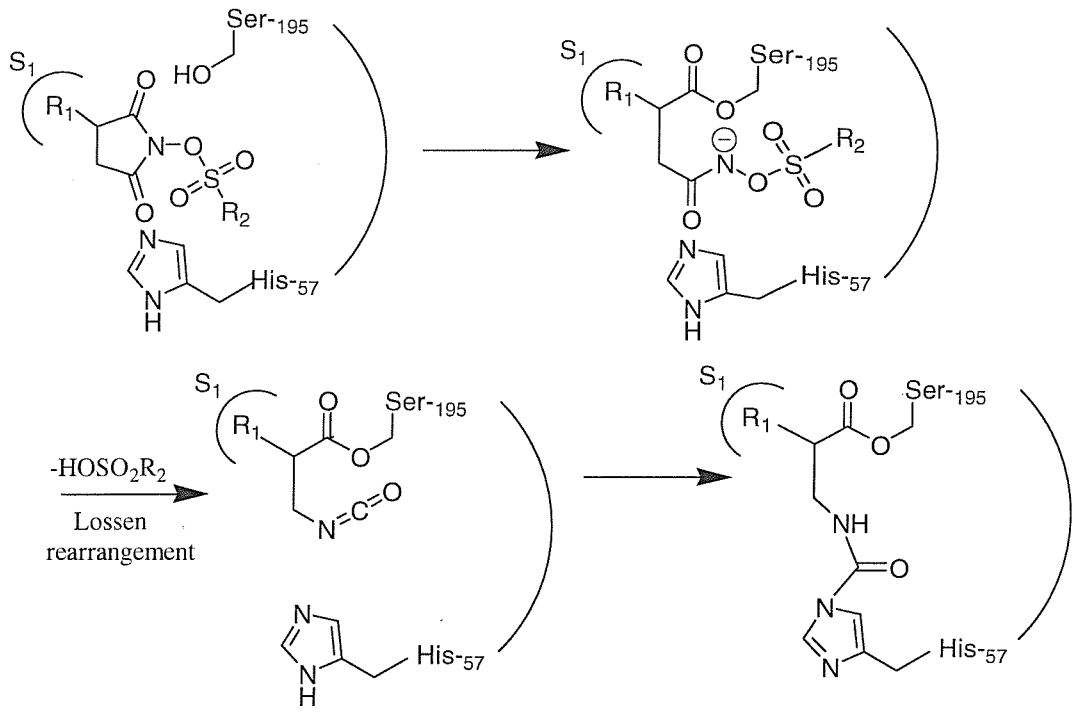


Figure 23. An example succinimide-based NEI.

Figure 23 shows the skeleton of an *N*-sulphonyl succinimide-based NEI. The proposed mechanism of inactivation[149] is shown in Scheme 8. The inhibitor sits in the active site of the enzyme and the serine hydroxyl group attacks the succinimide carbonyl group causing ring opening. A sulphonate group then leaves leading to a Lossen rearrangement[151] which produces an isocyanate. This acylates the histadine to give a doubly bound enzyme-inhibitor adduct.



Scheme 8. Literature proposed mechanism of inactivation of serine proteases by *N*-([alkylsulphonyl]oxy) succinimides.

The subsites in the active site in NE which have the most influence on substrate recognition are the ones nearest the scissile centre. The subsites which bind R_1 and R_2 are S_1 and S_1' respectively[¶]. The S_1 subsites on elastases are small, shallow and hydrophobic. They preferentially bind medium-sized aliphatic amino acids such as isoleucine, valine and alanine. This is why the R_1 group is required to be a short hydrophobic carbon chain with short branching units which act as a peptidomimetic group. The leaving group R_2 binds in the S_1' subsite which preferentially binds aromatic moieties.

There is a chiral centre on the succinimide skeleton shown in Figure 23. Although enzymes are stereoselective it has been shown that both enantiomers are effective inhibitors of NE[152].

2.3.3 *N*-Succinimydyl phosphate-based NEIs

Another class of compounds which has been found to act as NE inhibitors are *N*-succinimydyl phosphates (Figure 24). *N*-Phosphoryloximides are known to undergo a Lossen rearrangement upon treatment with nucleophiles[153].

R_3 = Ethyl, *n*-butyl, benzyl
R_4 = *n*-Propyl
* = Chiral center

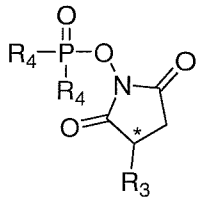
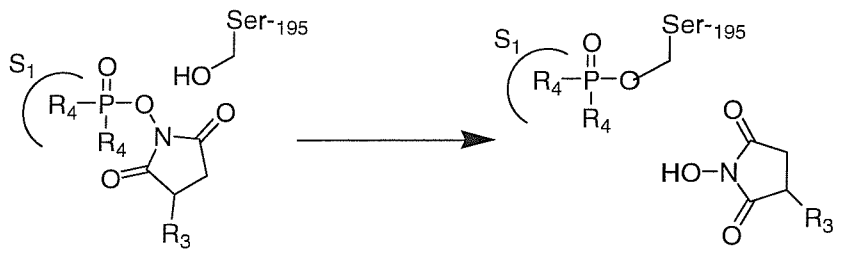


Figure 24. An example succinimydyl phosphate-based NEI.

When tested, compounds of the type shown in Figure 24 were found to be highly effective active-site directed inhibitors of NE[150]. The main mechanism of enzyme inactivation is the same as for *N*-sulphonyl succinimide-based NEIs however ^{31}P NMR studies have supported the idea that when in the active site, the inhibitor undergoes reverse binding. This leads to an alternative enzyme inactivation mechanism where the serine hydroxyl group attacks the phosphorus centre and the succinimide leaves, the phosphorus centre remains bound in the active site leading to enzyme inactivation (Scheme 9).



Scheme 9. Possible enzyme binding for succinimydyl phosphate-based inhibitors.

2.3.4 Investigation into *N*-hydroxysuccinimide-based NEIs

A short study was then undertaken to investigate to see how *N*-hydroxysuccinimide and its derivatives fragment when exposed to different nucleophiles. The aims of this were:

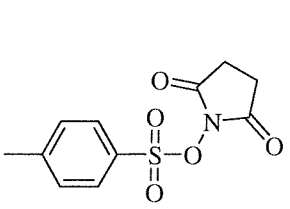
i) To provide confirmation of the literature proposed mechanism for enzyme deactivation which is derived from *in vitro* NMR studies of enzyme-substrate complexes[149].

ii) To gain insight into the factors that cause these succinimide-based derivatives to undergo a Lossen rearrangement, a key step in the enzyme inactivation mechanism.

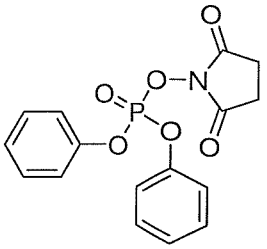
iii) To see under what conditions these compounds are stable as this will aid the design of an approach to the ultimate aim of their attachment to a PAMAM dendrimer.

2.3.5 Model compounds

Two model compounds were initially prepared for this study. *N*-Tosyloxy succinimide **38** was made as a model compound for *N*-sulphonyl succinimide inhibitors. It was prepared by the reaction of *N*-hydroxysuccinimide with *p*-toluene sulphonic acid in a 1:1 mixture of CH_2Cl_2 and pyridine in a yield of 78%. **39** was made as a model compound for *N*-succinimydyl phosphate inhibitors. It was prepared by the reaction of diphenyl chlorophosphate and *N*-hydroxysuccinimide in pyridine in a 47% yield.



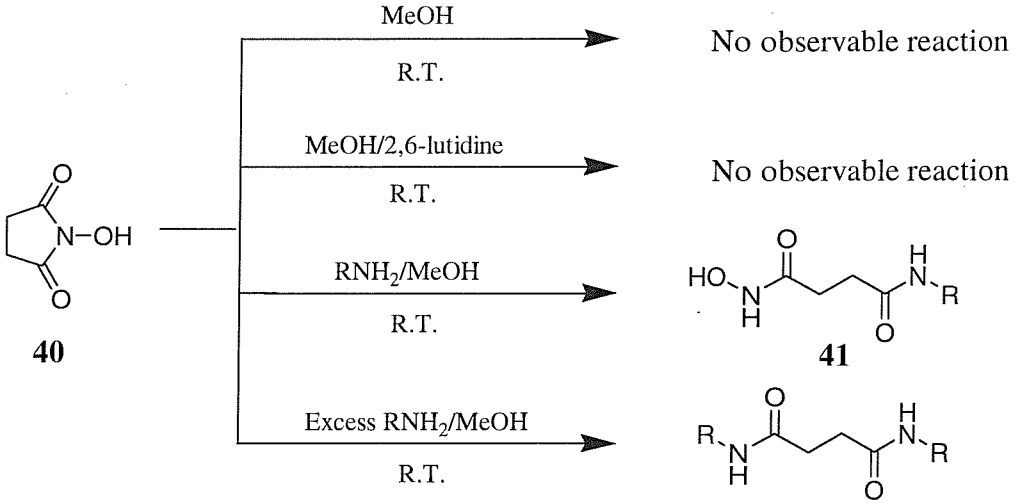
38



39

2.3.6 *N*-Hydroxysuccinimide

How *N*-Hydroxysuccinimide (**40**) reacts under different conditions provides a comparison to how **38** and **39** behave under similar conditions. As can be seen from Scheme 10, **5** shows no fragmentation when in the presence of a primary alcohol such as MeOH even when a base such as 2,6-lutidine is present. Primary amines attack the carbonyl group causing fragmentation of the succinimide ring to give **41** and, in the presence of excess amine[154], **42**.



40

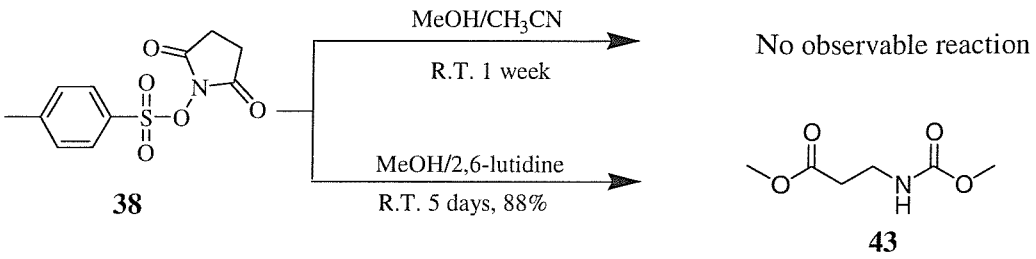
41

42

Scheme 10.

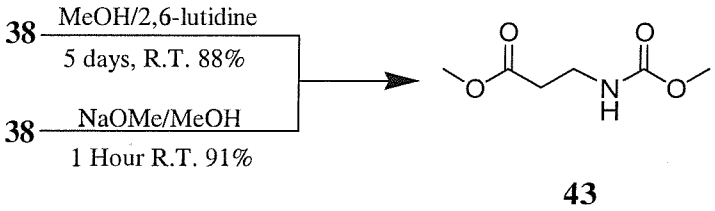
2.2.7 *N*-Sulphonyl succinimide model compound

Scheme 11 shows the behaviour of **38** under different conditions. As with **40** it shows no reaction with a primary alcohol such as MeOH. However this was not the case when **38** was put into a 1:1 mixture of MeOH and 2,6-lutidine (the 2,6-lutidine was being used as a substitute for the imidazole in the active site of the NE). Under these conditions **43** was isolated.



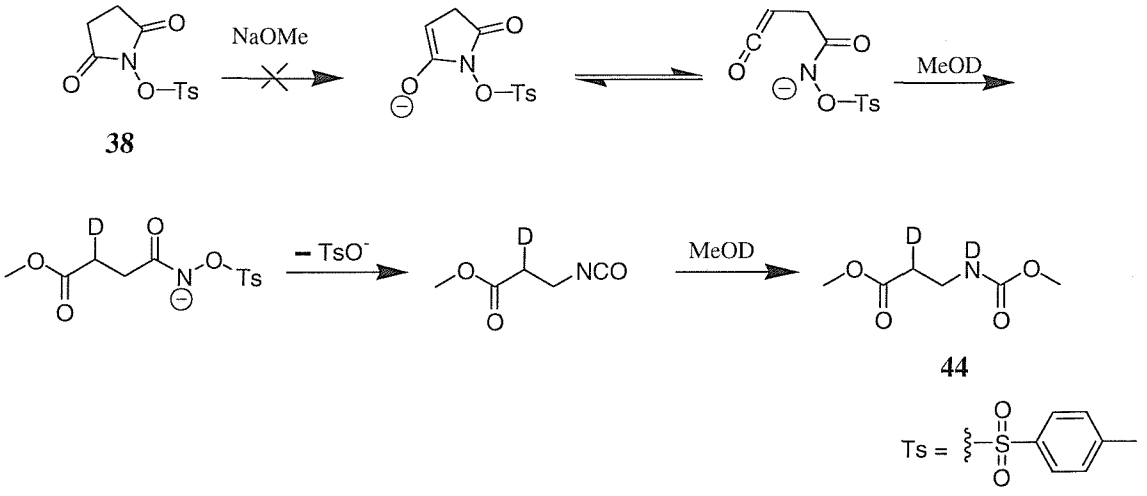
Scheme 11.

The ring opening reaction of **3** with MeOH and 2,6-lutidine takes several days at ambient temperature, but occurs much faster if sodium methoxide (NaOMe) is used as a base instead (Scheme 12).



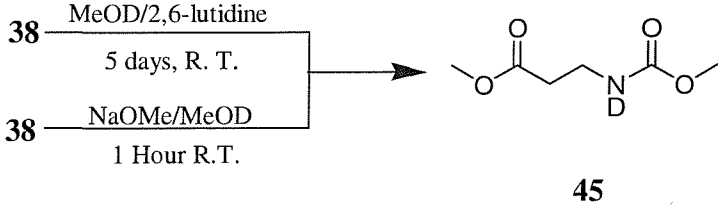
Scheme 12.

It was suggested that a possible reaction pathway for the transformation of **38** to **43** with MeOH and 2,6-lutidine or MeO^- could first involve deprotonation of the succinimide ring of **38** followed by fragmentation to a ketene intermediate. The ketene would then form an ester with MeOH. The loss of the tosylate leaving group would induce a Lossen rearrangement to form an isocyanate intermediate which would react with MeOH to form the carbamic acid methyl ester, **43**.



Scheme 13.

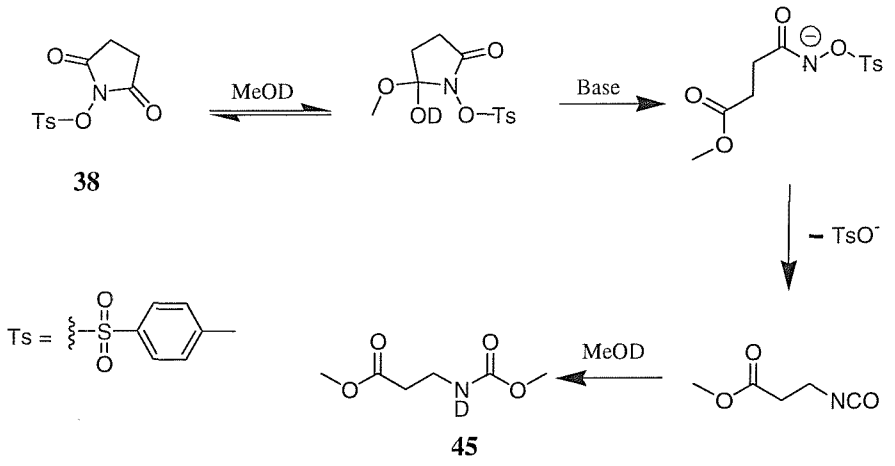
In order to probe this reaction pathway MeOD was used as a solvent instead of MeOH. If the suggested pathway was correct then deuterium would be incorporated into the α position of the methyl ester to form **44**, Scheme 13. That this was not the case as **45** was the only product isolated from this reaction, Scheme 14. The only deuterium incorporated into the molecule is attached to the nitrogen of the carbamic acid methyl ester. **45** cannot be isolated from the crude mixture as upon work up with H_2O or purification by column chromatography, the deuterium exchanges for hydrogen to form **43.**



Scheme 14.

It is likely that MeO^- or MeOD are involved in nucleophilic ring opening of **38** by first attacking one of the carbonyl groups on the succinimide ring. Subsequent deprotonation of the acetoxy acetimide nitrogen causes a Lossen rearrangement in

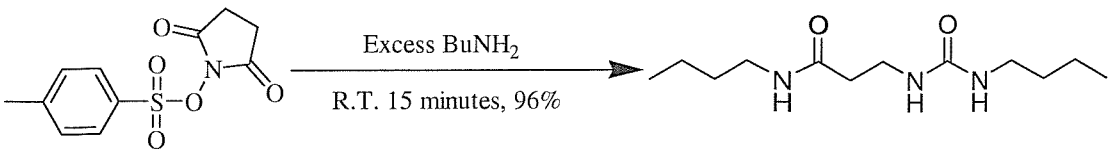
which loss of the tosylate leaving group gives an isocyanate intermediate which reacts with one equivalent of MeOD to form the carbamic acid **45** (Scheme 15). This reaction pathway also explains why no fragmentation of **38** is observed in MeOH without a base being present as deprotonation is a necessary step after attack by the MeOH on the carbonyl group of the succinimide ring.



Scheme 15.

This reaction pathway is consistent with the proposed literature mechanism of enzyme deactivation with succinimide-based NEIs, Scheme 8.

In order to assess the stability of *N*-sulphonyl succinimide-based NEIs in the presence of primary amines, **38** was added portion-wise to butylamine. The succinimide ring of **38** is opened by nucleophilic attack by butylamine on one of the succinimide rings' carbonyls followed by a Lossen rearrangement to give an isocyanate intermediate which reacts with another equivalent of butylamine to give **46**, (Scheme 16). When only one equivalent of butylamine was added to **38** in CH_2Cl_2 the crude NMR showed only the presence of **46** and starting material **38**. No intermediate products were seen. This suggests that the rate determining step is the ring opening process.



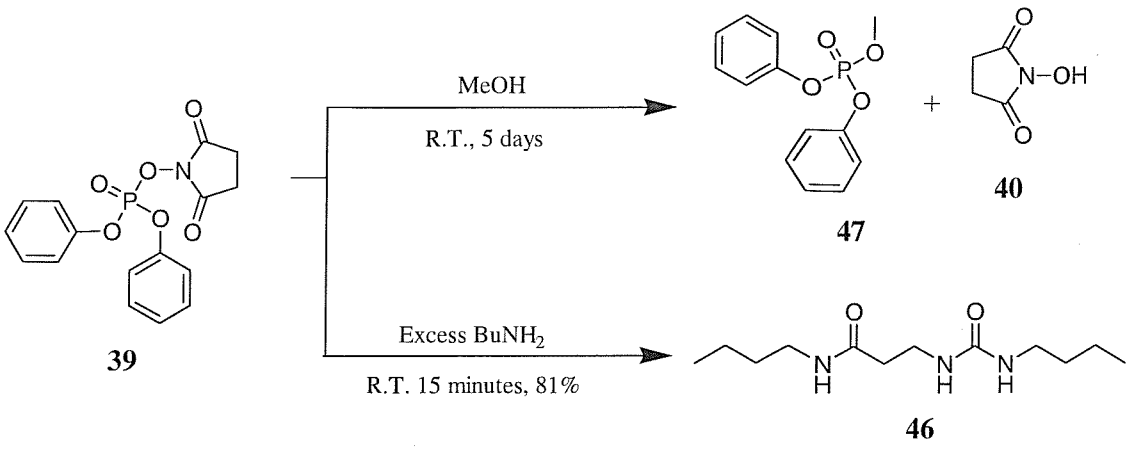
38 **46**

Scheme 16.

It was observed that there is rapid fragmentation of the succinimide ring of **38** in the presence of primary amines. Any reaction to join an *N*-sulphonyl succinimide-based NEI to the surface of an amine-terminated PAMAM dendrimer would be competing with this fragmentation reaction. As the fragmentation was observed to proceed rapidly, the overall yield of successfully linking an *N*-sulphonyl succinimide-based NEI to the surface of a dendrimer would likely be low. Therefore *N*-sulphonyl-succinimide based NEIs were judged to be unsuitable candidates for attachment to the surface of an amine-terminated PAMAM dendrimer.

2.3.8 *N*-Succinimydyl phosphate model compound

As with **38** MeOH does not cause the succinimide ring of **39** to fragment, but **39** does undergo nucleophilic attack by MeOH at the phosphorus centre to form **47** and give **40** as the leaving group, (Scheme 17). This agrees with the proposed alternative mechanism of enzyme deactivation[150] where the phosphorus moiety is bound to the active site after nucleophilic attack by the hydroxy group of the serine. In excess butylamine, phosphate **39** follows the same reaction path as **38** and forms **46.**



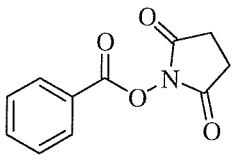
Scheme 17.

Over time the phosphate **39** decomposes at room temperature and because, like **38**, it rapidly fragments in the presence of butylamine to form **46** then *N*-succinimydyl phosphate based NEIs were also considered unsuitable candidates for attachment onto an amine-terminated PAMAM dendrimer.

2.3.9 Alternative succinimide based NEIs

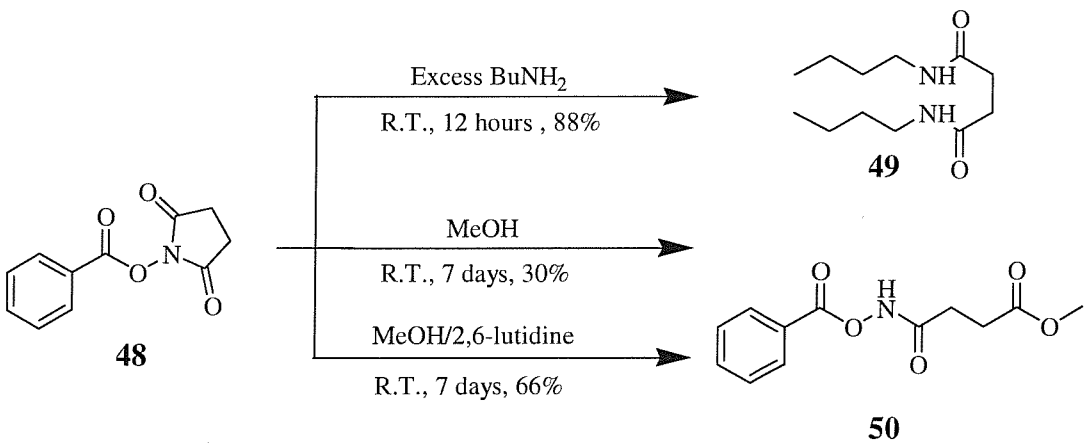
In view of their high reactivity with amines, both *N*-sulphonyl succinimide and *N*-succinimydyl phosphate-based NEIs were judged to be unsuitable candidates for attachment to an amine terminated PAMAM dendrimer. The Lossen rearrangement is a key step in the inactivation mechanism so it was decided to try and create *N*-hydroxysuccinimide derivatives which would not only undergo a Lossen rearrangement and so be potential inhibitors of NE but also show a higher degree of stability in the presence of amines than model compounds **38** and **39**.

N-(benzoyloxy) succinimide **48** was investigated to see whether it would follow similar reaction pathways as **38** and **39** when reacted under similar conditions. If the molecule were to form **43** in a 1:1 mixture of MeOH and 2,6-lutidine then this would mean it may act as a potential inhibitor of NE. Ester **48** was prepared by coupling *N*-hydroxysuccinimide to benzoyl chloride in CH_2Cl_2 in the presence of Et_3N with a yield of 64%.



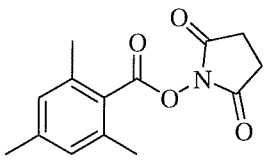
48

Unlike sulfonate **38**, benzoate ester **48** does not undergo a Lossen rearrangement in excess butylamine, (Scheme 18). Instead butylamine attacks the benzoyl carbonyl group to give a benzamide and *N*-hydroxysuccinimide is ejected. The succinimide ring is cleaved to give diamide **49**. **48** does not undergo a Lossen rearrangement in the presence of MeOH and 2,6-lutidine, but rather MeOH attacks one of the carbonyl groups on the succinimide ring causing a non-reversible ring opening to give **50**. The same reaction occurs if no 2,6-lutidine is present but the yield is decreased from 66% to 30%. It is thought that 2,6-lutidine does not act as a base in this reaction but hydrogen bonds to the MeOH increasing its nucleophilicity. As ester **48** did not display similar behaviour to **38** or **39** it was considered to not be a potential inhibitor of NE.



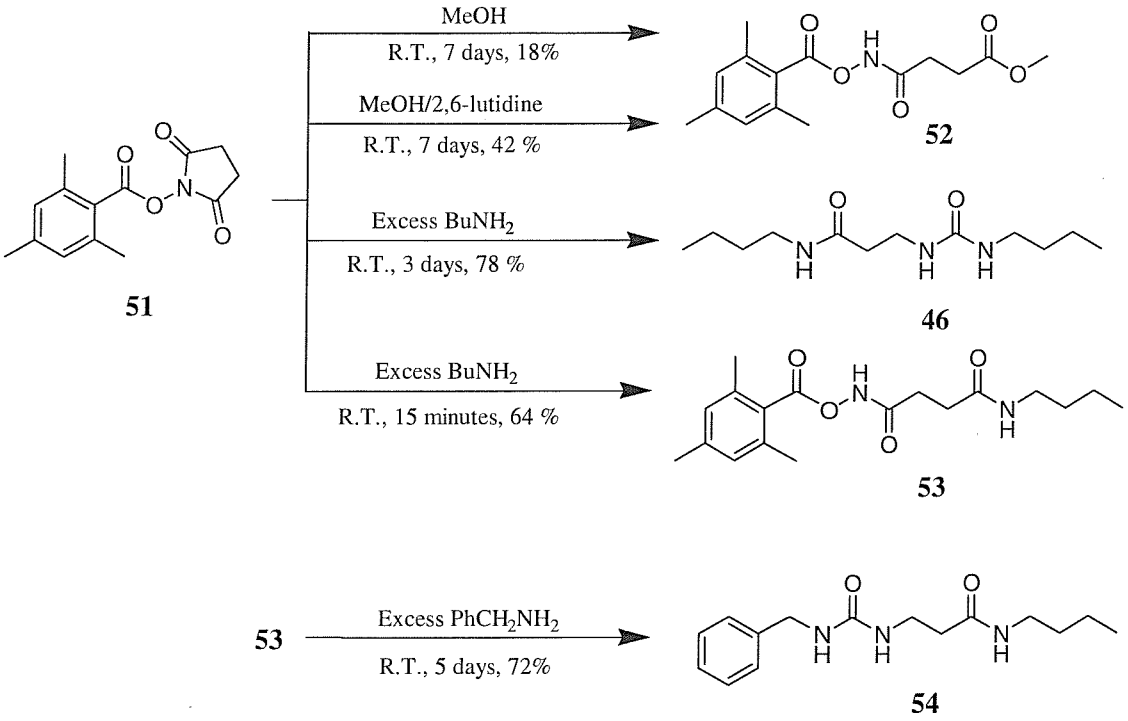
Scheme 18.

The sterically hindered ester **51** was prepared as an alternative to **48** by coupling *N*-hydroxysuccinimide with 2,4,6-trimethyl benzoyl chloride in CH_2Cl_2 in the presence of Et_3N with a yield of 69%.



51

As with **48**, MeOH causes the succinimide ring of **51** to open to give **52**, (Scheme 19). The yield of this reaction can be increased from 18% to 42% by the addition of 2,6-lutidine as a co-solvent. The ortho-methyl groups on the benzene ring block attack on **51** by butylamine on the benzoyl group, instead butylamine causes the succinimide ring to fragment and undergo a Lossen rearrangement to give **46**. This reaction occurs over 3 days rather than the 15 minutes observed for the complete decomposition of **38** or **39** in excess butylamine. By stopping the reaction of **51** with excess butylamine after 15 minutes **53** can be isolated. This can then be made to undergo a Lossen rearrangement with a different primary amine such as benzyl amine to form **54**. As **51** did not under go a Lossen rearrangement to form **43** in a 1:1 mixture of MeOH and 2,6-lutidine it was not considered to be a potential inhibitor of NE.



Scheme 19.

The difference in reactivity of tosylate **38** and phosphate **39** in comparison with the benzoates **48** and **51** most likely reflects the pKa values of the leaving groups attached to the succinimide derivatives. Compounds **38** and **39** both undergo a Lossen rearrangement when treated with excess butylamine and **38** also does so in a 1:1 mixture of methanol and 2,6-lutidine. Their corresponding leaving groups (*p*-toluene sulphonic acid and phosphoric acid diphenyl ester) both have low pKa values (-0.51 (30 °C, H_2O)[155] and 1.12 ± 0.20 (25 °C, H_2O)[156] respectively). Whilst **48** does not undergo a Lossen rearrangement when treated with excess butylamine, **51** does but at a much slower rate than with **38** and **39**. Neither **48** nor **51** undergo a Lossen rearrangement in a 1:1 mixture of methanol and 2,6-lutidine. The pKa values of benzoic acid and 2,4,6-trimethyl benzoic acid are 4.29 (25 °C, H_2O)[157] and 5.25 (25 °C, 1:1 (wt), H_2O/MeOH)[158] respectively. These values are significantly higher than those for the corresponding esters **38** and **39**.

It is likely that the factor that determines whether or not the compound will undergo a Lossen rearrangement is the pKa of the proton on the acetoxy acetimide nitrogen

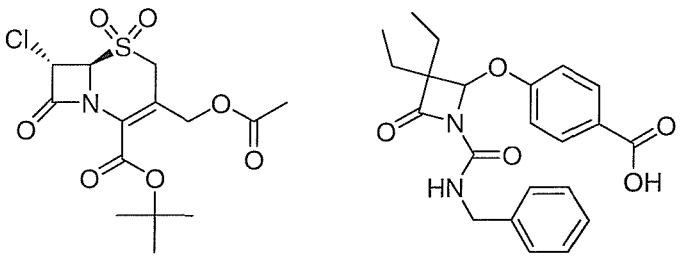
that is formed after the initial ring opening. The lower the pKa of the respective leaving group, the lower the pKa of this proton and the greater the susceptibility for the molecule to undergo a Lossen rearrangement.

2.3.10 Conclusion of the investigation into *N*-hydroxysuccinimide derivatives

The results obtained from this investigation have confirmed the proposed literature mechanism of enzyme inactivation, (Scheme 8). The results also suggest that the main factor which allows the inhibitor molecule to undergo a Lossen rearrangement, the key step in the inhibition mechanism, is the pKa of the leaving group. This investigation has also shown that succinimide based NEIs are not suitable for attachment to an amine terminated PAMAM dendrimer and consequently further development toward this aim was discontinued.

2.4.1 β-Lactam based NEIs

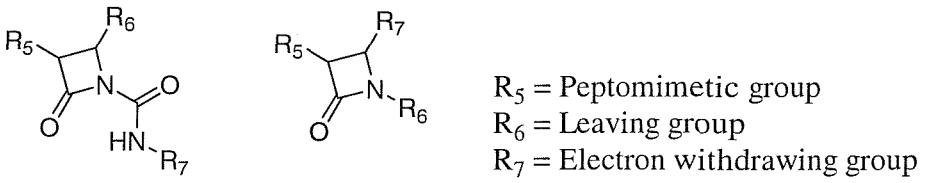
Another class of NEIs identified from the literature as potential candidates for attachment to an amine terminated PAMAM dendrimer are β-lactam based NEIs. β-Lactams are well known serine protease inhibitors that acylate the nucleophilic serine residue of a wide range of enzymes[159], including NE[160]. Cephalosporin sulfones such as **55**, (Figure 25), have been reported as one of the first β-lactam irreversible inhibitors of NE[161-163]. **55** and other cephalosporin sulfone analogues are bicyclic β-lactams that promote the acylation of the catalytic serine residue and alkylation of the histidine residue leading to enzyme inhibition[164].



55 **56**

Figure 25. Examples of ß-lactam based NEIs.

The need to improve oral bioavailability led to the development of several monocyclic β-lactam inhibitors[165-167] of which **56** is an example.

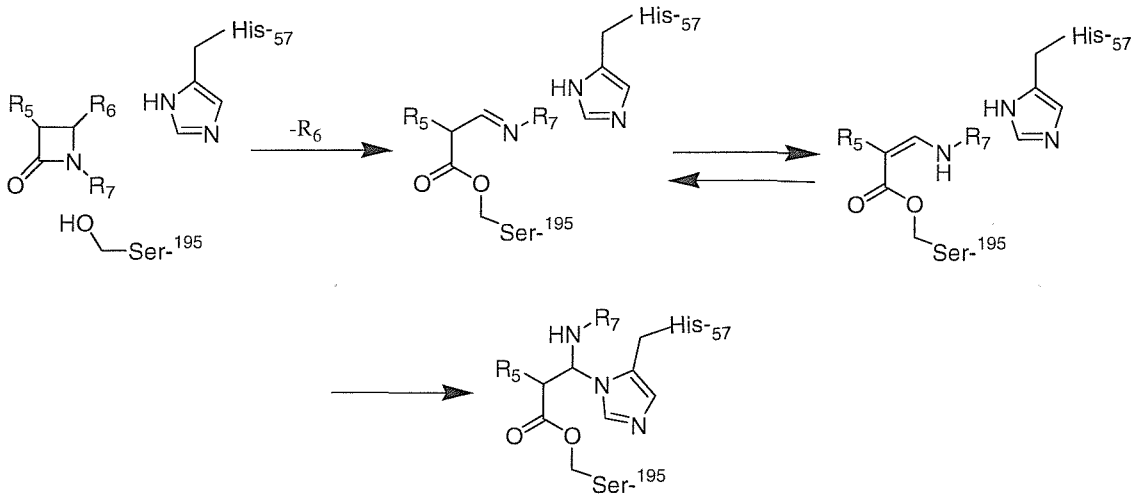


Type-I Type-II

R_5 = Peptomimetic group
R_6 = Leaving group
R_7 = Electron withdrawing group

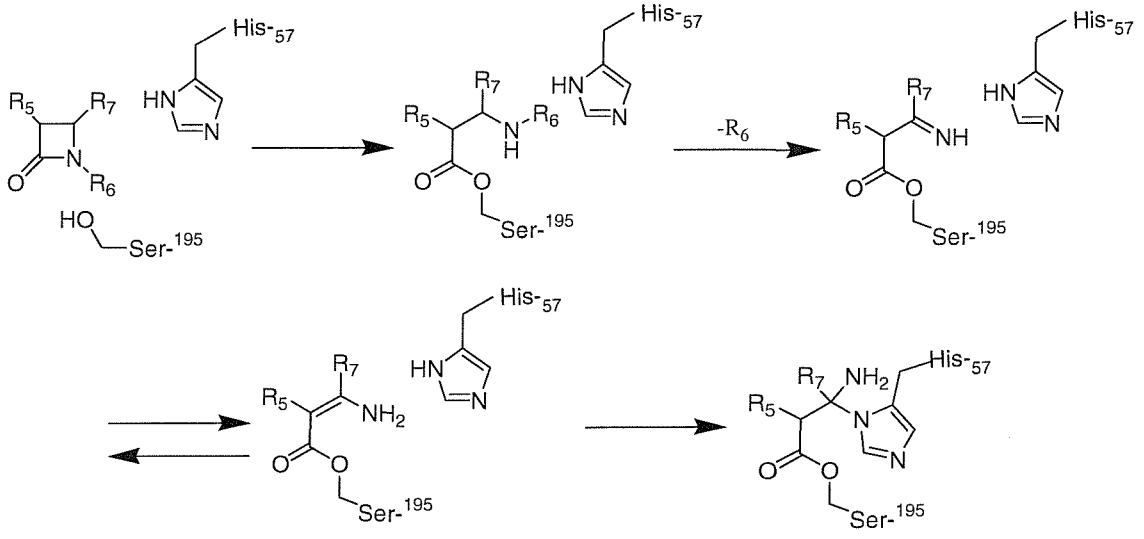
Figure 26. Skeleton of Type-I and Type-II ß-lactam-based NEIs.

There are two types of monocyclic β-lactam based NEIs, (Figure 26). Both types contain a peptomimetic group (R_5) in the same position which aids enzyme recognition of the substrate. The mechanism of inhibition of type-I inhibitors begins with acylation[168] of the enzyme Ser 195 by the lactam, (Scheme 20). This stimulates the departure of the leaving group R_6, creating an imine which acts as a Schiff base that could accept a nucleophile (His 57). Since monocyclic β-lactams are less strained than bicyclic ones, activation by an electron withdrawing group (R_7) is needed to achieve the initial acylation step, and also provides the reactive imine for the second covalent modification of NE.



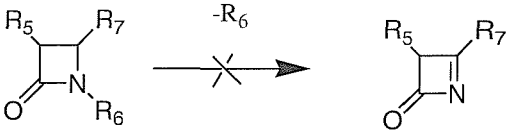
Scheme 20. Mechanisim of inhibition of NE with a type-I monocyclic ß-lactam-based NEI.

Type-II β-lactam-based NEIs have the functions of the *N*- and 4-substituents reversed. This places the leaving group (R_6) on the nitrogen atom and it departs via β-elimination[169], (Scheme 21). The electron withdrawing group (R_7) at C-4 facilitates the elimination by lowering the pKa of the C-4 proton. This creates a Schiff base which is activated by the electron withdrawing group to serve as an electrophile for the second NE modification.



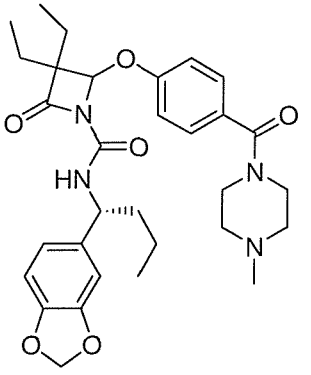
Scheme 21. Mechanism of inhibition of NE with a type-II monocyclic ß-lactam-based inhibitior.

In this case it is unlikely that R_6 would be eliminated prior to the first encounter with the enzyme which opens the lactam as this would create two new sp^2 centers in the four-membered ring resulting in greatly increased ring-strain, (Scheme 22).



Scheme 22.

Inhibitor **57** (Figure 27, DMP-77, Merck & Co. Inc.)[170] is an orally active monocyclic type-I β-lactam-based NEI. This compound had reached phase II clinical trials when further development was then discontinued.



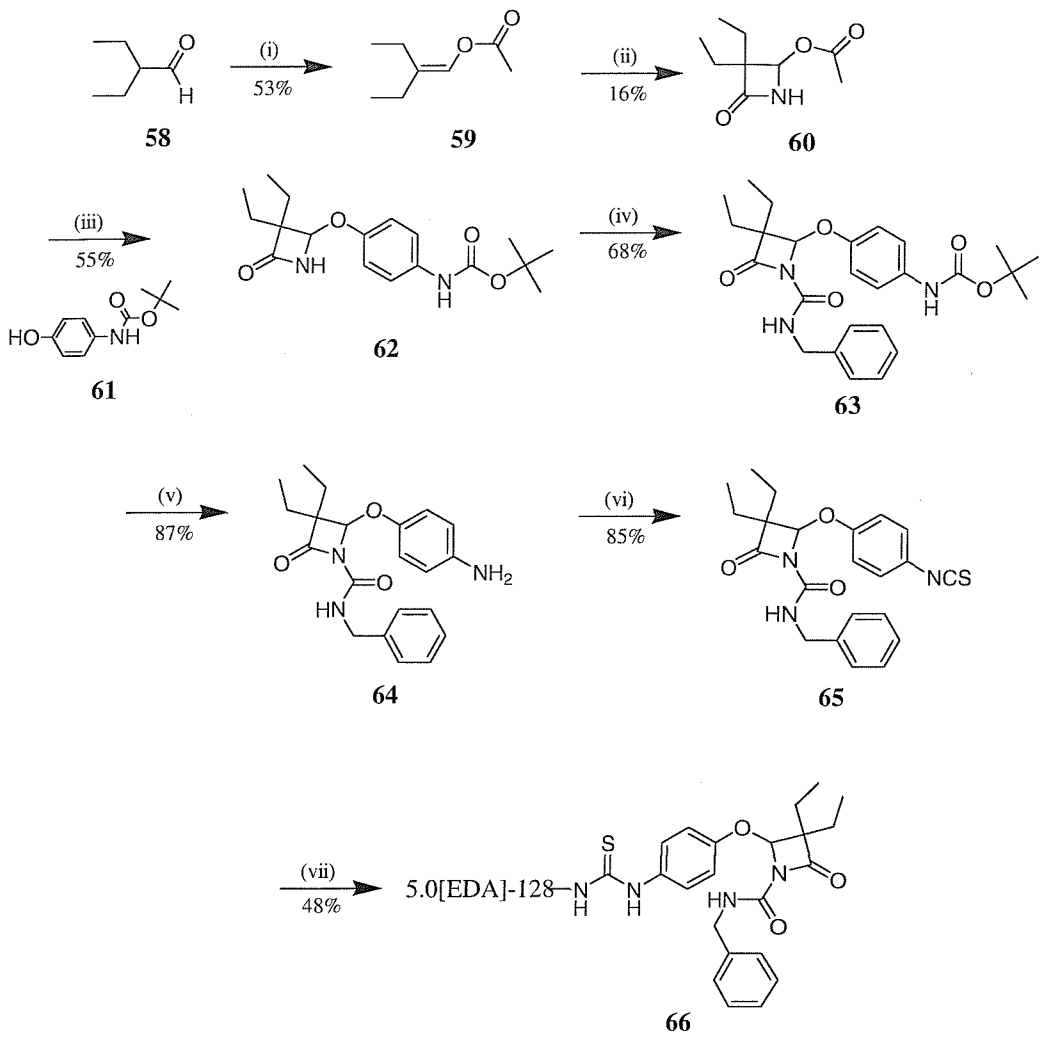
57

Figure 27. An example of a ß-lactam based inhibitor of NE.

2.4.2 Synthesis of β-lactam NEIs

It was decided to attach a type-I β-lactam-based NEI to the surface of an amine-terminated PAMAM dendrimer via a phenolic leaving group using a thiourea bond, (Scheme 23). 2-Ethyl butyraldehyde (**58**) was converted to **59** by stirring overnight in acetic anhydride and K_2CO_3. A [2+2] cycloaddition between chlorosulfonyl isocyanate and **59** gave **60**. Nucleophilic substitution of acetate by a previously prepared Boc-protected 4-aminophenol (**61**) gave **62** using NaOH in a mixture of

acetone and H_2O. The addition of a benzyl urea using benzyl isocyanate in CH_2Cl_2 gave **63**. The amine was deprotected using trifluoroacetic acid in CH_2Cl_2 to give **64** which was converted to the isothiocyante **65** using thiophosgene and $NaHCO_3$ in CH_2Cl_2 at 0 °C. This was then coupled to 5.0 [EDA]-128-amine by stirring overnight in DMSO at 40 °C to give the dendrimer-NEI conjugate **66**. It was found that dialysis was not a suitable method of purification, however the addition of Et_2O to the reaction mixture caused **66** to precipitate in the form of an off-white solid. Any excess **65** remained in solution and so **66** could be collected by filtration.



Scheme 23. (i) Ac_2O, K_2CO_3, overnight. (ii) a.) CH_2Cl_2, $ClSO_2NCO$, overnight. b.) H_2O, $NaHCO_3$, Na_2SO_3 (iii) NaOH, acetone, H_2O, 30 minutes. (iv) Benzyl isocyanate, DMAP, Et_3N, overnight. (v) CF_3COOH, 1 hour. (vi) thiophosgene, $NaHCO_3$, CH_2Cl_2, 0 °C, overnight. (vii) 5.0 [EDA]-128-amine, DMSO, $CHCl_3$, 40 °C, overnight.

IR and both ^1H and ^{13}C NMR spectra of **66** indicated the presence of inhibitor groups, but it was not possible to ascertain from these techniques the extent to which the surface sites had been filled by inhibitor molecules. An attempted measurement of the mass by MALDI mass spectroscopy failed. However, the percentage of sulphur as determined by elemental analysis indicated that the majority if not all of the surface sites had been filled by inhibitor molecules.

2.4.3 Testing 66 against NE

Dendrimer-NEI conjugate **66** was tested against isolated NE to measure its inhibitory properties, (Figure 28). It proved to be an effective inhibitor of NE with the percentage of NE activity reduced to 18.3% when the ratio of **66** to NE is 5:1 and 4.3% when the ratio is increased to 20:1.

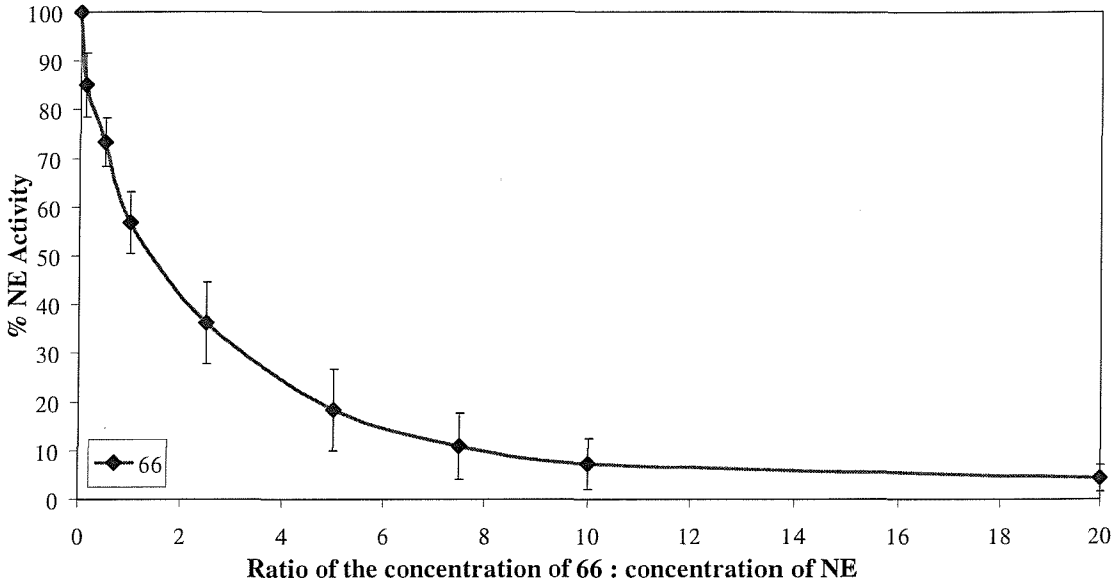


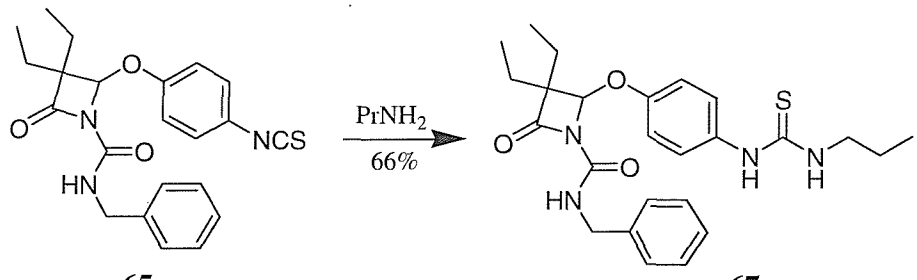
Figure 28. Plot showing the reduction in NE activity as the concentration of **66** is increased.

It was not possible to test **66** in whole blood solutions to see its effectiveness at inhibiting intracellular NE as its solubility in the buffer solution was too low.

2.4.4 Comparison of free β-lactam inhibitor with dendrimer bound inhibitor

It is unknown what effect the tethering of multiple inhibitors to a polymeric surface will have on inhibitory activity. It was postulated that the surface of the dendrimer would be crowded and there would be steric hindrance of the inhibitors accessing the active site of the NE by the neighboring inhibitor molecules attached to the dendrimer, as well as hindrance caused by the size of the dendrimer itself. One way

of measuring any effect on inhibitory activity would be to compare the performance of **66** with that of a free inhibitor.



65 **67**

Scheme 24.

Inhibitor **67**, (Scheme 24), was produced as a suitable model for the dendrimer-bound inhibitor. The thiourea should give similar electronic properties to the phenol leaving group as those of **66** and the propyl chain will act as a model for the alkyl chain that links the inhibitor molecules of **66** to the dendrimer. Compound **67** was prepared by stirring **65** with propylamine for 2 hours in CH_2Cl_2.

If it is assumed that **66** has complete surface coverage of 128 inhibitor groups per dendrimer molecule then the inhibitory properties **66** and **67** can be compared by plotting the decrease in NE activity against the concentration of inhibitor molecules to the concentration of NE, (Figure 29).

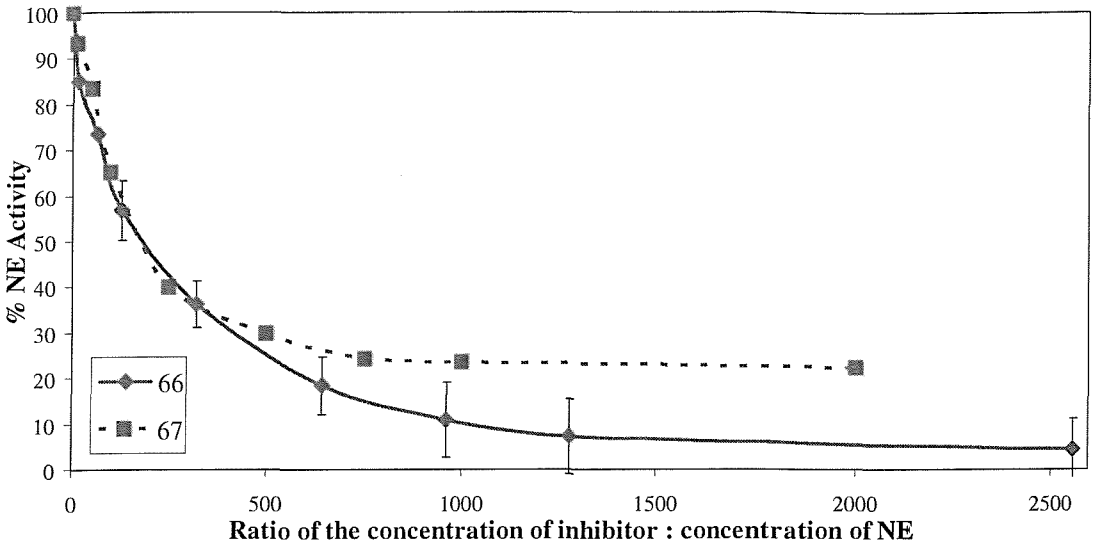
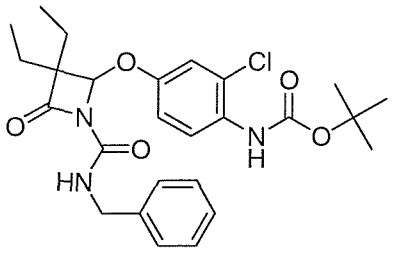


Figure 29. Plot comparing the reduction in NE activity as the concentrations of **66** and **67** are increased.

Both **66** and **67** show a similar inhibitory profile as the ratio of the concentration of inhibitor to the concentration of NE is increased from 0 to 300:1. After 300:1 the reduction in NE activity with **67** begins to plateau whilst the reduction in NE with **66** continues to decrease. When the ratio of the concentration of inhibitor to the concentration of NE is 2000:1 the NE activity with **66** present is 5.6% compared to 22.3% when **67** is present. These results indicate that there is an increase in inhibitory potency of the β-lactam-based inhibitors when attached to a 5th generation PAMAM dendrimer.

2.4.5 Alternative β-lactam-based NEIs

A short investigation was carried out in an attempt to improve potency. Variations of **63** have been explored by changing the phenolic leaving group. It was assumed that any difference in potency of the free inhibitors would remain similar if they were to then be attached to a dendrimer. Inhibitors **68** and **69** were produced by similar methodology to that used for **63** except that an alternative Boc-protected phenol was used in place of **61**.



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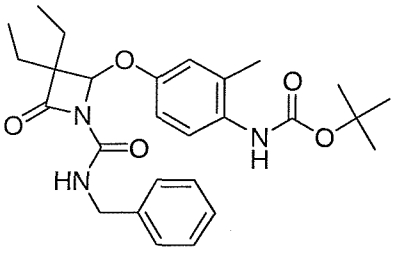
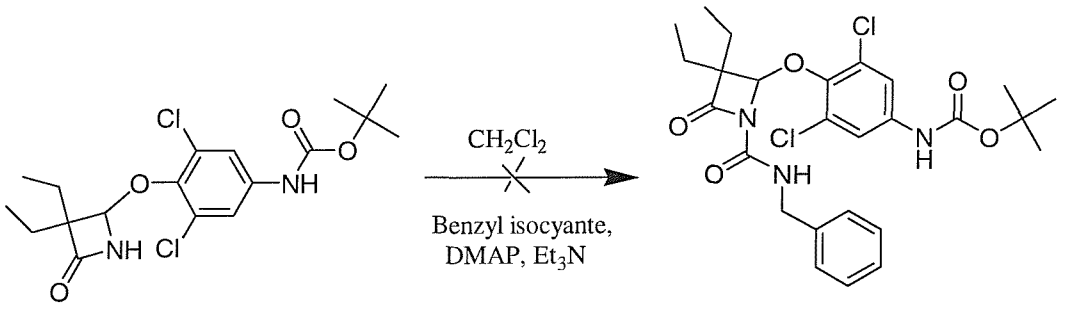


69

The synthesis of inhibitor **71** was also attempted. However the coupling of **70** with benzyl isocyanate to form **71** was unsuccessful as **71** decomposed to give a mixture of products, (Scheme 25).



70 **71**

Scheme 25.

Inhibitors **63**, **68** and **69** were tested against purified NE to measure their inhibitory potency, (Figure 30).

The enzyme inhibition assays were carried out at the Univeristy of Portsmouth, the inhibitory properties of each compound was tested against purified NE. The experiment was repeated twice and the result presented as an average. However, due to the cost of the assays, if after the first test the inhibitor had not shown to have caused a significant decrease in NE activity the experiment was not repeated. For these compounds the curve of the graph obtained is of lesser quality than for those compounds which had been tested three times.

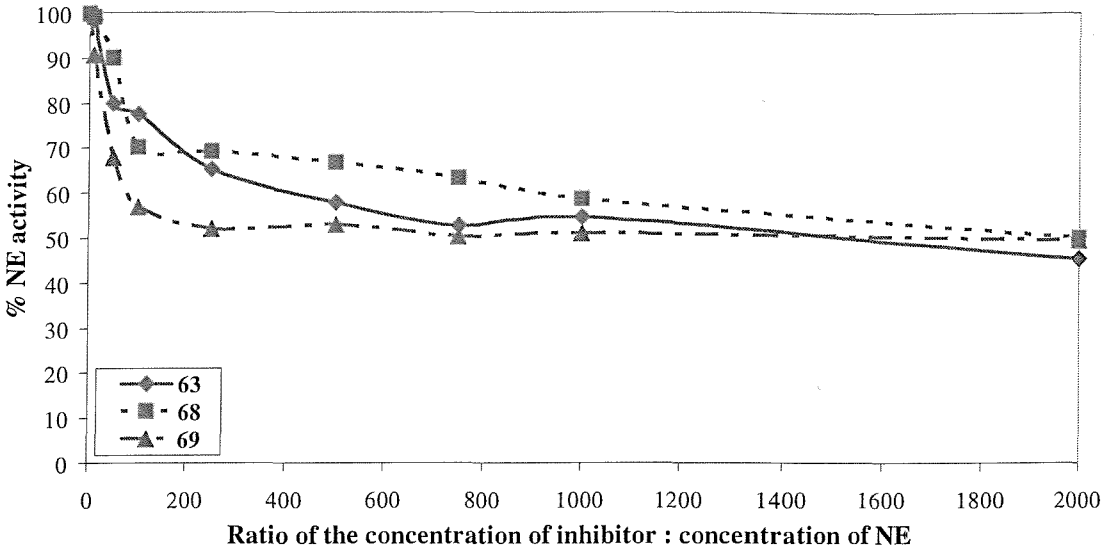
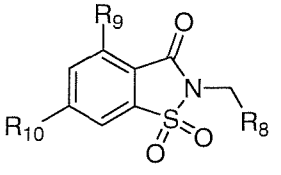


Figure 30. Plot comparing the reduction in NE activity as the concentrations of **63**, **68** and **69** are increased.

It was observed that there was little difference in the inhibitory properties of **63**, **68** and **69**. At the highest concentration tested, when the ratio of the concentration of inhibitor to the concentration of NE is 2000:1, the NE activity is reduced to 45.5% with **9** and 50.1% and 49.5% with **68** and **69** respectively. As no significant improvement in inhibitory activity has been achieved **68** and **69** were not developed further for attachment to a PAMAM dendrimer.

2.5.1 Saccharine-based NEIs

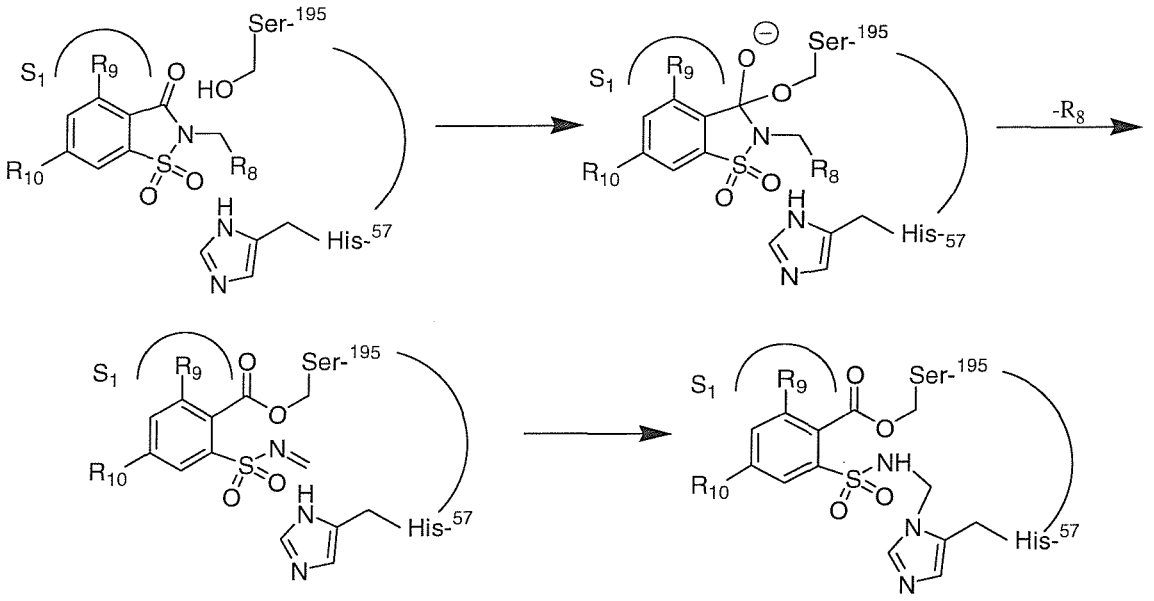
NEIs based on a saccharine skeleton were also identified from the literature as being potential candidates for attachment to an amine-terminated PAMAM dendrimer.



R_8 = Leaving group
R_9 = Peptimimetic group
R_{10} = Stabilising group

Figure 31. Skeleton of saccharine-based NEI.

Enzyme-activated inhibition of serine proteases by saccharine derivatives was first reported by Groutas *et al*[171], based on the premise that incorporation of a leaving group into known saccharine-based acylating agents would permit enzyme inactivation[172-174]. Groutas *et al* arrived at the design of inhibitors shown in Figure 31. The proposed mechanism of inactivation is shown in Scheme 26[174, 175].



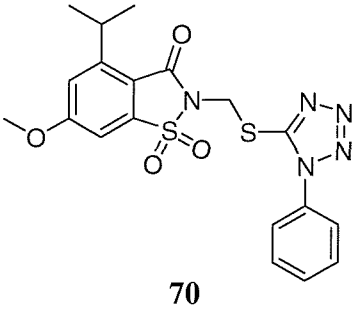
Scheme 26. Mechanism of the inactivation of proteases by saccharine derivatives.

The proposed mechanism of inactivation proceeds via a Gabriel-Colman rearrangement[176]. This involves nucleophilic attack by Ser 195, leading to the opening of the heterocyclic ring and elimination of the leaving group (R_8), thereby turning the substrate into an *N*-sulfonyl imine (a Michael acceptor). A second nucleophilic attack by His 57 on the terminal $=CH_2$ group produces the doubly bound enzyme-inhibitor adduct and therefore causes inactivation of the enzyme.

The potency of this class of inhibitor has been shown to be dependant on the electronic properties of the leaving group and the substitution pattern of the saccharine unit as this is involved in enzyme recognition. R_9 is a peptidomimetic group that sits in the S^1 subsite and as with succinimide-based NEIs, the groups found to have optimum recognition are short chain hydrocarbons such as an

isopropyl group[177]. *In vivo* activity of saccharine proteases by oral administration has been difficult to achieve because of their poor hydrolytic and metabolic stability[178-181]. This is the result of elastase-catalysed hydrolysis[189] at the carbonyl end of the saccharin nucleus. Increasing electron density or steric hindrance around the carbonyl group would increase stability[180]. This can be achieved by the introduction of a short alkoxy residue at R_{10}.

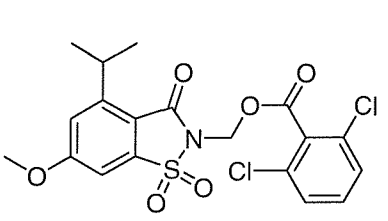
2.5.2 Examples of saccharine-based NEIs



70

Figure 32. An example of a saccharine-based NEI.

Hlasta *et al* have described **70** (Figure 32), a saccharine-based skeleton with a phenylmercaptotetrazole leaving group as being a potent NEI[182]. However, compounds of this type have proved to be particularly lipophilic with poor aqueous solubility and hence shown poor *in vivo* activity.



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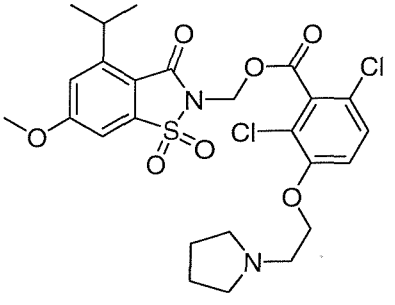


72

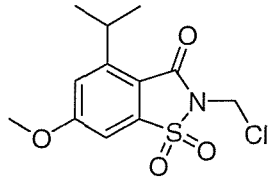
Figure 33. Examples of saccharine-based NEIs.

Compound **71**, (Figure 33), was also found to be potent inhibitor[183, 184], yet like **70** it proved to have poor *in vivo* activity because of its lipophilicity. A series of compounds was then prepared which contained aqueous solubilising groups (amines and carboxylic acids e.g. **72**) in an attempt to improve aqueous solubility and therefore *in vivo* activity.

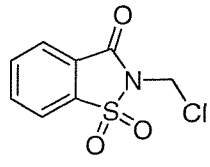
2.5.3 Model saccharine-based NEIs

As an initial investigation into saccharine-based NEIs it was decided to prepare two model compounds and test their inhibitory activity against NE. This would allow assessment of the general synthetic pathways used in preparing this class of inhibitors and their general level of inhibitory activity. This information could then be used to decide whether to pursue saccharine-based NEIs as candidates for attachment to amine-terminated PAMAM dendrimers.

The literature reports that, for optimum *in vivo* stability and enzyme recognition, the saccharine unit should contain an alkoxy and isopropyl group on its aryl ring. For the synthesis of saccharine-based NEIs this would require the preparation of **73** as a precursor. The synthesis of **73** is lengthy and expensive[185-187], as an alternative the chloromethyl saccharine unit **74** was used as it can be prepared in bulk in a one-step synthesis from saccharine which is in itself inexpensive and widely available[188]. Compound **74** can be prepared in 81% yield by refluxing saccharine overnight in $SOCl_2$ with excess formaldehyde sodium bisulphite.

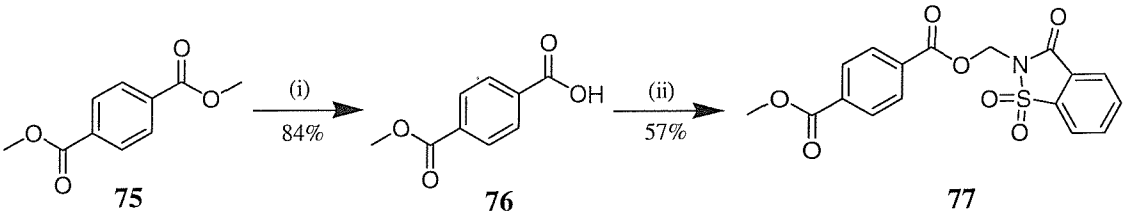


73



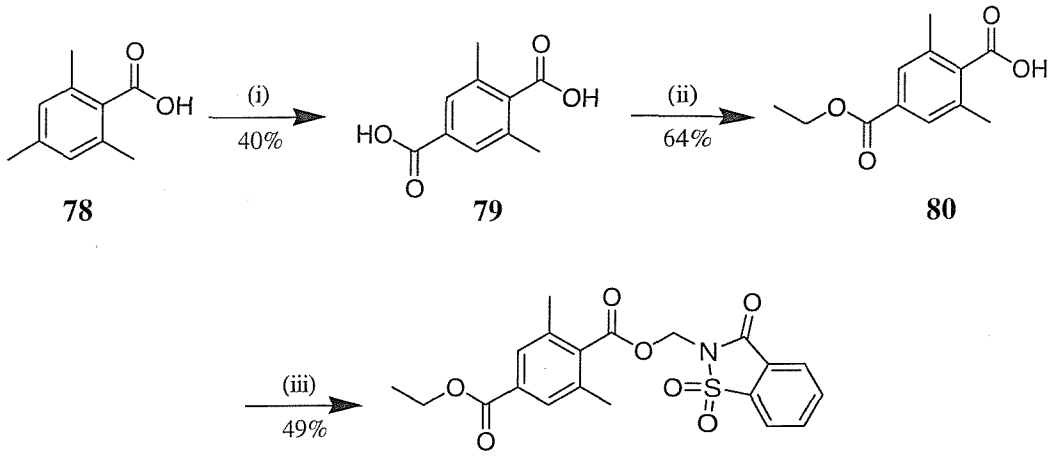
74

Compound **77** (Scheme 27) was the first model compound prepared. Dimethyl terephalate (**75**) was half hydrolysed in a mixed solvent system of MeOH, toluene and H_2O with KOH to give **76**. The chloromethyl saccharine unit **74** could then be coupled to **76** by stirring overnight in DMF with K_2CO_3 and NaI to give **77**.



Scheme 27. (i) MeOH, PhMe, H_2O, KOH, overnight. (ii) **74**, DMF, K_2CO_3, NaI, overnight.

The sterically crowded analogue **81**, (Scheme 28), was also prepared to act as a model compound for comparison with **77**. This was chosen as a target in order to see what effect the electron donating and sterically crowding methyl groups on the 2 and 6 positions of the benzoic acid leaving group have on inhibitor potency. The *para* methyl group of 2,4,6 trimethyl benzoic acid (**78**) was first oxidised to a benzoic acid using $KMnO_4$ and KOH to give **79**. The newly formed benzoic acid group can be selectively esterified by refluxing **79** in EtOH with a catalytic amount of $SOCl_2$ to give **80**. The chloromethyl saccharine unit **74** can then be coupled to **80** by stirring them together overnight in DMF with K_2CO_3 and NaI to give **81**.



Scheme 3. (i) 1.) $KMnO_4$, KOH, H_2O. 2.) 50% H_2SO_4 solution. 3.) $NaHSO_3$. (ii) EtOH, cat. $SOCl_2$, 5 hours. (iii) **74**, DMF, K_2CO_3, NaI, overnight.

The inhibitory activity of both **77** and **81** were tested using purified NE, (Figure 34). Both showed inhibitory activity. When the ratio of the concentration of inhibitor to the ratio of the concentration of NE was 2000:1 then the NE activity with **81** was 8.9% which is lower than the value obtained for **77** which was 24.6%. Since **81** was shown to be a better inhibitor, it appears that the methyl groups in 2 and 6 positions about the benzoic acid leaving group have a positive effect on inhibitor potency.

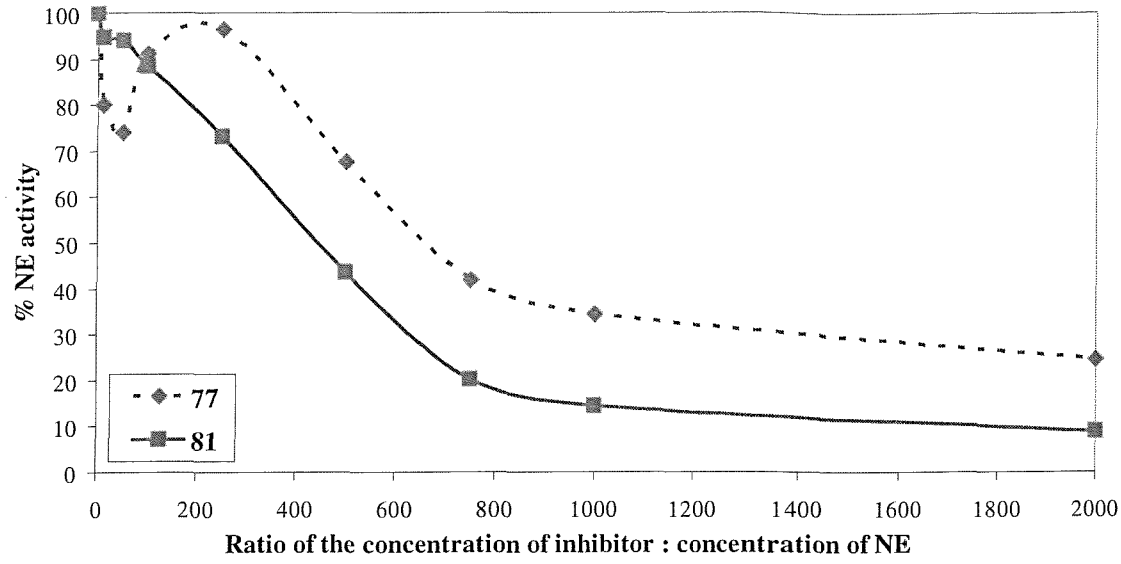
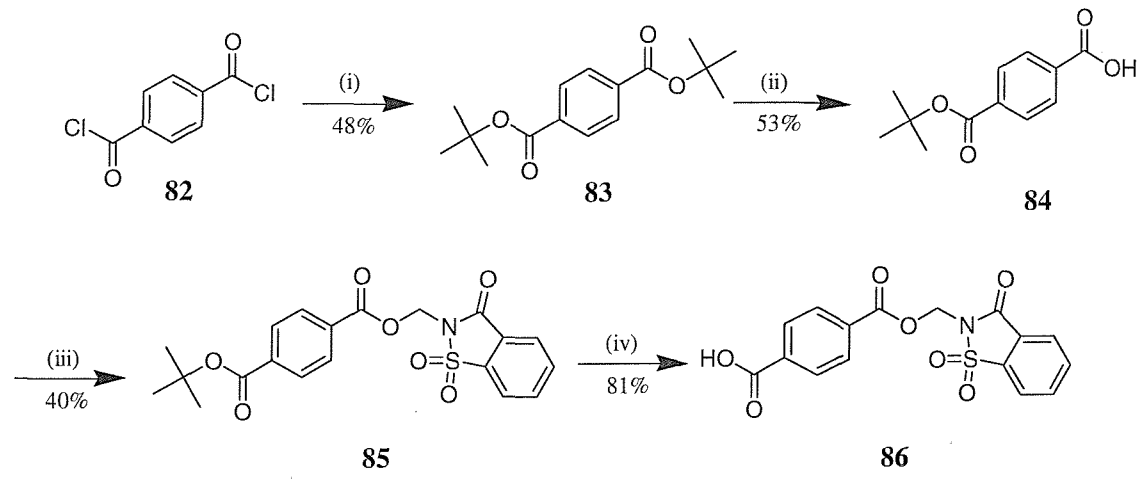


Figure 34. Plot comparing the reduction in NE activity as the concentration of **77** and **81** is increased.

In view of the successful synthesis of the model compounds and their ability to effectively inhibit NE, it was decided that saccharine-based NEIs were suitable candidates for attachment to an amine-terminated PAMAM dendrimer.

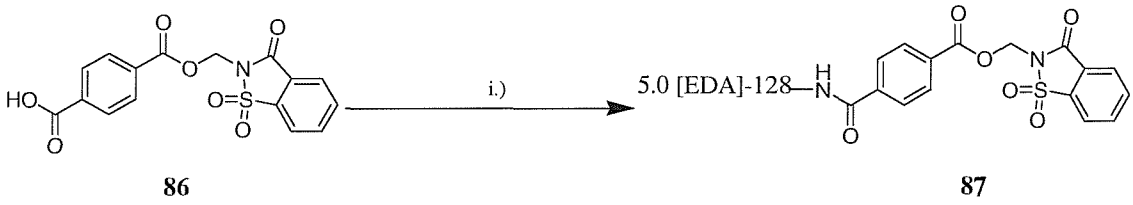
2.5.4 First approach to creating a dendrimer-NEI conjugate

The initial approach decided upon was to try and link a saccharine-based NEI to the surface of a 5.0 [EDA]-128-amine using a DCC mediated peptide coupling. Derivative **86** (Scheme 29), was identified as a suitable target. It was prepared by coupling *t*-BuOH to terephthaloyl dichloride (**82**) in pyridine to give **83**. Hydrolysis of one of the *t*-butyl esters of **83** by reflux in *t*-BuOH with KOH gave **84**. The chloromethyl saccharine unit **74** can then be coupled to **84** by stirring them together overnight in DMF with K_2CO_3 and NaI to give **85**. The *t*-butyl ester of **85** can be cleaved to give target molecule **86** by stirring for one hour in CH_2Cl_2 with trifluoroacetic acid.



Scheme 29. (i) pyridine, *t*-BuOH, 3 hours. (ii) *t*-BuOH, KOH, reflux, 3 hours. (iii) **74**, DMF, K_2CO_3, NaI, overnight. (iv) CF_3COOH, CH_2Cl_2, 1 hour.

Several attempts were made to couple **86** to the surface of 5.0 [EDA]-128-amine to give **87** using DCC as a peptide coupling reagent (Scheme 30).



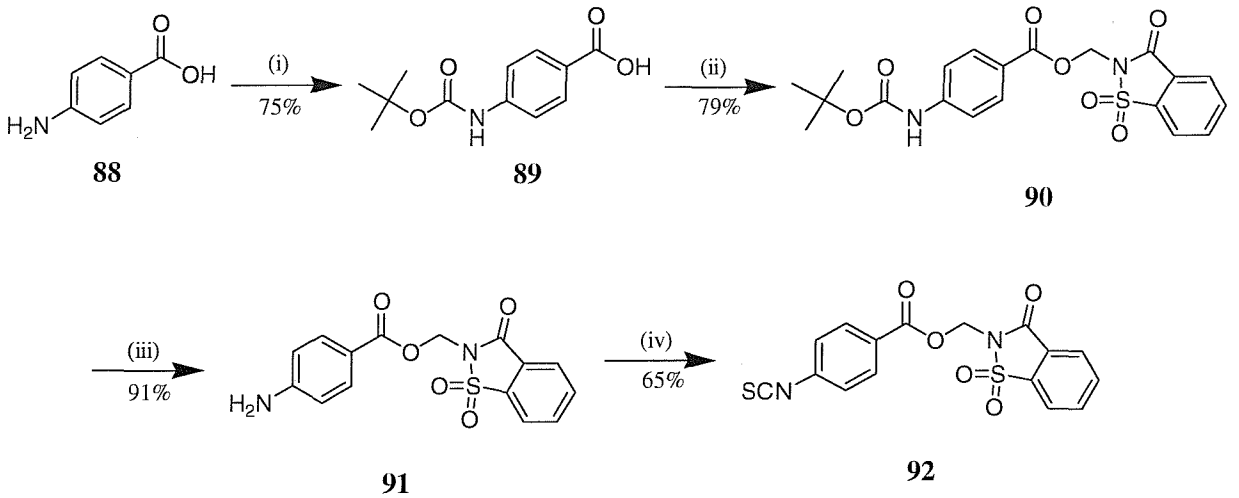
Scheme 30. i.) 1.) THF/CH_2Cl_2, DCC, 0 °C. 2.) DMSO, 5.0 [EDA]-128-amine.

A crude solid product was obtained from this reaction upon the addition of Et_2O to the reaction mixture. The NMR and IR of the crude material showed the presence of both dendrimer and inhibitor groups which suggested that the reaction had been at least partially successful. However, it was not possible to estimate the amount of surface sites which had been filled by inhibitor molecules. The NMR spectra of the crude material also showed the presence of the urea by-product from the DCC coupling reaction. Purification of the solid and removal of residual DMSO and the urea by-product by dialysis was not possible because of the low solubility of the crude solid in most common solvents except DMSO.

As a result of the large excesses of reagents required, dendrimer synthesis can only be carried out on a small scale and each step can require several weeks to complete. In order to conserve the limited stocks of dendrimer available it was decided that rather than repeating the DCC coupling reaction or try an alternative coupling reagent, it would be more appropriate to try an approach which had already been shown to be successful.

2.5.5 Second approach to creating a dendrimer-NEI conjugate

Previous work had demonstrated that the coupling of isothiocyanates **30** and **65** to the surface of 5.0 [EDA]-128-amine to give a dendrimer capable of binding $NaTcO_4$ (**31**) and a β-lactam based dendrimer-NEI conjugate (**66**) as being successful. It was decided to use the same approach for creating a dendrimer-NEI conjugate as there are no byproducts or additional reagents involved in the reaction. Isothiocyanate **92** (Scheme 31), was identified as a suitable target molecule.

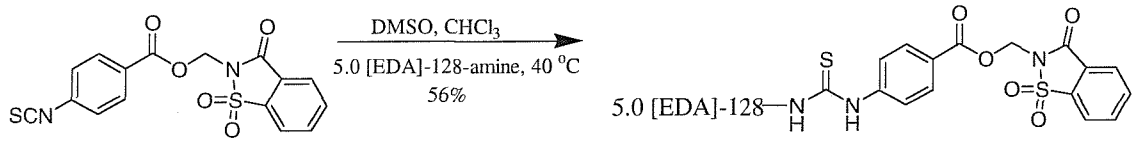


Scheme 31. (i) THF, H_2O, di-*tert*-butyl dicarbonate, Et_3N, overnight. (ii) **74**, DMF, K_2CO_3, NaI, overnight. (iii) CF_3COOH, CH_2Cl_2, 1 hour. (iv) CH_2Cl_2, thiophosgene, Na_2CO_3, 0 °C, overnight.

The amino group of 4-amino benzoic acid (**88**) was first Boc-protected by stirring overnight in a mixture of THF and H_2O (2:1) with Et_3N and di-*tert*-butyl dicarbonate to give **89**. The chloromethyl saccharine unit **74** was coupled to **89** by stirring both

together in DMF overnight with K_2CO_3 and NaI to give **90**. The amino group of **90** was then deprotected by stirring in CH_2Cl_2 for 1 hour with trifluoroacetic acid to give **91**. The resulting amine derivative could then be converted to an isothiocyanate by slow addition of **91** to a solution of thiophosgene in CH_2Cl_2 with Na_2CO_3 at 0 °C to yield **92**.

The dendrimer-NEI conjugate **93** was prepared by heating 5.0 [EDA]-128-amine at 40 °C overnight in a 1:1 mixture of $CHCl_3$ and DMSO in the presence of 180 equivalents of **92**, (Scheme 32).



92 **93**

Scheme 32.

Purification using dialysis against $CHCl_3$ to remove DMSO and remaining **92** was unsuccessful. But **93** was isolated by shaking the reaction mixture in a solution of H_2O and $CHCl_3$ as the product precipitated from the solution and could be collected by filtration. The IR and both ^1H and ^{13}C NMR spectra indicated the presence of the inhibitor groups. It was not however, possible to ascertain from these techniques the extent to which the surface amine sites had inhibitor molecules attached. An attempted measurement of the overall molecular mass using MALDI mass spectrometry was unsuccessful. The percentage of sulphur found by elemental analysis of **93** was reasonably close to the theoretical value so it was assumed that most, if not all, of the surface dendrimer sites were filled with inhibitor molecules.

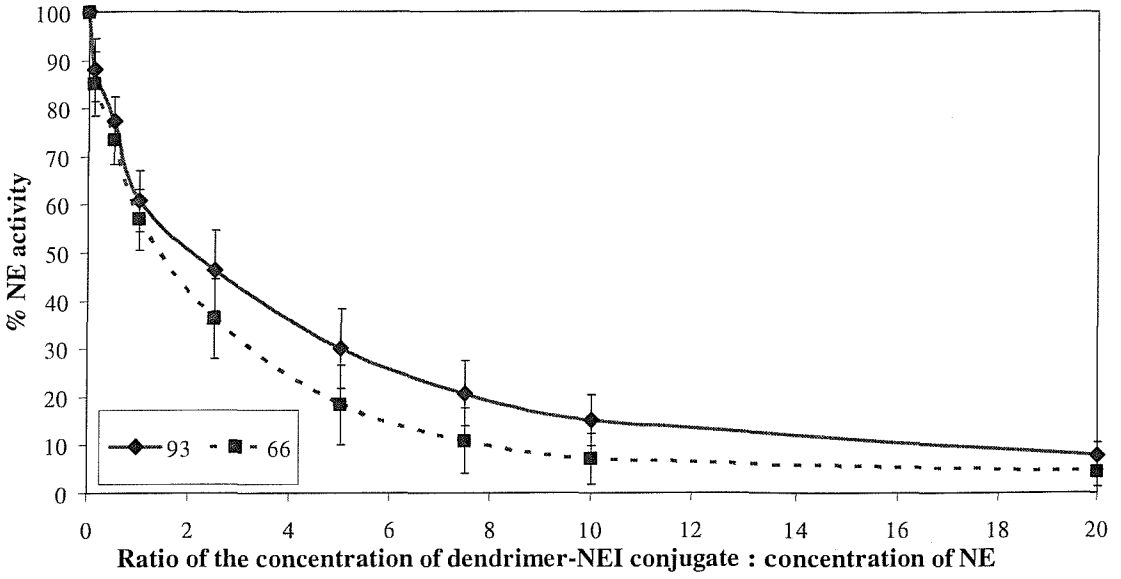


Figure 35. Plot comparing the reduction in NE activity as the concentration of **66** and **93** is increased.

Dendrimer-NEI conjugate **93** was tested against isolated NE to measure its inhibitory properties, (Figure 35). It proved to be an effective inhibitor of NE with the percentage of NE activity reduced to 30.1% when the ratio of **93** to NE is 5:1 and 7.8% when the ratio is increased to 20:1. It is similar in potency to the β-lactam-based dendrimer-NEI conjugate **66**. However the activity with **66** present is lower at all the concentrations tested than with **93** present, making **66** the more effective NEI.

The dendrimer-NEI conjugate **66** was tested to investigate whether it would inhibit intracellular NE. Varying concentrations of **66** were dissolved into an aqueous buffer solution and mixed with blood samples, the neutrophils were then isolated from the blood samples and lysed to release the intracellular NE. The activity of the NE could then be measured. It was observed that as the concentration of **66** increased then the NE activity decreased, this implied that **66** was being absorbed by the neutrophils and inhibiting the intracellular NE. However, **66** displayed low solubility in the aqueous buffer solution and it was not possible to increase the concentration past 0.271 μmol. At this concentration the NE activity had decreased to 62.0%,

(Figure 36). In order to increase effectiveness a dendrimer-NEI conjugate with greater solubility in the buffer solution needs to be produced.

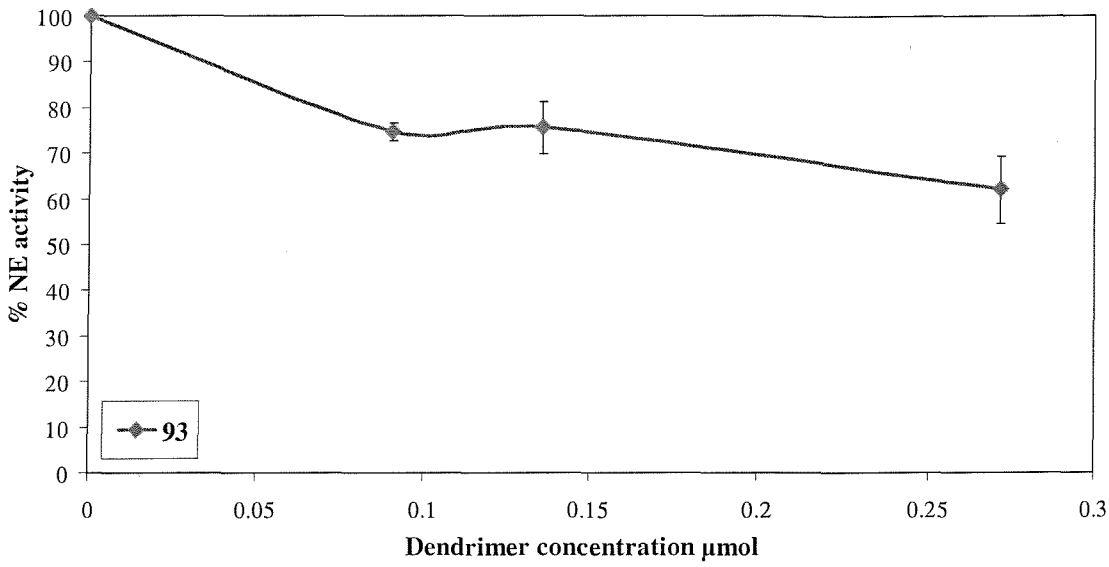
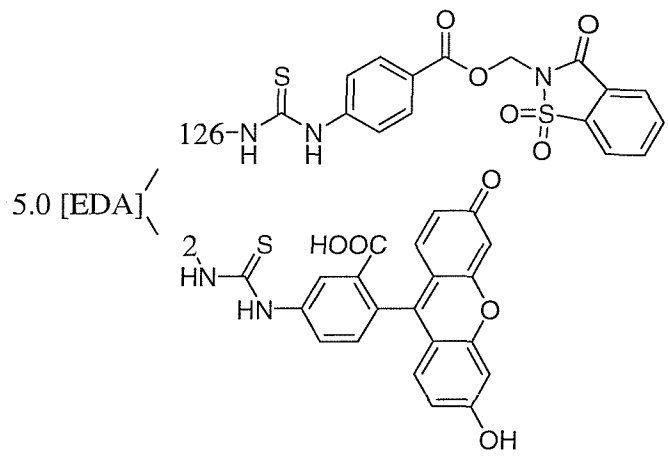


Figure 36. Plot to show the decrease in NE activity in whole blood as the concentration of **93** is increased.

2.5.6 FITC tagged dendrimer-NEI conjugate

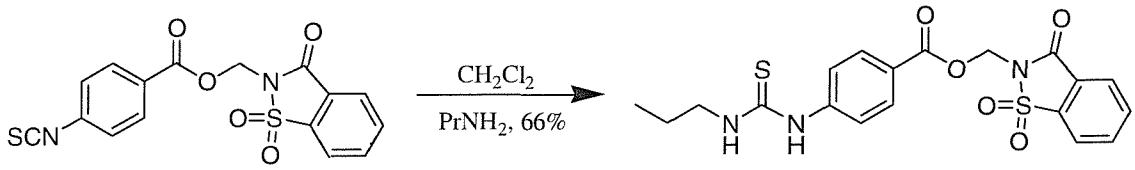
In order to confirm that the dendrimer-NEI conjugate was absorbed inside the neutrophil a FITC-tagged dendrimer-NEI conjugate **94** was prepared using similar methodology to that used for the other FITC-tagged dendrimers **33-35**.



94

Using confocal fluorescent microscopy it was observed that when mixed with a sample of white blood cells, as with the other FITC-tagged dendrimers, the neutrophils absorbed **94** inside the cell. Uptake by any other class of monocyte was not observed.

2.5.7 Comparison of dendrimer bound inhibitor to free inhibitor



92 **95**

Scheme 33.

95 (Scheme 33), was prepared to act as a free mono-functional model for comparison with the inhibitor when attached to the surface of the dendrimer **93**. It was prepared by stirring **92** for 2 hours in CH_2Cl_2 with propylamine. The thiourea substituted benzoate should show similar leaving group ability as those of the inhibitor groups of **93**. The propyl chain will act as a model for the alkyl chain that links the inhibitor molecules of **93** to the dendrimer surface. If it is assumed that **93** has complete surface coverage of 128 inhibitor groups per dendrimer molecule then the inhibitory properties **93** and **95** can be compared by plotting the decrease in NE

activity versus the concentration of inhibitor molecules relative to the concentration of NE, (Figure 37).

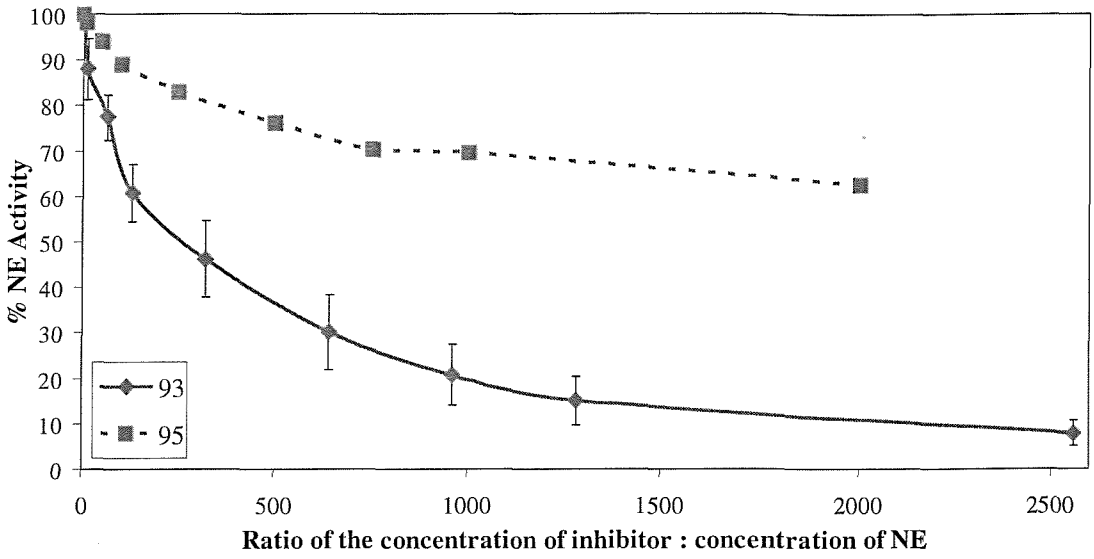


Figure 37. Plot comparing the reduction in NE activity as the concentration of **93** and **95** is increased.

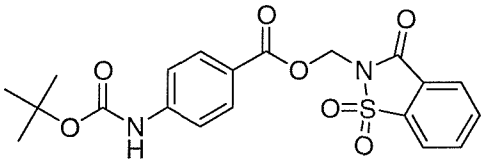
It can be seen that there is a large increase in potency of the dendrimer-bound inhibitor **93** to the free inhibitor **95**. When the ratio of the concentration of inhibitor to the concentration of NE is 2000:1 then with **93** the NE activity drops to 11.2%, however as is the case with model inhibitor **95** it only drops to 62.4%. These results indicate that there is an increase in inhibitory potency of the saccharine-based inhibitors when attached to a 5th generation PAMAM dendrimer.

2.5.8 Variations of the benzoic acid leaving group

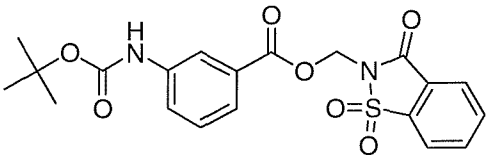
Other saccharine-based NEIs have been explored in an attempt to increase inhibitory potency. Inhibitory potency would be judged to have been increased when the NE activity is reduced at lower ratios of the concentration of inhibitor to the concentration of NE. **90** (Scheme 31), was used as a basis for comparison as it represents the dendrimer-bound inhibitors of **93**. It was decided that the variations

should be incorporated into the benzoic acid portion of the molecule rather than the saccharine portion. All of the variations contain a Boc-protected amine which could potentially be cleaved and the amine could subsequently be converted to an isothiocyanate for attachment to an amine-terminated dendrimer. It was assumed that the differences in inhibitory potency of the Boc-protected forms of the inhibitors would be the same if they were then attached to a dendrimer.

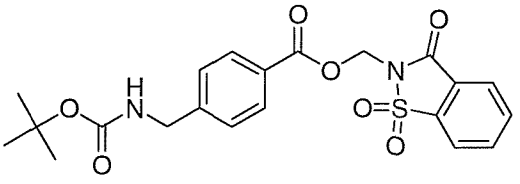
Inhibitor **96,** in which the Boc-protected amine is placed on the *meta* position of the aromatic ring instead of in the *para* position as on **90**. Inhibitor **97** which has an additional methylene group between the benzoate ring and Boc-protected amine, and **98**, which has two saccharine units coupled to one benzoic acid leaving group. They were all produced by first Boc-protecting the corresponding amino-benzoic acid and then coupling the product to **74** by stirring with K_2CO_3 and NaI in DMF overnight.



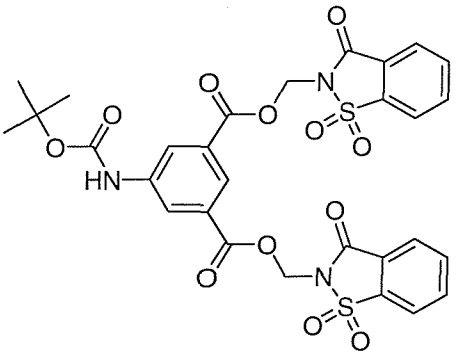
90



96



97



98

Inhibitors **96**, **97** and **98** were tested against purified NE to measure their effect on NE activity and the results were compared with those for **90**, (Figure 38). Derivative

96 displayed negligible inhibitory activity and it was not possible to test **97** as it precipitated out of the buffer solution used in the assay.

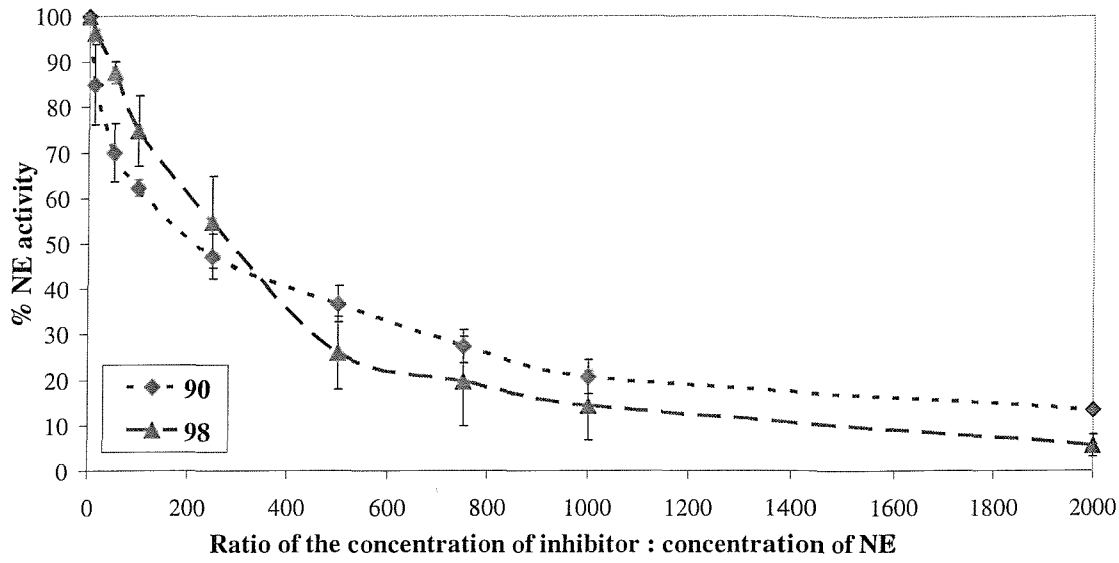


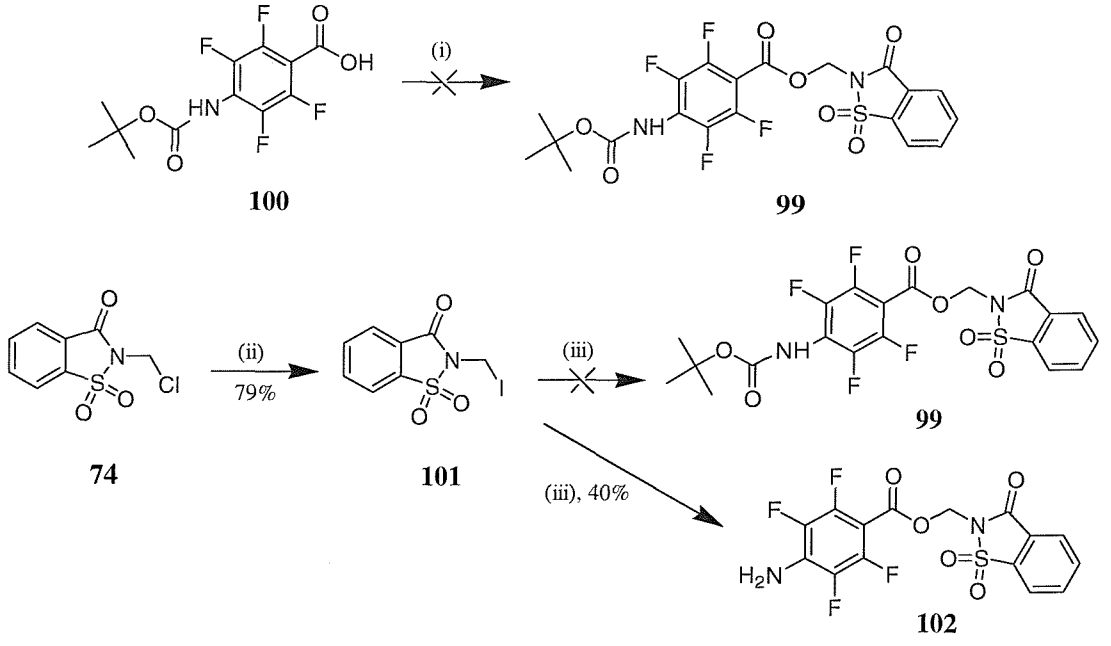
Figure 38. Plot comparing the reduction in NE activity as the concentration of **90** and **98** is increased.

When the ratio of the concentration of **98** to the concentration of NE was 2000:1, the NE activity dropped to 5.5%; the corresponding NE activity with **90** at this ratio was 13.3%. **98** was therefore shown to be a more potent inhibitor than **90**. However there was a large decrease in the solubility of **98** from **90** in most common laboratory solvents and because of this it was decided not to continue to develop **98** and attempt to attach the corresponding amine to the surface of a dendrimer.

2.5.9 Changing the electronic properties of the benzoic acid leaving group

The next variation changed the electronic properties of the benzoic acid leaving group. It had previously been shown that incorporation of electron withdrawing groups such as chlorine into the benzoic acid leaving group had improved inhibitory potency[183, 184]. It was thought that the incorporation of fluorine into the leaving group would increase the NE inhibitory properties of the molecule. Inhibitor **99**

(Scheme 34), was identified as a suitable target molecule. However it was not possible to make inhibitor **99** directly, the electron withdrawing properties of the fluorine atoms reduce the effectiveness of the Boc-protected 2,3,5,6-tetrafluoro-benzoic acid (**100**) as a nucleophile so that the coupling of **74** to **100** using K_2CO_3 and NaI in DMF to form **99** was unsuccessful. An alternative method was proposed whereby **74** is first converted into **101** by stirring overnight with NaI in acetone, **101** is then coupled to **100** by stirring overnight DBU in CH_2Cl_2. This reaction was unsuccessful as during the reaction the amine is deprotected, yielding **100** instead of **99** (Scheme 34). Although this is useful in the respect that **100** is the next step in a potential route to join this inhibitor to a dendrimer it is unfortunate that **99** could not be successfully isolated as a direct comparison of its behaviour with that of **90** has not been possible.

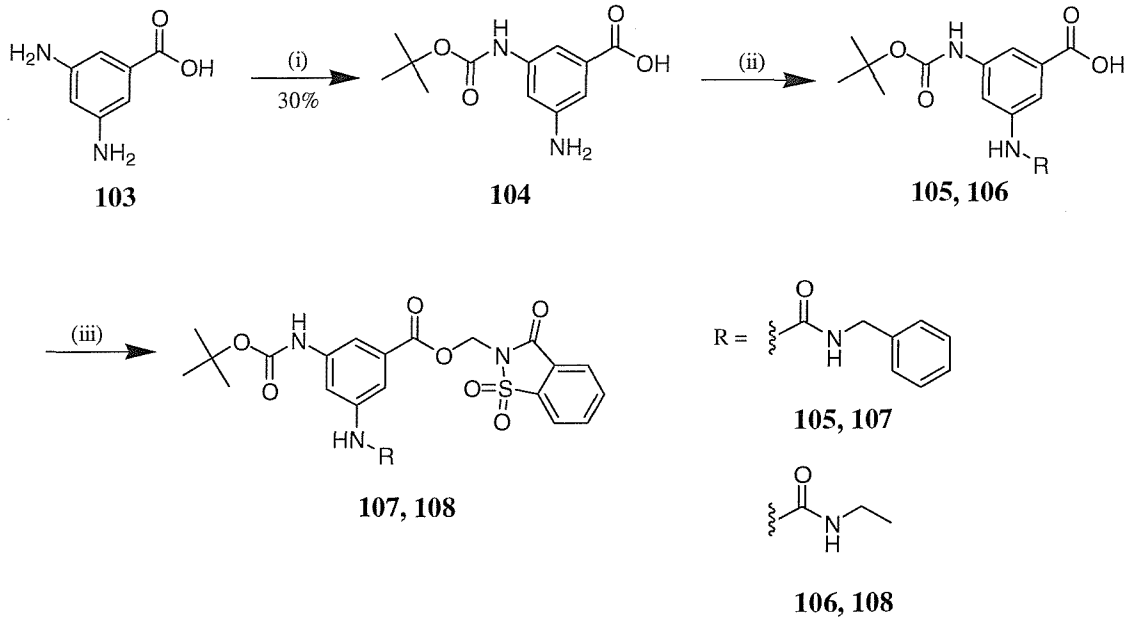


Scheme 34. (i) **74**, DMF, K_2CO_3, NaI, overnight. (ii) NaI, acetone, overnight. (iii) **100**, CH_2Cl_2, DBU, overnight.

When **102** was tested with NE there was negligible reduction in NE activity as the concentration of **102** was increased, because of this **102** was considered an unsuitable candidate for further development for attachment to a dendrimer.

2.5.10 Introducing other functionality into the benzoic acid leaving group

Three other variations were explored in which additional functionality was introduced into the benzoic acid leaving group. Additional functionality could be used to potentially increase enzyme recognition of the substrate which may in turn increase inhibitory potency.



103 **104** **105, 106**

107, 108

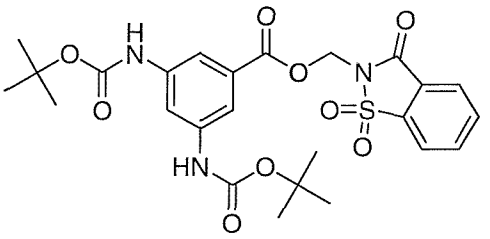
105, 107

106, 108

Scheme 35. (i) di-*tert*-butyl dicarbonate, Et_3N, THF, H_2O, overnight. (ii) R-Isocyanate, THF, overnight. (iii) **74**, K_2CO_3, NaI, DMF, overnight.

One way of introducing additional functionality is to mono Boc-protect 3,5-diamino benzoic acid (**103**) to give **104** then couple a functional group to the unprotected amine group via a urea to give **105** and **106** in yields of 48% and 87% respectively, (Scheme 35). This was carried out successfully and the functionalised benzoic acid was then coupled to a saccharine unit using K_2CO_3 and NaI in DMF. In this way both a benzyl and an ethyl unit were introduced to produce **107** and **108** in yields of

43% and 64% respectively. A bis-Boc protected analogue **109** was also produced as a comparison.



109

Inhibitors **107**, **108** and **109** were tested against purified NE to measure their inhibitory potency, (Figure 39), the results were compared to those obtained for **90**. At the highest concentration tested where the ratio of the concentration of inhibitor to the concentration of NE was 2000:1 then **107** proved to be the most effective inhibitor as the NE activity was 7.2%. This was lower than the NE activity found for **90**, **108** and **109** at this concentration (which was 13.3%, 25.7% and 32.6% respectively). However, at lower concentrations **90** was shown to be the most effective NEI. When the ratio of the concentration of inhibitor to the concentration of NE is 250:1 then the NE activity shown with **90** is 47.2%. This is lower than the NE activity of **107**, **108** and **109** at this concentration (65.0%, 85.9% and 82.3% respectively).

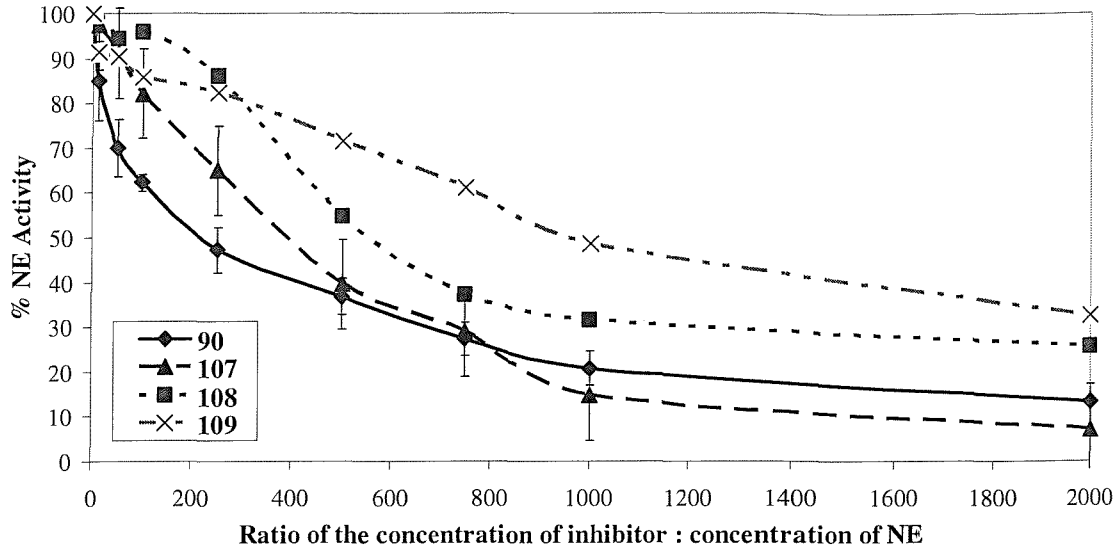


Figure 39. Plot comparing the reduction in NE activity as the concentration of **90**, **107**, **108** and **109** is increased.

Neither **107**, **108** or **109** proved to be a significant improvement on **90** and so were judged unsuitable candidates for further development for attachment to a PAMAM dendrimer. However these results did show that it is possible to introduce additional functionality into the benzoic acid leaving group of the saccharine inhibitor. The results obtained for **107** show that it is also possible for this additional functionality to improve inhibitory potency.

NE is just one of many serine proteases and it has been shown that saccharine-based compounds can also be used to inhibit human leukocyte cathepsin G[189] and human mast cell tryptase[190]. The ability to introduce additional functionality into the inhibitor design will aid in giving the inhibitor a greater specificity in towards NE. This is an important factor to consider for future development as the other serine proteases have important physiological functions and inhibiting them may result in detrimental effects. As yet none of the compounds in this thesis have been tested against any other serine proteases except NE and so no information on their specificity has been obtained.

2.5.11 Increasing solubility of the dendrimer-NEI conjugate in whole blood

The dendrimer-NEI conjugate **93** was found to have low solubility when tested in whole blood and so it was only possible to test **93** at low concentrations. In order for a dendrimer-NEI conjugate to be used as an effective drug then its solubility in whole blood needs to be increased.

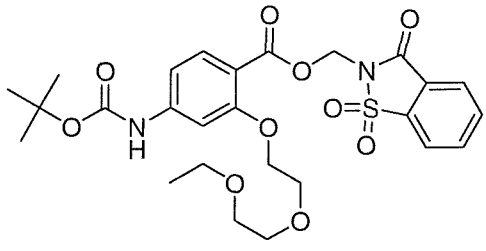
Linking PEG chains to therapeutic agents such as polypeptides[191] has been shown to have several beneficial affects including increased solubility. PEG conjugation (PEGylation) masks the protein's surface and increases the molecular size of the polypeptide which reduces renal ultrafiltration, prevents the approach of antibodies or antigen processing cells and reduces degradation by proteolytic enzymes. PEG conveys to molecules its physico-chemical properties and therefore modifies biodistribution of peptide and non-peptide drugs.

PEGylated 4th generation PAMAM dendrimers have previously been produced to increase solubility of lipophilic drugs. These PEGylated dendrimers have displayed improved entrapment efficiency of the sparingly water soluble anticancer agent 5-Fluorouracil[192]. Two other anticancer drugs Methotrexate which is practically water insoluble and another hydrophobic drug Adriamycin were succesfully encapsulated in the hydrophobic interior of a PAMAM dendrimer which had poly(ethylene glycol) monomethyl ether (MPEG) chains of different molecular weights on its surface[192].

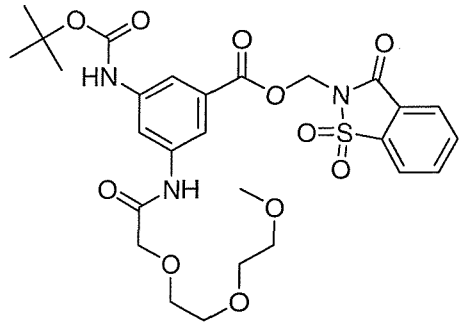
It was proposed that the introduction of a short polyethylene glycol (PEG) chain would aid solubility. Two strategies are available for the introduction of PEG chains into the dendrimer-NEI conjugate. The first is to first fill a proportion of the dendrimer surface sites with PEG chains and then fill the rest with inhibitor molecules, or alternatively attach the inhibitor molecules first followed by the PEG chains. However the major disadvantage with this approach is that it would create a polydisperse sample.

It was decided to follow the second strategy to retain mondispersity of the sample. The PEG chain could be attached to the inhibitor then both the PEG-inhibitor molecule could be attached to the dendrimer. It was decided initially to use a short PEG chains of low molecular weight such as diethylene glycol mono-ethyl ether as they are commercially available as a monodisperse liquid. Higher molecular weight PEG chains are available but are polydisperse, this polydispersity is then conferred on any dendrimer-NEI conjugate produced. Longer PEG chains would also have steric effects which may impede attachment of the inhibitor to the dendrimer and may reduce inhibitor activity by preventing access to the active site of NE. It was decided to attach the PEG chain to the leaving group section of the inhibitor as this was identified as being easier to modify than the inhibitor section and less likely to have detrimental effects on inhibitor potency.

Inhibitors **110** and **112** were identified as target compounds. They are both saccharine-based inhibitors which include a short PEG chain and have a Boc-protected amine which has the potential to be cleaved and converted to an isothiocyanate for attachment to an amine-surfaced PAMAM dendrimer.



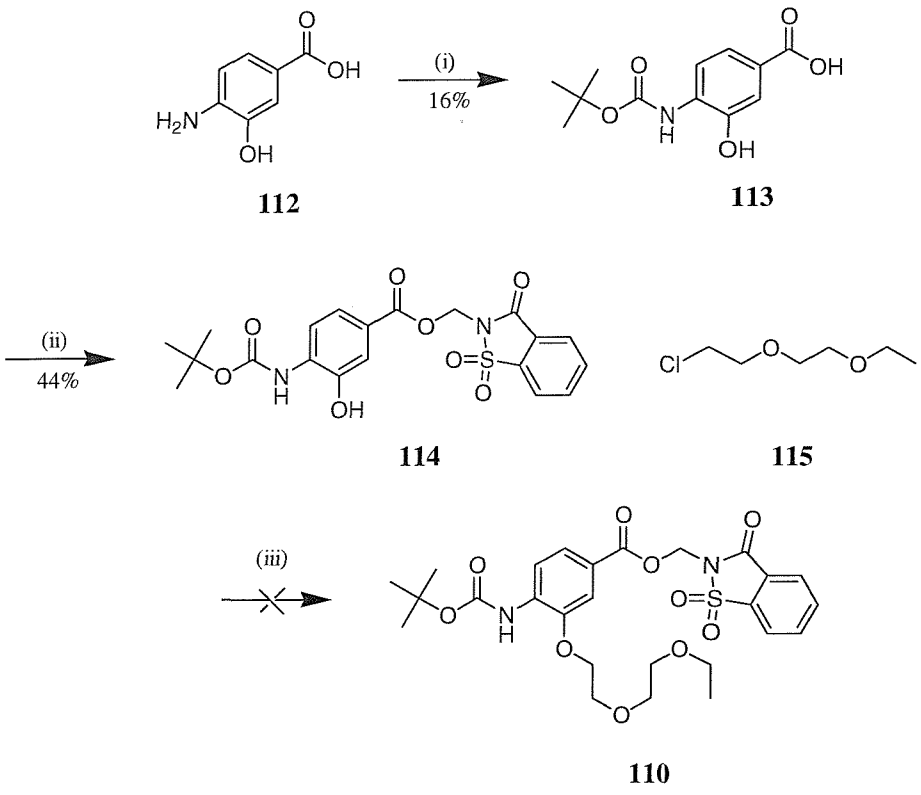
110



112

The initial strategy for the preparation of **110** was to first Boc-protect 2-hydroxy, 4-amino benzoic acid (**112**) to give **113** which could then be coupled to a saccharine inhibitor unit **74** using $KHCO_3$ and NaI in DMF to give **114**. The attempted coupling

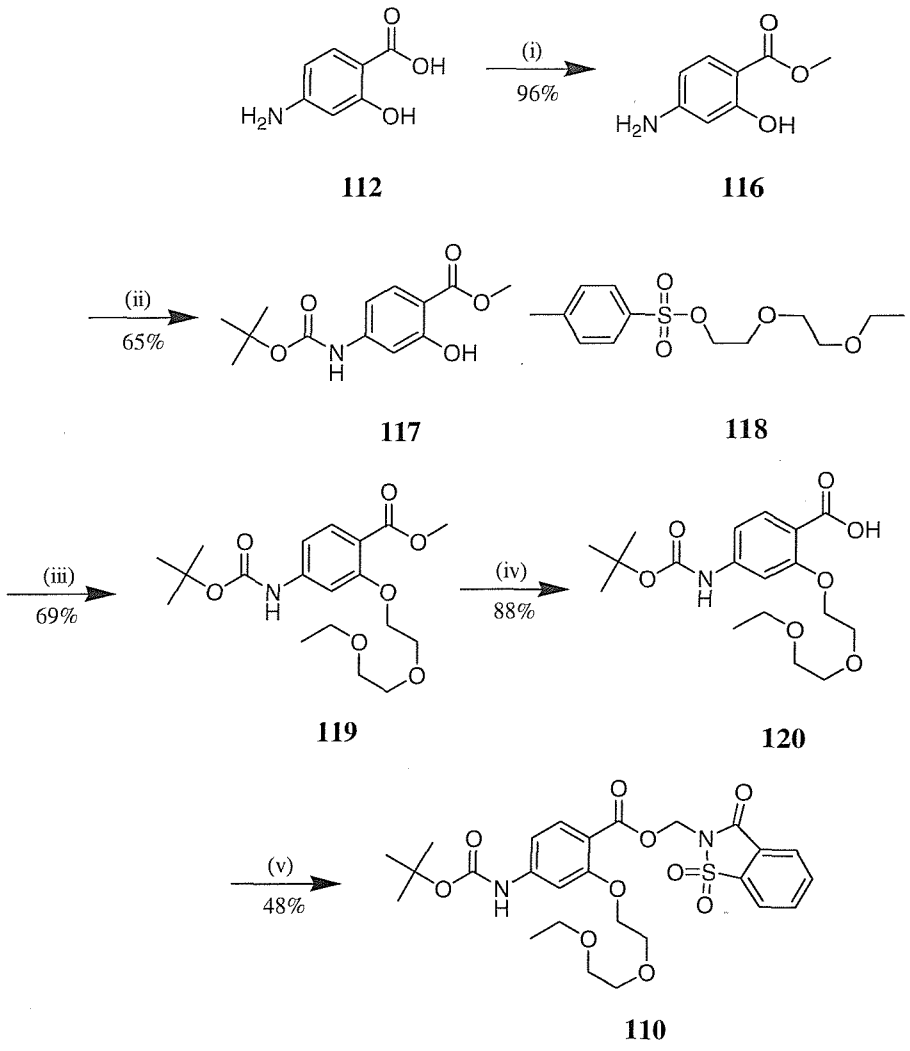
of **114** with a short PEG chain **115** to produce **110** via a nucleophilic substitution by heating in DMF with K_2CO_3 was unsuccessful as decomposition occurred, (Scheme 36).



112 **113** **114** **115** **110**

Scheme 36. (i) di-*tert*-butyl dicarbonate, Et_3N, THF, overnight. (ii) **74**, $KHCO_3$, NaI, DMF, overnight. (iii) **115**, K_2CO_3, NaI, DMF, 70 °C, 2 days.

An alternative route was proposed as shown in Scheme 37, whereby 2-hydroxy-4-amino benzoic acid (**112**), was first converted to the methyl ester **116** by refluxing for four hours in MeOH with H_2SO_4. The amino group of **116** was then Boc-protected to give **117**. This could then be coupled to **118** via a nucleophilic substitution by heating in DMF with K_2CO_3 and NaI to give **119**. **118** was prepared separately from 2-(2-ethoxy-ethoxy)-ethanol by reacting it with *p*-toluene sulphonyl chloride with Et_3N in THF overnight. The methyl ester of **119** was then hydrolysed to the acid **120** using NaOH in H_2O and Et_2O. This can was then coupled to saccharine unit **74** by stirring overnight in DMF with K_2CO_3 and NaI to give **110**.



112 **116**

117 **118**

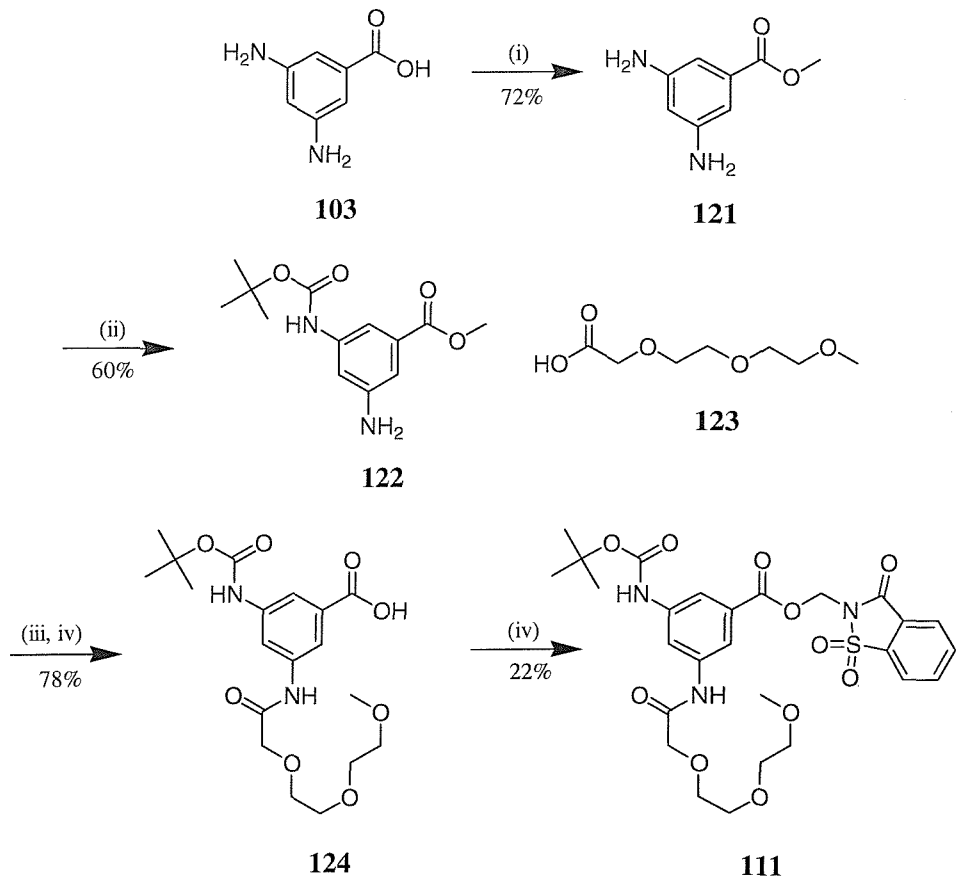
119 **120**

110

Scheme 37. (i) MeOH, H_2SO_4, reflux, 4 hours. (ii) di-*tert*-butyl dicarbonate, THF, overnight (iii) **118**, K_2CO_3, NaI, DMF 70 °C, overnight. (iv) NaOH, H_2O, 60 °C, 30 minutes. (v) **74**, K_2CO_3, NaI, DMF, overnight.

Inhibitor **111** (Scheme 38), was prepared by first converting 3,5-diamino benzoic acid (**103**) to the methyl ester **121** by reflux in MeOH and H_2SO_4 for 4 hours. **121** was mono-Boc-protected by stirring with one equivalent of di-*tert*-butyl dicarbonate in THF overnight to give **122**. PEG chain **123** was then coupled to the amine of **122** by a DCC-mediated coupling. The crude product from this reaction was hydrolysed by heating at 60 °C for 1 hour in 1:1 mixture of MeOH and H_2O with KOH. Any urea by product from the DCC coupling reaction was easily removed from the aqueous reaction mixture by filtration before acidification with 2M HCl allowed **124**

to be extracted into an organic solvent. **74** was then coupled to **124** by stirring them both together in DMF overnight with K_2CO_3 and NaI to give **111**.



103 **121** **122** **123** **124** **111**

Scheme 38. (i) MeOH, H_2SO_4, reflux, 4 hours. (ii) di-*tert*-butyl dicarbonate, THF, overnight. (iii) **123**, CH_2Cl_2, DCC, 1 hour. (iv) MeOH/H_2O, KOH, 60 °C, 1 Hour. (v) **74**, K_2CO_3, NaI, DMF, overnight.

Inhibitors **110** and **111** were tested with purified NE to see their effect on NE activity, (Figure 40). **110** was shown to be the most effective inhibitor. When the ratio of the concentration of inhibitor to the concentration of NE was 500:1 the residual NE activity with **110** was 47.9% compared to 89.0 % for **111**. At a ratio of 2000:1 the NE activity with **110** was 38.2% compared to 54.0% for **111**.

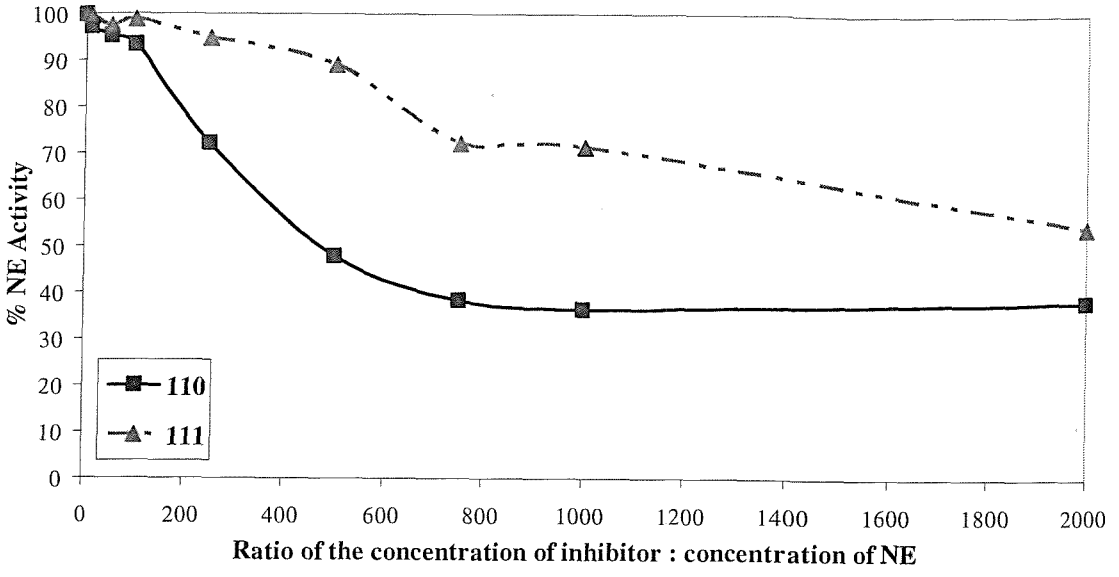
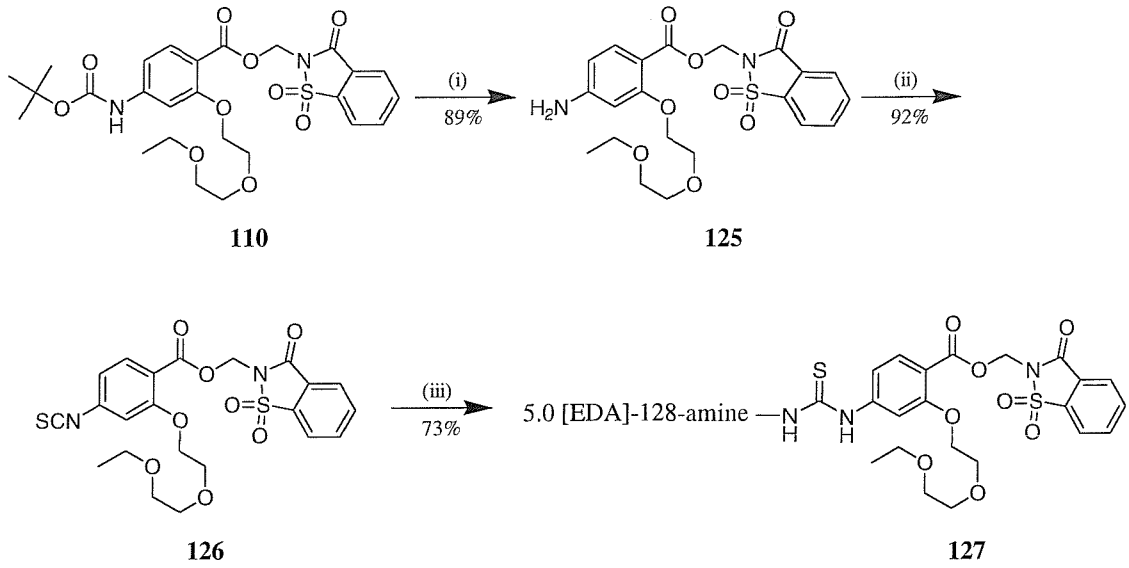


Figure 40. Plot comparing the reduction in NE activity as the concentration of **110** and **111** is increased.

Out of the two inhibitors **110** was judged to be the best candidate for further development for attachment to a PAMAM dendrimer, (Scheme 39). The amino group of **110** was deprotected by stirring in CH_2Cl_2 for 1 hour with trifluoroacetic acid to give **125**. The amino group could then be converted to an isothiocyante by slow addition of **125** to a solution of thiophosgene in CH_2Cl_2 with Na_2CO_3 at 0 °C to give **126**. The dendrimer-NEI conjugate **127** was prepared by heating 5.0 [EDA]-128-amine at 40 °C overnight in a 1:1 mixture of $CHCl_3$ and DMSO with over 150 equivalents of **126**. **127** was precipitated from the reaction mixture upon addition of Et_2O and could then be collected by filtration in the form of an off-white solid.



Scheme 39. (i) CF_3COOH, CH_2Cl_2, 1 hour. (ii) CH_2Cl_2, $CSCl_2$, Na_2CO_3, 0 °C, overnight. (iii) 5.0 [EDA]-128-amine, DMSO, $CHCl_3$, 40 °C, overnight.

The IR and both ^1H and ^{13}C NMR spectra of **127** indicated the presence of inhibitor groups, but it was not possible to ascertain from these techniques the extent to which the surface amine sites had been filled by inhibitor molecules. A measurement of the mass was unable to be obtained using MALDI mass spectrometry. The percentage of sulphur found by elemental analysis of **127** was reasonably close to the theoretical value so it was assumed that most, if not all of the surface dendrimer sites were filled with inhibitor molecules. **127** was tested to see its effectiveness at inhibiting NE in whole blood, the results were compared with those obtained for dendrimer-NEI inhibitor conjugate **93**, (Figure 41).

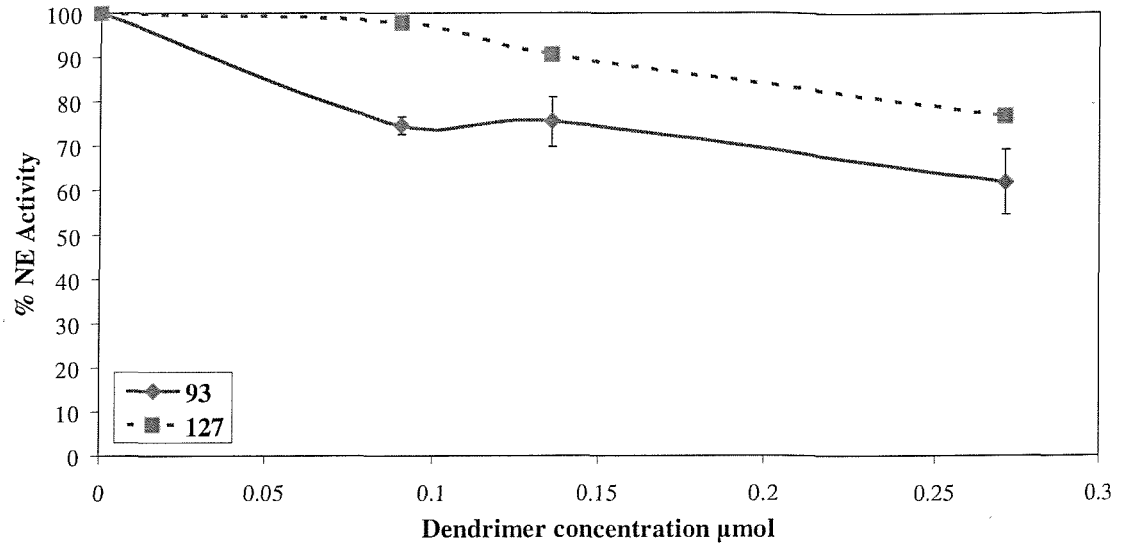


Figure 41. Plot comparing the decrease in NE activity in whole blood as the concentration of **93** and **127** is increased.

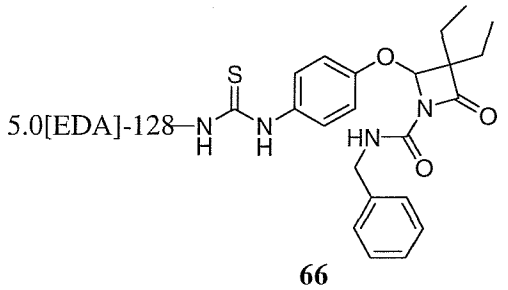
The addition of a short PEG chain did not significantly increase the solubility of **127** in whole blood and as with **93** it was not possible to increase the concentration of **127** past 0.271 µmol. At the highest concentration tested **93** was shown to be the better inhibitor of intracellular elastase as the NE activity was reduced to 62.0% compared with 77.0% with **127**.

3. Conclusions and future work

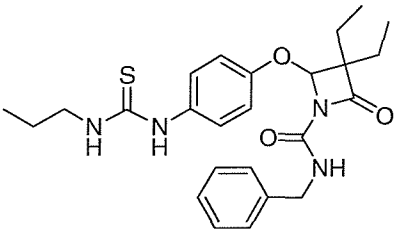
The work outlined in this thesis has demonstrated that with further development a dendrimer-NEI conjugate has potential to offer an effective treatment for CF.

It has been shown that in a blood sample neutrophils selectively uptake 4th and 5th generation PAMAM dendrimers regardless of surface functionality. The precise method of dendrimer recognition and uptake by neutrophils is currently unknown. Although neutrophils primarily recognise oponised pathogens, sometimes it is because they recognise the surface properties as being unusual (compared to the host tissues)[114] and this may be the mechanism by which neutrophils choose to absorb dendrimers. Although it has been observed that neutrophils are the only white blood cell to absorb the dendrimer-NEI conjugate, further experiments need to be undertaken to see if there is uptake by other tissues.

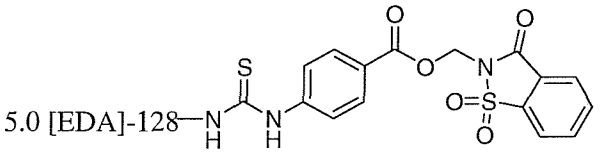
The dendrimer-NEI conjugates **66** and **93** both inhibited purified NE more effectively than the model NEIs **67** and **95**. It is thought that the reason potency is increased is because when bound to the dendrimer the NEIs are presented to the NE active site in a more favorable way.



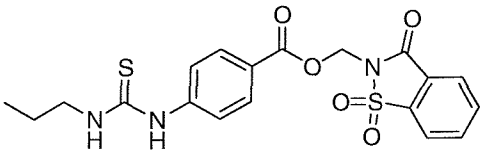
66



67



93



95

Further work on dendrimer-NEI conjugates needs to focus on three main areas; namely potency, enzyme specificity and solubility.

All of the saccharine-based NEIs prepared have used non-functionalised saccharine units. Future work towards improving enzyme specificity and potency could involve creating dendrimer-NEI conjugates in which the aryl ring of the saccharine is optimised for recognition and potency with NE as well as *in vivo* stability. This would be achieved incorporating isopropyl and methoxy groups[194] in the 4 and 6 positions respectively, (Figure 42).

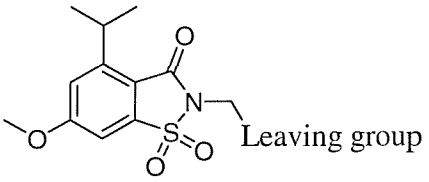


Figure 42. Saccharine unit optimised for NE recognition and potency

Future work could also include using NEIs based on a 1,2,5-thiadiazolidin-3-one 1,1-dioxide scaffold[195-198]. The literature reports an inhibitor unit which has been optimised for recognition and potency with NE, (Figure 43).

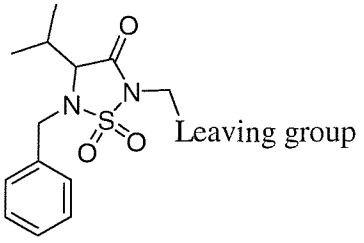
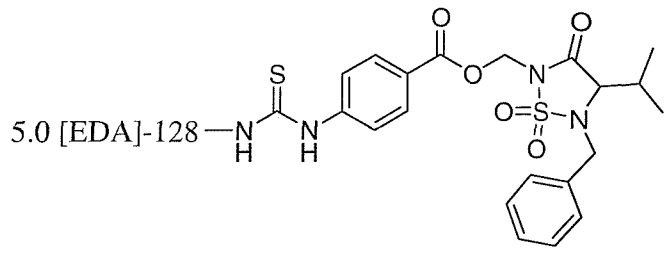


Figure 43. 1,2,5-thiadiazolidin-3-one 1,1-dioxide unit optimised for NE recognition and potency

The mechanism of inhibition is the same as that proposed for the saccharine-based NEIs[174, 175]. Attack by the Ser 195 stimulates loss of a leaving group creating an imine which binds to His 57 leading to enzyme inhibition. As with the saccharine-

based NEIs, benzoic acid makes an effective leaving group and the work outlined in this thesis could be directly transferred into attaching this type of NEI to the surface of a PAMAM dendrimer. A dendrimer-NEI conjugate such as **128** could be prepared using similar methodology used for **93**.



128

The solubility of β-lactam-based dendrimer-NEI conjugate **66** was too low to test its potency at inhibiting intracellular NE in whole blood and saccharine based dendrimer-NEI conjugates **92** and **127** could only be evaluated at low concentrations. For a dendrimer-NEI conjugate to be used as an effective drug it needs to have a high degree of solubility in the blood. The incorporation of a short PEG chain in **127** did little to improve solubility. However longer PEG chains could be incorporated using similar methodology, but to greater effect. This work has shown that it is possible to incorporate other functionality into the benzoic acid leaving group, the inclusion of water solubilising groups such as amines or carboxylic acids may also increase solubility in the blood. Increasing the solubility of the β-lactam based dendrimer-NEI conjugates may be achieved by including water solubilising groups into the phenolic leaving group or the electron withdrawing group, (Figure 26).

The original intention of creating a dendrimer-NEI conjugate was to reduce the amount of extracellular NE present in the lungs of a CF patient. This work shows that dendrimer-NEI conjugates could be used in a far more effective way. It has been shown that they have potential to inhibit intracellular NE whilst the neutrophils are still in the blood. This means that if delivered systemically then they could significantly reduce the NE payload of the neutrophils before their recruitment to the

lungs. This means the levels of NE in the lungs of a CF patient would be reduced which would lead to a reduction in the extent of NE mediated damage to the lung. This would therefore both increase the life expectancy and quality of life of a CF patient.

The ability to inhibit intracellular NE whilst the neutrophils are still in the blood also means that dendrimer-NEI conjugates have the potential to not only be used to treat CF but other diseases where NE-mediated damage is a factor, for example, rheumatoid arthritis, pulmonary emphysema and chronic bronchitis[199-201].

4. Experimental

4.1.1 General experimental for the NE inhibition studies

All of the NE studies were performed at the University of Portsmouth, School of Pharmacy and Biomedical Sciences by Sarah L. Clarke under the supervision of Dr Janis K. Shute.

4.1.2 Neutrophil isolation

Blood from healthy adult volunteers (~6mL) was collected in heparinised tubes and mixed with Macrodex™ in a 2:1 ratio at room temperature for 45 minutes, to allow red blood cell aggregation. The red cells were discarded and the remaining plasma layer (~5 mL) was transferred to a plastic centrifuge tube and an equal volume of Lymphoprep™ (Nycomed) was layered underneath using a long necked Pasteur pipette. Tubes were centrifuged for 30 minutes at 1500 rpm (450 g) at 20 °C. The supernatant containing mononuclear cells was carefully aspirated and discarded, leaving the neutrophil pellet. The cell pellet was re-suspended in 0.2 % NaCl (5 mL) for 45 seconds, followed by 1.6 % NaCl (5 mL) resulting in 0.9 % solution to lyse remaining red blood cells. Neutrophils were centrifuged at 2000 rpm (800g) for 10 minutes at 4 °C. Red cell lysis was repeated, by re-suspending pellet in 0.2 % NaCl (5 mL) for 30 seconds, before the addition of 1.6% NaCl (5 mL), Neutrophils were pelleted at 1000 rpm (200g) for 5 minutes. The final neutrophil pellet was re-suspended in 1mL of appropriate buffer and cell counts performed using a haemocytometer slide. Cell viability was shown to be >95% using Trypan Blue exclusion.

4.1.3 NE activity in whole blood

Blood from healthy adult volunteers (6 mL) was collected in heparinised tubes and treated with a neutrophil elastase inhibitor and a suitable control. Treated blood was incubated at 37 °C for 2 hours. Neutrophils were isolated and re-suspended in 1mL

of neutrophil elastase assay buffer (0.3 M Tris-HCl, pH 8.0). 100 µL of each cell suspension (5 x 10^6/mL) was lysed for 10 minutes with Triton-X-100 (2 %). 10 µL of cell suspension (lysates of 2.5 x 10^4 cells) was added in triplicate to a 96-well plate, and samples warmed to 37° C for 1 minute. 90 µL of 0.555mM neutrophil elastase substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (AAPVpNA) prepared in neutrophil elastase assay buffer was added to each well. Optical density at 405nm was measured at 5-minute intervals for 5 hours. The change in optical density was measured between 50 and 80 minutes, within this range it was consistently linear. The optical density of the treatment groups was taken as a percentage of that observed from the control/no treatment group.

4.1.4 Fluorescent confocal microscopy

Blood from healthy adult volunteers (6 mL) was collected in heparinised tubes and treated with FITC tagged dendrimers. The treated blood was incubated at 37 °C for 2 hours. Neutrophils were isolated and diluted to 10^6cells/ml. Cytospins (1500rpm (250g), 5 minutes) were produced using cell suspensions (100µL), and mounting medium and cover slips applied. Slides were analysed using fluorescent confocal microscopy (Zeiss LSM 510 META, Hertfordshire, UK), (excitation 488nm, emission 543).

4.1.5 Isolated NE activity assay

Purified human neutrophil elastase (50 µL containing 10 munits) was incubated in 96 well plates with the inhibitor, over a range of molar ratios (2000-10:1, inhibitor to neutrophil elastase), for 2 minutes at 37 °C. A control group with no inhibitor was also included. Neutrophil elastase inhibitors were initially diluted in DMSO, and adjusted to the required molarity with 0.3 M Tris 0.15 M NaCl, pH 8.0 buffer. The substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (AAPVpNA), (50µL of 1.25 mmol prepared in 0.3 M Tris 0.15 M NaCl, pH 8.0 buffer) was added to all wells, and the optical density measured at 410 nm for 10 minutes at 37 °C. Inhibition of neutrophil elastase activity was expressed as a percentage of the enzyme activity

in the absence of inhibitor. A standard curve of DMSO against neutrophil elastase activity was also produced.

4.1.6 Materials

Tris (methoxymethyl) methylamine, sodium chloride, 96-well plates were obtained from Fisher Scientific, Loughborough, UK.
N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide, dimethyl sulfoxide, purified elastase from human leukocytes, triton-X-100, trypan blue were obtained from Sigma-Aldrich Co., Poole, UK.
Heparinised tubes were obtained from Griener Bio-one Ltd., Stonehouse, UK.
Phosphate buffered saline (PBS) (with calcium and magnesium), Hanks' balanced salt solution (HBSS) (with calcium and magnesium) were obtained from Invitrogen Ltd, Paisley UK.
Lymphoprep™ (Nycomed) were obtained from Axis-Shield Diagnostics Ltd., Huntingdon, UK.
6% w/v dextran 70 was obtained from Baxter Healthcare, Thetford UK.

4.2.1 Instrumentation

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker DPX 400 or a Bruker Avance 300 spectrometer. Chemical shifts (δ) are quoted relative to residual solvent peaks.

Infrared spectra were obtained using a golden gate sampling attachment on a Nicolet 380 IR spectrometer FTIR instrument or between KBr discs on a Nicolet 400 Infrared spectrometer.

Masses are quoted as the lowest isotopic mass (for instance ^{35}Cl or ^{79}Br if these elements are involved). For compounds that were soluble in acetonitrile, electrospray mass spectra were recorded on a Micromass Platform II single quadrupole spectrometer. For compounds soluble in methanol, electrospray mass

spectra were recorded on a Micromass ZMD spectrometer. High resolution mass spectrometry was carried out on a Bruker Apex III FT-ICR spectrometer.

Elemental analyses were performed by Medac Ltd of Brunel Science Centre, Coopers Hill Lane, Englefield, Egham, Surrey, TW20 0JZ.

Melting points were measured on an Electrothermal apparatus and are uncorrected.

Thin layer chromatography was carried out on aluminium-backed Merck Si 60/F_{254} sheets.

4.2.2 Chemicals

All chemicals were used as supplied by Aldrich Chemical Company, Lancaster or Avacado.

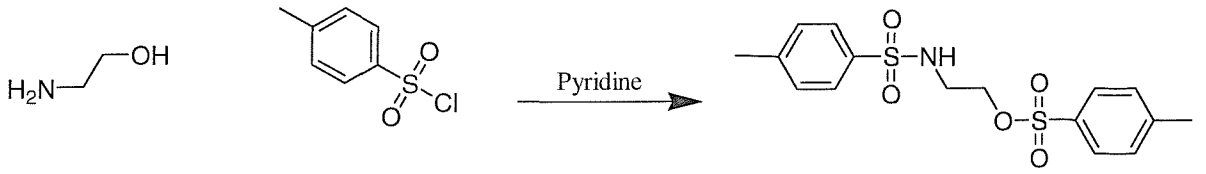
4.2.3 Solvent purification

Where necessary solvents were purified and dried according to known literature procedures[202] and wherever possible, solvents were distilled immediately before use.

4.2.4 Chemical names

The names of all the chemicals produced in this report were predicted by AutoNom Version 2.1 under license from Informationsysteme GmBH copyright © 1988-1998, Beilstien Institut Fuer Lituratur der Organischen Chemie liscensed to Beilstein Chemiedatun und software GmBH and Beilstein Informationsysteme GmBH. All Rights Reserved.

4.3.1 Preparation of 2-(toluene-4-sulfonylamino)-ethyl toluene-4-sulfonate (24)



M.W. = 61.1 g Mol^{-1} M.W. = 190.7 g Mol^{-1} M.W. = 369.5 g Mol^{-1}

C_2H_7NO $C_7H_7ClO_2S$ $C_{16}H_{19}NO_5S_2$

23 **24**

Using a method by Chandrasekhar *et al*[203], *p*-toluene sulphonyl chloride (34.50 g, 0.18 mol) was suspended in pyridine (40 mL) and cooled to -15 °C. A solution of ethanolamine (**23**) (5.00 g, 81.2 mmol, 4.9 mL) in pyridine (20 mL) was cooled to -15 °C and added drop-wise over 20 minutes. The solution was then kept cold and stirred for 5 hours under nitrogen; pyridine hydrochloride precipitated, this was filtered off and the reaction mixture was then concentrated *in vacuo*. Any remaining pyridine was removed by azeotropic distillation with toluene (3 x 50 mL). The residue was purified by column chromatography (5% MeOH/95% CH_2Cl_2, Silica 60) followed by crystallisation (EtOH/H_2O) to give **24** as white crystals (4.69 g, 12.7 mmol, 38%), (R_f = 0.68 (5% MeOH/95% CH_2Cl_2)), (m.p. 83-85 °C (EtOH/H_2O), (Lit.[203] 86 °C)).

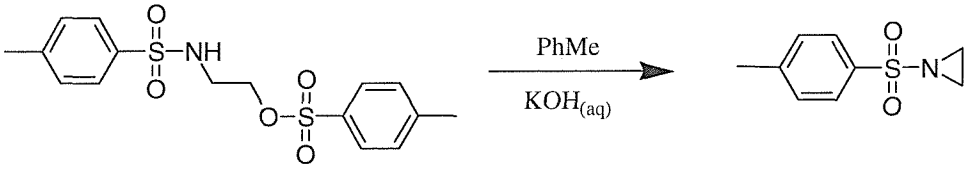
^{1}H NMR (300 MHz, $CDCl_3$): 7.79 (d, 4H, *J* = 8.5 Hz, $SO_2Ar\underline{H}$), 7.34 (d, 4H, *J* = 8.5 Hz, $CH_3Ar\underline{H}$), 3.52 (t, 2H, *J* = 6.0 Hz, $OC\underline{H}_2$), 3.32 (t, 2H, *J* = 6.0 Hz, $NHC\underline{H}_2$), 2.47 (s, 3H, $C\underline{H}_3Ar$) ppm.

^{13}C NMR + DEPT (75 MHz, $CDCl_3$): δ 143.8, 136.9 (<u>Ar</u>), 129.9, 127.0 (<u>Ar</u>H), 44.7 ($O\underline{C}H_2$), 43.6 ($N\underline{C}H_2$), 21.5 ($Ar\underline{C}H_3$) ppm.

IR (neat) ν_{max}: 3272 (s), 3033 (w), 2962 (w), 2978 (w), 1598 (w), 1492 (w), 1449 (w), 1426 (m), 1314 (s, SO_2-O), 1301 (s), 1288 (s), 1151 (s, SO_2-O), 1088 (s), 1031 (m), 912 (m), 808 (s), 786 (m) cm^{-1}.

LRMS (ES^+): *m/z* 392.3 ($[M+Na]^+$, 100%, 393.3, 10%).

4.3.2 Preparation of 1-(toluene-4-sulfonyl)-aziridine (25)



M.W. = 369.5 g Mol^{-1}
$C_{16}H_{19}NO_5S_2$
24

M.W. = 197.3 g Mol^{-1}
$C_9H_{11}NO_2S$
25

This was prepared using a method by Martin *et al*[204], A solution of KOH (20 mL, 20% w/v) was added to a stirred solution of **24** (2.14 g, 5.8 mmol) in toluene (60 mL). The reaction was stirred at room temperature overnight then concentrated *in vacuo* and the product was partitioned between CH_2Cl_2 (50 mL) and H_2O (50 mL). The organic layer was isolated, dried (anhydrous $MgSO_4$) and concentrated *in vacuo* to give **25** in the form of a white crystalline solid (1.06 g, 5.4 mmol, 93%), (R_f = 0.74 (5% MeOH/95% CH_2Cl_2)), (m.p. 50-52 °C (Lit.[205] 52-53 °C (EtOH))).

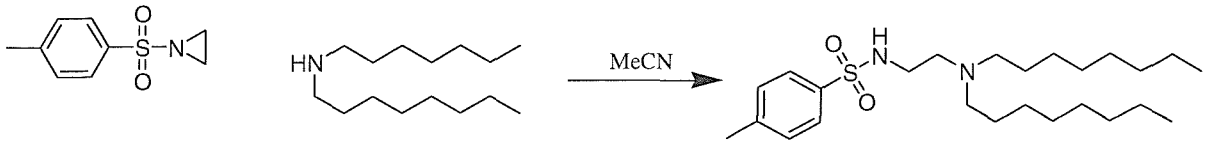
^{1}H NMR (400MHz, $CDCl_3$): δ 7.76 (d, 2H, *J* = 8.3 Hz, $CH_3Ar\underline{H}$), 7.28 (d, 2H, *J* = 8.3 Hz, $SO_2Ar\underline{H}$), 2.38 (s, 3H, $ArC\underline{H}_3$), 2.30 (s, 4H, $NC\underline{H}_2$) ppm.

^{13}C NMR + DEPT (100MHz, $CDCl_3$): δ 144.6, 134.9 (**Ar**), 129.7, 128.0 (**Ar**H), 25.9, 27.4 ($Ar\underline{C}H_3$), 21.6 ($N\underline{C}H_2$) ppm.

IR (neat) ν_{max}: 1595 (w), 1453 (w), 1318 (s, SO_2-N), 1304 (s), 1293 (m), 1234 (m), 1154 (s, SO_2-N), 1084 (m), 895 (s), 811 (s) cm^{-1}.

LRMS (ES^+): *m/z* 210.3 ($[M+Na]^+$, 100%, 211.3, 10%).

4.3.3 Preparation of *N*-(2-dioctylamino-ethyl)-4-methyl-benzenesulfonamide (26)



M.W. = 197.3 g Mol^{-1} M.W. = 241.5 g Mol^{-1} M.W. = 438.7 g Mol^{-1}

$C_9H_{11}NO_2S$ $C_{16}H_{34}N$ $C_{25}H_{46}N_2O_2S$

25 **26**

25 (0.78 g, 4.0 mmol) was dissolved in MeCN (20 mL) and added drop-wise to di-*n*-octylamine (0.91 g, 3.8 mmol, 1.1 mL) in MeCN (40 mL) under nitrogen. The reaction mixture was refluxed overnight and then concentrated *in vacuo*. The residue was purified by column chromatography (5% MeOH/95% CH_2Cl_2, Silica 60) to give **26** as a yellow oil (1.11 g, 2.5 mmol, 67%), (R_f = 0.35 (5% MeOH/95% CH_2Cl_2)), (b.p.154 °C at 0.1 mmHg).

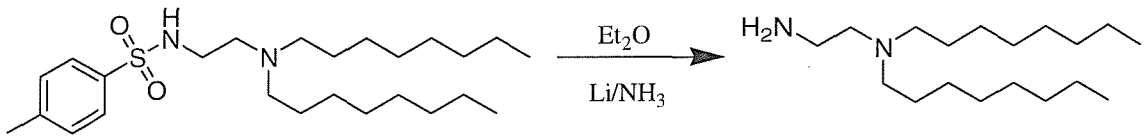
^{1}H NMR (300MHz, $CDCl_3$): δ 7.75 (d, 2H, *J* = 8.4 Hz, CH_3ArH), 7.29 (d, 2H, *J* = 8.4 Hz, SO_2ArH), 2.93 (t, 2H, *J* = 6.0 Hz, $NHCH_2$), 2.45 (t, 2H, *J* = 6.0 Hz, $NHCH_2CH_2$), 2.43 (s, 3H, $ArCH_3$), 2.23 (t, 4H, *J* = 7.5 NCH_2), 1.1-1.4 (m, 24H, *n*-CH_2), 0.90 (t, 6H, *J* = 6.9 Hz, *n*-CH_2CH_3) ppm.

^{13}C NMR + DEPT (75MHz, $CDCl_3$): δ 143.2, 136.8 (Ar), 129.6, 127.2 (ArH), 53.6, 52.2 (NCH_2), 40.3 ($NHCH_2$) 31.9, 29.5, 29.3, 27.4, 26.9, 22.6 (*n*-CH_2), 21.5 ($ArCH_3$), 14.5 (*n*-CH_2CH_3) ppm.

IR (film) ν_{max}: 2925 (s), 2855 (m), 1787 (w), 1588 (w), 1465 (w) 1330 (s, SO_2-N), 1162 (s, SO_2-N) 1093 (w) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{25}H_{47}N_2O_2S$ $(M+H)^+$ 439.3352, found 439.3357.

4.3.4 Preparation of 2(amino) ethyl di-*n*-octylamine (27)



M.W. = 438.7 g Mol^{-1}
$C_{25}H_{46}N_2O_2S$
26

M.W. = 284.5 g Mol^{-1}
$C_{18}H_{40}N_2$
27

Excess NH_3 (approx. 5.0 g, 0.29 mol) was condensed into Et_2O (20 mL) at -78 °C, **26** (0.83 g, 1.9 mmol) was then added. Lithium (0.30 g, 43.2 mmol) was added slowly and the reaction was stirred for 30 minutes. NH_4Cl (2.30 g, 43.2 mmol) was then added to quench the reaction. The reaction mixture changed from a pale yellow colour to white. The remaining ammonia was allowed to evaporate out of the reaction mixture overnight. The remaining white solid was dissolved in CH_2Cl_2 and the LiCl was removed by filtration. The filtrate was concentrated *in vacuo* to give a yellow oil. Purification by distillation (b.p. 109 °C at 0.1 mmHg) gave **27** as a colourless oil (0.38 g, 1.3 mmol, 70%), (R_f = 0.57 (20% MeOH/80% CH_2Cl_2)), (b.p. 109 °C at 0.1 mmHg).

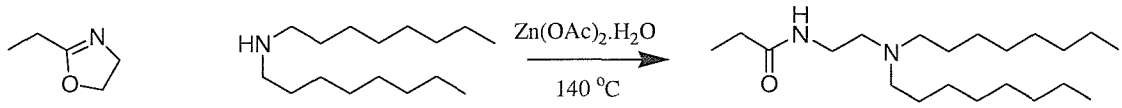
^{1}H NMR (300MHz, $CDCl_3$): δ 3.2-3.3 (br, 2H, $N\underline{H}_2$), 2.72 (t, 2H, J = 6.0 Hz, $NH_2C\underline{H}_2$), 2.39 (t, 2H, J = 6.0 Hz, $NH_2CH_2C\underline{H}_2$), 2.36 (t, 4H, J = 7.5 Hz, $NC\underline{H}_2$), 1.2–1.4 (m, 24H, *n*-$C\underline{H}_2$), 0.89 (t, 6H, J = 6.5 Hz, *n*-$CH_2C\underline{H}_3$) ppm.

^{13}C NMR + DEPT (75MHz, $CDCl_3$): δ 55.9, 54.2 ($N(\underline{C}H_2)$), 39.2 ($H_2N\underline{C}H_2$), 31.8, 29.5, 29.3, 27.5, 27.0, 22.6 (*n*-$\underline{C}H_2$), 14.0 (*n*-$\underline{C}H_3$) ppm.

IR (film) ν_{max}: 2957 (m), 2928 (m), 2854 (m), 1467 (w), 1378 (w), 1093 (w) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{18}H_{41}N_2$ 285.3264 $(M+H)^+$, found 285.3264.

4.3.5 Preparation of *N*-(2-dioctylamino)-ethyl propionamide (29)



M.W. = 99.13 g Mol^{-1}
C_5H_9NO
28

M.W. = 241.5 g Mol^{-1}
$C_{16}H_{34}N$

M.W. = 340.6 g Mol^{-1}
$C_{21}H_{44}N_2O$
29

Di-*n*-octylamine (3.50 g, 14.5 mmol, 4.4 mL) and zinc acetate dihydrate (0.45g 0.2 mmol) was heated at 140 °C in 2-ethyloxazoline (**28**) (5.90 g, 59.5 mmol, 6.0 mL) for ten days. The reaction mixture turned black upon heating. The excess 2-ethyloxazoline was removed under vacuum. Purification of the residue by column chromatography (5% MeOH/95% CH_2Cl_2, Silica 60) gave **29** as a yellow oil (3.38 g, 9.9 mmol, 71%), (R_f = 0.54 (10% MeOH/90% CH_2Cl_2)), (b.p. 134 °C at 0.1 mmHg).

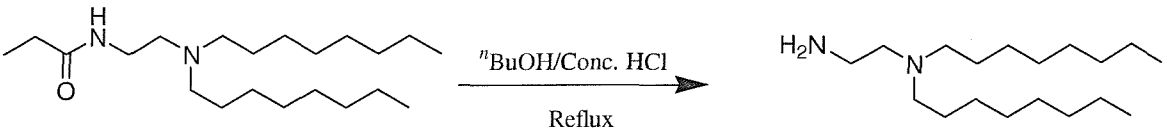
^{1}H NMR (300 MHz, $CDCl_3$): 3.27 (dt, 2H, J = 5.7 Hz, CONHC**H**$_2$), 2.50 (t, 2H, J = 5.7 Hz, CONHCH$_2$C**H**$_2$N), 2.38 (t, 4H, J = 7.5 Hz, NC**H**$_2$), 2.20 (q, 2H, J = 7.5 Hz, COC**H**$_2$), 1.2–1.5 (m, 24H, *n*-C**H**$_2$), 1.15 (t, 3H, J = 7.5 Hz, COCH$_2$C**H**$_3$), 0.88 (t, 6H, J = 6.5 Hz, *n*-CH$_2$C**H**$_3$) ppm.

^{13}C NMR + DEPT (75 MHz, $CDCl_3$): 173.6 (**C**ONH), 53.8, 52.6 (**C**H$_2$N), 36.8 (CONH**C**H$_2$), 31.8, 29.8, 29.6, 29.3, 27.5, 27.1, 22.6 (*n*-**C**H$_2$), 14.0, 9.9 (*n*-**C**H$_3$) ppm.

IR (film) ν_{max}: 2930 (s), 2855 (s), 2806 (w), 1697 (s) (CONH), 1552 (m), 1465 (m), 1377 (w) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{21}H_{45}N_2O$ 341.3526 $(M+H)^+$, found 341.3526.

4.3.6 Preparation of 2(amino) ethyl di-*n*-octylamine (27)



M.W. = 340.6 g Mol^{-1}
$C_{21}H_{44}N_2O$
29

M.W. = 284.5 g Mol^{-1}
$C_{18}H_{40}N_2$
27

29 (0.71 g, 2.1 mmol) was refluxed in conc. HCl (30mL) and *n*-BuOH (15mL) overnight. The reaction mixture turned from yellow to orange. The reaction mixture was cooled to room temperature and 2M NaOH was added until the solution was pH 14. The product was extracted with CH_2Cl_2 (3 x 50 mL). The organic phases were combined, washed with brine (100 mL), dried (anhydrous Mg_2SO_4) and concentrated *in vacuo*. Purification of the brown oil by distillation (109 °C at 0.1 mmHg) gave **27** in as a colourless oil (0.55 g, 1.9 mmol, 93%), (R_f = 0.57 (20% MeOH/80% CH_2Cl_2)), (109 °C at 0.1 mmHg).

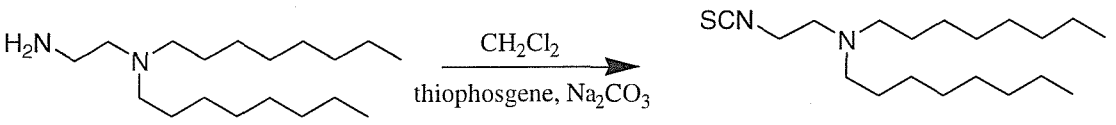
^{1}H NMR (300MHz, $CDCl_3$): 2.72 (t, 2H, *J* = 6.0 Hz, $NH_2C\underline{H}_2$), 2.39 (t, 2H, *J* = 6.0 Hz, $NH_2CH_2C\underline{H}_2$), 2.36 (t, 4H, *J* = 7.5 Hz, $NC\underline{H}_2$) 1.2–1.4 (m, 24H, *n*-$C\underline{H}_2$), 0.89 (t, 6H, *J* = 6.5 Hz, *n*-$CH_2C\underline{H}_3$) ppm.

^{13}C NMR + DEPT (75MHz, $CDCl_3$): δ 55.9, 54.2 (N($\underline{C}H_2$)), 39.2 ($H_2N\underline{C}H_2$), 31.8, 29.5, 29.3, 27.5, 27.0, 22.6 (*n*-$\underline{C}H_2$), 14.0 (*n*-$\underline{C}H_3$) ppm.

IR (film) ν_{max}: 2957 (m), 2928 (m), 2854 (m), 1467 (w), 1378 (w), 1093 (w) cm^{-1}.

LMRS (ES^+): *m/z* 285.4 ($[M+H]^+$, 100%, 286.5, 18%).

4.3.7 Preparation of (2-isothiocyanatoethyl)-di-*n*-octylamine (30)



M.W. = 284.5 g Mol^{-1}
$C_{18}H_{40}N_2$
27

M.W. = 326.6 g Mol^{-1}
$C_{19}H_{38}N_2S$
30

27 (2.70 g, 9.5 mmol) was dissolved in CH_2Cl_2 (50 mL) and added drop-wise to a solution of thiophosgene (2.18 g, 19.0 mmol, 1.5 mL) and Na_2CO_3 (19.8 mmol) in CH_2Cl_2 (50 mL) at 0 °C over 1 hour. The reaction was left stirring at 0 °C for another hour and then allowed to warm to room temperature overnight. H_2O was slowly added and the organic phase was separated, dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. The resulting brown oil was then purified using column chromatography (CH_2Cl_2, Silica 60) to give **30** as a yellow oil (1.24 g, 3.8 mmol, 78%), (R_f = 0.35 (CH_2Cl_2)), (b.p. 145 °C at 0.1 mmHg).

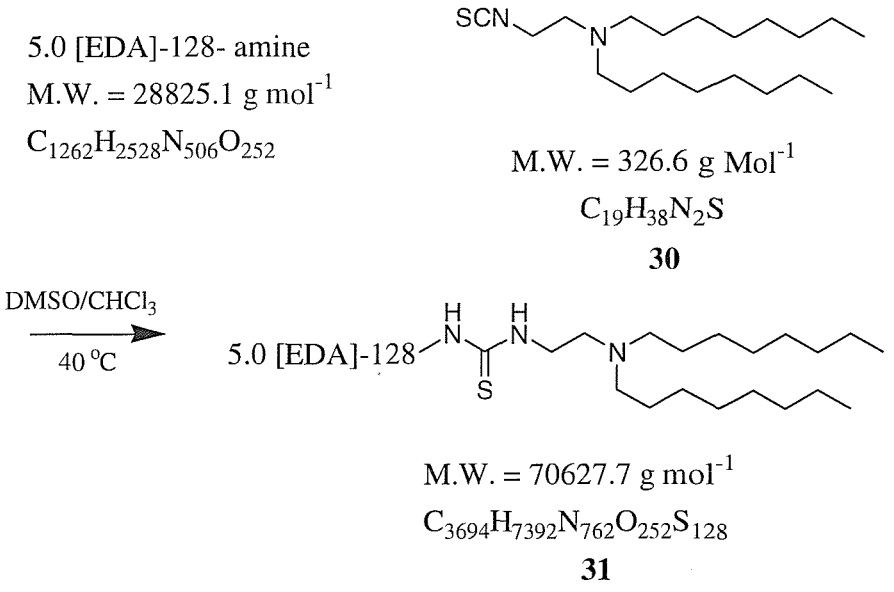
^{1}H NMR (300MHz, $CDCl_3$): δ 3.50 (t, 2H, *J* = 6.6 Hz, SCNC**H**$_2$), 2.74 (t, 2H, *J* = 6.6 Hz, SCNCH$_2$C**H**$_2$), 2.45 (t, 4H, *J* = 7.5, NC**H**$_2$), 1.2-1.4 (m, 24H, *n*-C**H**$_2$), 0.89 (t, 6H, *J* = 6.5 Hz, *n*-CH$_2$C**H**$_3$) ppm.

^{13}C NMR + DEPT (75MHz, $CDCl_3$): δ 54.5, 53.8 (N**C**H$_2$), 43.9 (SCN**C**H$_2$), 31.9, 29.6, 29.3, 27.4, 27.4, 22.7 (*n*-**C**H$_2$), 14.1 (*n*-CH$_2$**C**H$_3$) ppm.

IR (film) ν_{max}: 2928 (m), 2853 (m), 2095 (s, -N=C=S), 1510 (w), 1465 (w), 1377 (w), 1343 (w), 1299 (w), 1151 (w), 1096 (w) cm^{-1}

HRMS (ES^+): Calcd. for $C_{19}H_{39}N_2S$ 327.2828 $(M+H)^+$, found 327.2828.

4.3.8 Preparation of 5.0-[EDA]-128-[1-(2-dioctylamino-ethyl)-thiourea] (31)



5.0 [EDA]-128-amine (0.174 g, 6.0 x 10^{-6} mol) and **30** (0.35 g, 1.1 mmol) were stirred at 40 °C overnight in a mixture of DMSO (10 mL) and $CHCl_3$ (5 mL). Purification by dialysis against $CHCl_3$ (2 x 2.5 L) gave **31** as a yellow oil (0.456 g, 6.4 x 10^{-6}, 106%).

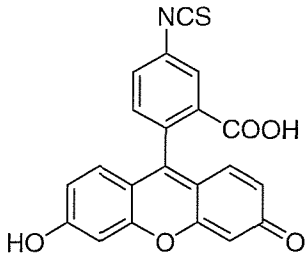
^{1}H NMR (400MHz, $CDCl_3$): δ 3.90-2.20 (m, *n*-C**H**$_2$), 3.55 (t, J = 6.0 Hz, CSNHC**H**$_2$), 2.59 (t, J = 6.0 Hz, CSNHCH$_2$C**H**$_2$), 2.41 (t, J = 7.0 Hz, NC**H**$_2$), 1.43-1.39 (m, *n*-C**H**$_2$), 1.20-1.30 (m, *n*-C**H**$_2$), 0.85-0.89 (m, *n*-C**H**$_3$) ppm.

^{13}C NMR + DEPT (75MHz, $CDCl_3$): δ 182.2 (**C**S), 173.6, 172.9 (**C**O), 54.8, 54.1, 44.2, 32.0, 29.7, 29.5, 29.4, 27.7, 27.6, 27.5, 22.8 (**C**H$_2$), 14.2, 14.1 (**C**H$_3$) ppm.

IR (neat) ν_{max}: 2923 (s), 2853 (s), 1643 (s), 1547 (s, CS-NH), 1464 (m), 1376 (m), 1342 (m), 1152 (s, CS), 1093 (m), 1026 (m), 952 (w), 756 (m), 721 (m) cm^{-1}.

Anal. Calcd. for $C_{3694}H_{7392}N_{762}O_{252}S_{128}$: C, 62.82; H, 10.55; N, 15.11; S, 5.81 Found: C, 63.62; H, 10.68; N, 14.80; S, 6.39.

4.4.1 Preparation of 4.0-[N]-62-[amine]-2-[fluorescein thiourea] (33)



M.W. = 389.4 g Mol^{-1}

$C_{18}H_{11}NO_4S$

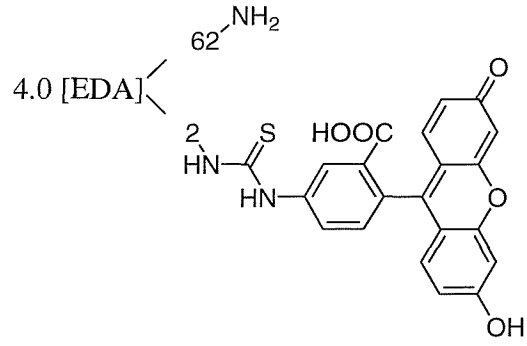
4.0 [EDA]-64-amine

M.W. = 14214.2 g Mol^{-1}

$C_{622}H_{1248}N_{250}O_{124}$

$CHCl_3$/DMSO

40 °C



M.W. = 14603.3 g Mol^{-1}

$C_{643}H_{1259}N_{251}O_{129}S$

33

Fluorescein isothiocyanate (6.3 mg, 1.6 x 10^{-5} mol) was dissolved in $CHCl_3$ (5 mL) and added to a solution of 4.0 [N]-64-amine (115 mg, 8.1 x 10^{-6} mol) in DMSO (5 mL). The reaction mixture was stirred at 40 °C overnight and then dialysed first against H_2O (2.5 L) and then MeOH (2.5 L). The remaining MeOH was concentrated *in vacuo* to give **33** as an orange oil (71 mg, 4.9 x 10^{-6} mol, 60%).

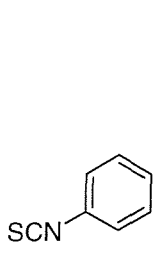
^{1}H NMR (300 MHz, CD_3OD): δ 3.6-3.4 (m), 3.35-3.10 (m), 3.05-2.95 (m), 2.85-2.20 (m), 1.80-1.50 (m), 1.35-1.25 (m), 0.90-0.80 (m) ppm.

^{13}C NMR + DEPT 135 (75 MHz, CD_3OD): δ 175.3, 174.7 (**C**O), 65.8, 65.2, 64.1, 53.5, 51.3, 49.9, 44.3, 40.5, 40.2, 38.8, 35.0, 32.5, 30.8, 27.2, 23.6, 21.0, 14.5 (**C**H_2) ppm.

IR (neat) ν_{max}: 3269 (m), 3067 (w), 2930 (w), 2870 (w), 1648 (s, C=O), 1553 (s), 1466 (m), 1387 (m), 1340 (m), 1165 (w, C=S), 1026 (w) cm^{-1}.

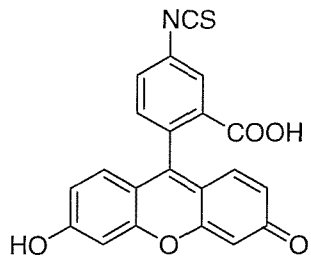
Anal. Calcd. for $C_{643}H_{259}N_{251}O_{129}S_1$: C, 56.81; H, 1.92; N, 25.86; S, 0.23. Found: C, 55.92; H, 2.23; N, 24.62; S. 0.32.

4.4.2 Preparation of 4.0-[N]-62-[phenyl thiourea]-2-[fluorescein thiourea] (34)



M.W. = 135.2 g Mol^{-1}

C_7H_5NS



M.W. = 389.4 g Mol^{-1}

$C_{18}H_{11}NO_4S$

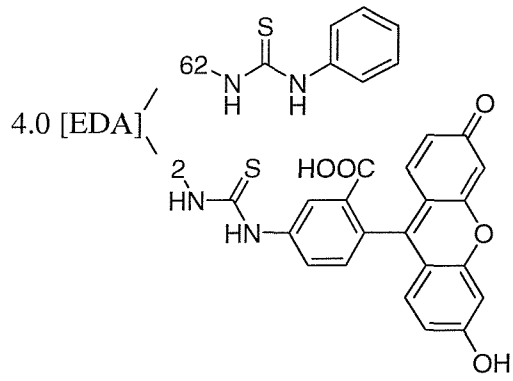
4.0 [EDA]-64-amine

M.W. = 14214.2 g Mol^{-1}

$C_{622}H_{1248}N_{250}O_{124}$

$CHCl_3$/DMSO

40 °C



M.W. = 24024.4 g Mol^{-1}

$C_{1098}H_{1580}N_{374}O_{124}S_{64}$

34

Phenyl isothiocyanate (101 mg, 7.51 x 10^{-4} mol, 61 μL) and fluorescein isothiocyanate (6 mg, 1.54 x 10^{-5} mol) were dissolved in $CHCl_3$ (5 mL) and then added to a solution of 4.0 [N]-64-amine (111 mg, 7.8 x 10^{-6} mol) in DMSO (5 mL). The reaction mixture was stirred at 40 °C overnight and then dialysed against MeOH (2 x 2.5 L). The remaining MeOH was concentrated *in vacuo* to give **34** as an orange oil (121 mg, 5.0 x 10^{-6} mol, 64%).

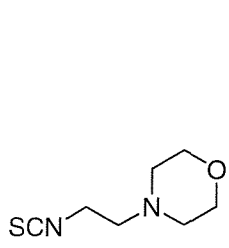
^{1}H NMR (400 MHz, CD_3OD): δ 7.24 (d, J = 7.8 Hz, Ar**H**), 7.08 (t, J = 7.8 Hz, Ar**H**), (t, J = 7.8 Hz, Ar**H**), 3.51-3.30 (m), 3.10-2.84 (m), 2.22-2.13 (m), 1.57-1.35 (m), 1.15-1.12 (m), 0.72-0.68 (m) ppm.

^{13}C NMR + DEPT 135 (100 MHz, CD_3OD): δ 174.5, 173.9, 154.6 (**C**O), 140.0 (**Ar**), 130.4, 124.5, 120.8 (**Ar**H), 65.0, 64.4, 52.8, 50.6, 43.6, 39.9, 39.5, 37.9, 34.0, 31.8, 26.5, 22.9, 20.3, 13.8 (**C**H_2) ppm.

IR (neat) ν_{max}: 3264 (m), 3075 (w), 2953 (w), 2359 (s), 2342 (m), 1652 (s, C=O), 1550 (s), 1313 (m), 1233 (m, C=S), 1033 (w), 757 (w), 669 (w) cm^{-1}.

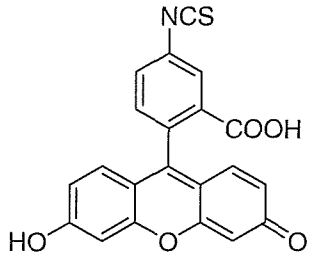
Anal. Calcd for $C_{1098}H_{1580}N_{374}O_{124}S_{64}$: C, 54.83; H, 6.62; N, 21.78; S, 8.53. Found: C, 53.92; H, 6.12; N, 22.02; S. 8.84.

4.4.3 Preparation of 4.0-[N]-62-[2-(4-morpholino)ethyl thiourea]-2-[fluorescein thiourea] (35)



M.W. = 160.2 g Mol^{-1}

$C_7H_{12}N_2OS$



M.W. = 389.4 g mol^{-1}

$C_{18}H_{11}NO_4S$

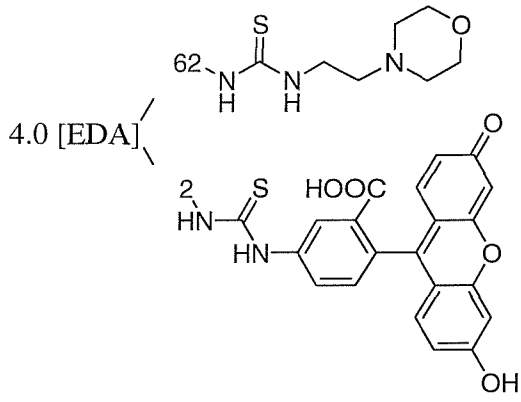
4.0 [EDA]-64-amine

M.W. = 14214.2 g Mol^{-1}

$C_{622}H_{1248}N_{250}O_{124}$

$CHCl_3$/DMSO

40 °C



M.W. = 26318.6 g Mol^{-1}

$C_{1098}H_{2014}N_{436}O_{186}S_{64}$

35

2-(4-Morpholino)ethyl isothiocyanate (79 mg, 4.96 x 10^{-4} mol, 70µL) and fluorescein isothiocyanate (4 mg, 1.02 x 10^{-5} mol) were dissolved in $CHCl_3$ (5 mL) and added to a solution of 4.0 [N]-64-amine (73 mg, 5.1 x 10^{-6} mol) in DMSO (5 mL). The reaction mixture was stirred at 40 °C overnight and then dialysed against MeOH (2 x 2.5 L). The remaining MeOH was concentrated *in vacuo* to give **35** as an orange oil (95 mg, 3.6 x 10^{-6} mol, 71%).

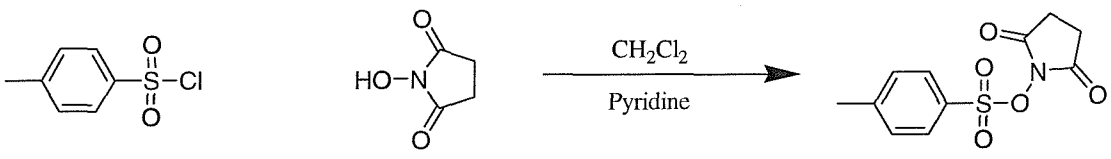
^{1}H NMR (400 MHz, CD_3OD): δ 3.60 (t, *J* = 5.0 Hz, $CH_2C\underline{H}_2O$), 3.57 (t, *J* = 6.0 Hz, $CSNHC\underline{H}_2CH_2N$), 3.3-3.15 (m), 3.05-2.95 (m), 2.56 (t, *J* = 6.0 Hz, $CSNHCH_2C\underline{H}_2N$), 2.42 (t, *J* = 5.0 Hz, $CH_2C\underline{H}_2O$), 2.38-2.20 (m), 1.80-1.30 (m), 1.35-1.20 (m), 0.85-0.78 (m) ppm.

^{13}C NMR + DEPT 135 (100 MHz, CD_3OD): δ 174.1, 173.5 ($\underline{C}O$), 66.9, 64.7, 64.1, 57.6, 53.6, 48.8, 42.6, 39.5, 37.5, 33.8, 31.4, 26.1, 22.5, 19.9, 13.4 ($\underline{C}H_2$) ppm.

IR (neat) ν_{max}: 3264 (w), 3067 (w), 2953 (w), 2859 (w), 2360 (s), 2341 (s), 1652 (m, C=O), 1542 (s), 1457 (m), 1339 (m), 1250 (m), 1115 (m, C=S), 669 (m) cm^{-1}.

Anal. Calcd. for $C_{1098}H_{2014}N_{436}O_{186}S_{64}$: C, 50.04; H, 7.70; N, 23.17; S, 7.79. Found: C, 49.51; H, 8.65; N, 22.62; S. 8.42.

4.5.1 Preparation of toluene-4-sulfonic acid 2, 5-dioxo-pyrrolidin-1-yl ester (38)



M.W. = 190.7 g Mol^{-1}
$C_7H_7O_2SCl$

M.W. = 115.1 g Mol^{-1}
C_4H_5NO
40

M.W. = 269.3 g Mol^{-1}
$C_{11}H_{11}NO_5S$
38

N-Hydroxysuccinimide (**40**) (3.40 g, 29.5 mmol) was added portion-wise to *p*-toluene sulphonyl chloride (5.91 g, 31.0 mmol) dissolved in pyridine (50 mL) and CH_2Cl_2 (50 mL) at -15 °C. The reaction mixture was stirred at -15 °C for two hours, then at 0 °C overnight. The reaction mixture was then poured into 2M HCl (100 mL) at 0 °C and extracted with CH_2Cl_2 (50 mL). The organic phase was washed further with 2M HCl (2 x 50 mL) at 0 °C and H_2O (2 x 50 mL). The organic phase was dried (anhydrous $MgSO_4$) and concentrated *in vacuo* to give a white solid which was crystallised (CH_2Cl_2/petroleum ether 40-60 °C) to give **38** as white needles (6.09 g,

22.6 mmol, 78%), (R_f = 0.63 (EtOAc)), (m.p. 142°C (CH_2Cl_2/petroleum ether 40-60 °C), (Lit.[206] 142-144°C)).

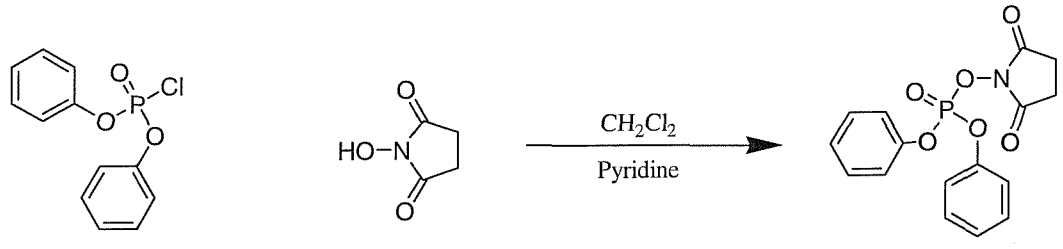
^{1}H NMR (300MHz, $CDCl_3$): δ 7.93 (d, 2H, *J* = 8.8 Hz, $CH_3Ar\underline{H}$), 7.40 (d, 2H, *J* = 8.8 Hz, $SO_2Ar\underline{H}$), 2.80 (s, 4H, $COC\underline{H}_2$), 2.48 (s, 3H, $ArC\underline{H}_3$) ppm.

^{13}C NMR + DEPT (75MHz, $CDCl_3$): δ 168.6 ($\underline{C}O$) 147.1, 131.1 ($\underline{Ar}$), 130.0, 129.4 ($\underline{Ar}H$), 25.4 ($CO\underline{C}H_2$), 21.9 ($Ar\underline{C}H_3$) ppm.

IR (neat) ν_{max}: 1735 (s, C=O), 1592 (w), 1375 (m, SO_2-O), 1346 (w), 1190 (s, SO_2-O), 1089 (w), 1054 (s), 995 (m), 813 (m), 743 (s), 702 (m) cm^{-1}.

LRMS (ES^+): *m/z* 292.3 ($[M+Na]^+$, 100%, 293.3, 10%).

4.5.2 Preparation of phosphoric acid 2, 5-dioxo-pyrrolidin-1-yl ester diphenyl ester (39)



M.W. = 268.6 g Mol^{-1} — $C_{12}H_{10}O_3PCl$

M.W. = 115.1 g Mol^{-1} — C_4H_5NO — **40**

M.W. = 347.3 g Mol^{-1} — $C_{16}H_{14}NO_6P$ — **39**

Diphenyl chlorophosphate (6.48 g, 24.1 mmol, 5.0 mL) was added drop-wise to *N*-hydroxysuccinimide (**40**) (2.50 g, 21.7 mmol) in pyridine (20 mL) at -15 °C. The reaction mixture was stirred for five hours at 0 °C and then poured into 2M HCl (100 mL) at 0 °C. The resulting precipitate was extracted with EtOAc (100 mL). The organic phase was washed with 2M HCl (2 x 100mL) and then H_2O (2 x 100 mL)

and then dried (anhydrous $MgSO_4$). It was then concentrated *in vacuo* to give an oil. Trituration of the oil with petroleum ether 40-60 °C gave a white solid. Purification by column chromatography (40% EtOAc/60% petroleum ether 40-60°C, Silica 60) gave an oil which was triturated with petroleum ether 40-60 °C to give a white solid which was recrystallised ($CHCl_3$/petroleum ether 40-60 °C) to give **39** as white flakes (3.56 g, 10.3 mmol, 47%), (R_f = 0.50 (70% EtOAc/30% petroleum ether 40-60 °C)), (m.p. 88 °C ($CHCl_3$/petroleum ether 40-60 °C), (Lit.[207] 88-90 °C)).

^{1}H NMR (300MHz, $CDCl_3$): δ 7.43 - 7.36 (m, 8H, Ar**H**), 7.29 - 7.24 (m, 2H, Ar**H**), 2.77 (s, 4H, COC**H**$_2$), ppm.

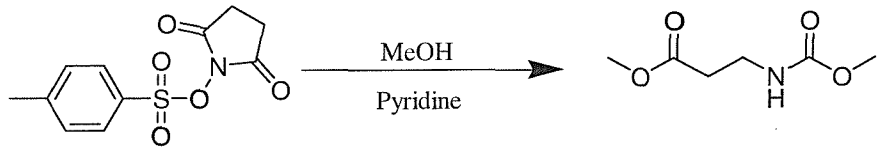
^{13}C NMR + DEPT (75MHz, $CDCl_3$): δ 168.2 (N**C**O), 150.2 (**Ar**), 129.8, 126.1, 120.3 (**Ar**H), 25.3 (CO**C**H$_2$) ppm.

^{31}P NMR (121MHz, $CDCl_3$): δ -12.5 (s, Ar$_2$**P**(O)O) ppm.

IR (neat) ν_{max}: 1796 (w), 1737 (m, C=O), 1585 (w), 1486 (m), 1304 (m), 1199 (m, P-O-Ar), 1156 (m), 1064 (m), 940 (s), 855 (m), 769 (m), 751 (m) cm^{-1}.

LRMS (ES$^+$): *m/z* 370.3 ([M+Na]$^+$, 100%, 370.3, 10%).

4.5.3 Preparation of 3-methoxy carbonylamino-propionic acid methyl ester (43)



M.W. = 269.3 g Mol^{-1}
$C_{11}H_{11}NO_5S$
38

M.W. = 161.3 g Mol^{-1}
$C_6H_{11}NO_4$
43

38 (0.50 g, 1.9 mmol) was stirred in MeOH (10 mL) and pyridine (10 mL) for one week. The reaction mixture was then concentrated *in vacuo*. Purification by distillation (b.p. 110 °C at 0.4 mmHg) gave **43** in the form of a waxy solid (0.27 g, 1.7 mmol, 88%), (R_f = 0.35 (5% MeOH/95% CH_2Cl_2), (m.p 30 °C (Lit.[208] 29-31°C)).

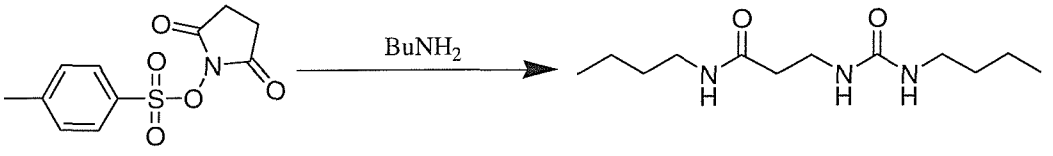
^{1}H NMR (300MHz, $CDCl_3$): δ 3.69, 3.75 (s, 3H, OC**H**$_3$), 3.44 (dt, *J* = 6.0 Hz, NHC**H**$_2$), 2.54 (t, 2H, J = 6.0, COC**H**$_2$) ppm.

^{13}C NMR + DEPT (75MHz, $CDCl_3$): δ 172.8 (**C**OCH_2), 156.9 (**C**ONH) 52.1, 51.8 (O**C**H_3), 36.5 (NH**C**H_2), 34.2 (CO**C**H_2) ppm.

IR (neat) ν_{max}: 1701 (s, C=O), 1528 (m), 1439 (m), 1368 (m), 1325 (m), 1248 (s), 1194 (s), 1177 (s), 1146 (m), 1078 (m), 1009 (m), 780 (m) cm^{-1}.

LMRS (ES^+): *m/z* 184.1 ($[M+Na]^+$, 100%).

4.5.4 Preparation of *N*-butyl-3-(3-butyl ureido) propionamide (46)



M.W. = 269.3 g Mol^{-1}
$C_{11}H_{11}NO_5S$
38

M.W. = 243.3 g Mol^{-1}
$C_{12}H_{25}N_3O_2$
46

38 (0.25 g, 0.93 mmol) was added portion-wise to butylamine (10 mL) and stirred for fifteen minutes at room temperature. The reaction mixture was then concentrated *in vacuo* and the resulting white solid was crystallised (CH_2Cl_2) to give **46** as white crystals (0.22 g, 0.88 mmol, 96%), (R_f = 0.36 (CH_2Cl_2)), (m.p. 191 °C (CH_2Cl_2)).

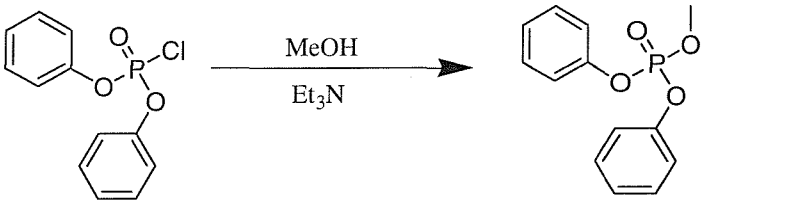
^{1}H NMR (300MHz, CD_3OD): δ 3.26 (t, 2H, J = 6.6 Hz, NHCOCHC**H**$_2$), 3.07 (t, 2H, J = 7.1 Hz, CH_2CONHC**H**$_2$), 2.99 (t, 2H, J = 7.1 Hz, NHCONHC**H**$_2$), 1.43 - 1.18 (m, 8H, *n*-C**H**$_2$), 0.83 (t, 3H, J = 7.1 Hz, *n*-C**H**$_3$), 0.82 (t, 3H, J = 7.1 Hz, *n*-C**H**$_3$) ppm.

^{13}C NMR + DEPT (75MHz, CD_3OD): δ 173. 4 (**C**ONH), 161.1 (NH**C**ONH), 40.7, 40.1, 37.7, 37.6, 33.5, 32.5 (**C**H_2), 14.2, 14.1 (*n*-**C**H_3) ppm.

IR (neat) ν_{max}: 3281 (m), 3226 (s, CONH), 1628 (s, N-CO-N), 1562 (s), 1464 (m), 1302 (s), 1227 (m), 1186 (m), 1077 (s), 1048 (s), 953 (s), 895 (s), 801 (m) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{12}H_{26}N_3O_2$ 244.2020 $(M+H)^+$, found 244.2019.

4.5.5 Preparation of phosphoric acid methyl ester diphenyl ester (47)



M.W. = 268.6 g Mol^{-1}
$C_{12}H_{10}O_3PCl$

M.W. = 264.6 g Mol^{-1}
$C_{13}H_{13}O_4P$
47

Diphenyl chlorophosphate (1.30 g, 4.8 mmol, 1.0 mL) was added to drop-wise to a solution of Et_3N (0.51, 5.1 mmol, 0.71 mL) in CH_2Cl_2 (10 mL) and MeOH (5 mL) at 0 °C. The reaction mixture was stirred for two hours at room temperature and then concentrated *in vacuo*. CH_2Cl_2 (5 mL) was added then Et_2O (50 mL). The resulting Et_3NHCl precipitate was removed by filtration and the filtrate was concentrated *in vacuo*. Purification of the residue by distillation (110 °C at 0.4 mmHg) gave **47** as a colourless liquid (0.93 g, 3.5 mmol, 73%), (R_f = 0.56 (CH_2Cl_2)).

^{1}H NMR[209, 210] (300MHz, $CDCl_3$): δ 7.33 - 7.40 (m, 4H Ar**H**), 7.28 - 7.18 (m, 6H, Ar**H**), 3.96 (d, 3H, J = 11.5 Hz, P(O)OC**H**$_3$) ppm.

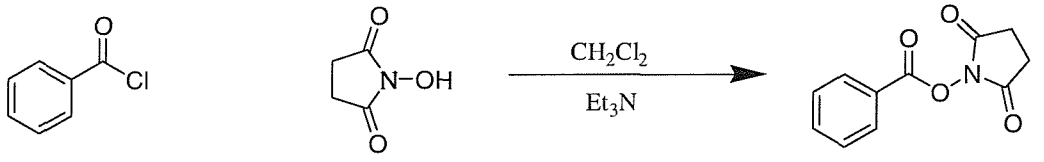
^{13}C NMR + DEPT (75MHz, $CDCl_3$): δ 150.5 (d, J = 7.2 Hz, **Ar**OP), 129.8, 125.4 (**Ar**H), 120.0 (d, J = 4.7 Hz, **Ar**H), 55.4 (d, J = 6.5 Hz, O**C**H$_3$) ppm.

^{31}P NMR (121MHz, CD_3OD): δ -10.9 (s, Ar$_2$**P**(O)O) ppm.

IR (neat) ν_{max}: 1590 (w), 1486 (m), 1456 (w), 1290 (m, PO), 1186 (s, P-Ar), 1162 (m), 1044 (s, P-O-CH_3), 1008 (w), 936 (s), 906 (m), 820 (m), 754 (s) cm^{-1}.

LMRS (ES^+): *m/z* 287.2 ($[M+Na]^+$, 100%, 288.2 15%).

4.5.6 Preparation of benzoic acid 2, 5-dioxo-pyrrolidin-1-yl ester (48)



M.W. = 140.6 g Mol^{-1} M.W. = 115.1 g Mol^{-1} M.W. = 219.3 g Mol^{-1}

C_7H_5OCl C_4H_5NO $C_{11}H_9NO_4$

40 **48**

N-Hydroxysuccinimide (**40**) (1.50 g, 13.0 mmol) was added portion-wise to benzoyl chloride (3.65 g, 26.0 mmol, 3.0 mL) and Et_3N (2.28 g, 32.5 mmol, 4.5 mL) in CH_2Cl_2 (50 mL). The reaction mixture was stirred for forty five minutes, washed with H_2O (3 x 50 mL), dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. The resulting solid was crystallised (CH_2Cl_2/petroleum ether 40-60 °C) to give **48** as white crystals (1.85 g, 8.4 mmol, 64%), (R_f=0.43 (CH_2Cl_2)), (m.p. 139 °C (CH_2Cl_2/petroleum ether 40-60 °C), (Lit.[211] 138-140°C)).

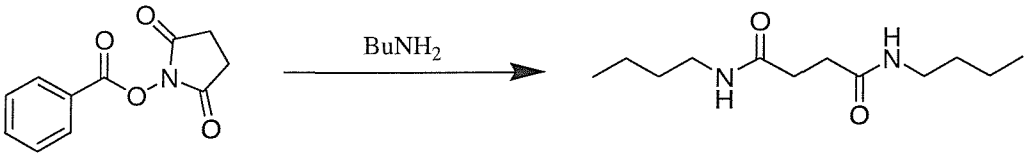
^{1}H NMR (300MHz, $CDCl_3$): δ 8.13 (d, 1H, *J* = 8.8, Hz, Ar**H**), 7.68 (t, 2H, *J* = 8.8 Hz, Ar**H**), 7.52 (t, 2H, *J* = 8.8 Hz, Ar**H**), 2.88 (s, 4H, COC**H**$_2$) ppm.

^{13}C NMR + DEPT (75MHz, $CDCl_3$): δ 169.3 (N**C**O), 161.9 (Ar**C**OO) 134.9, 130.5, 128.9 (**Ar**H), 125.2 (**Ar**), 25.7 (CO**C**H$_2$) ppm.

IR (neat) ν_{max}: 1764 (s, C=O), 1726 (s, C=O), 1598 (w), 1455 (w), 1426 (w), 1367 (w), 1252 (m), 1231 (m), 1203 (s), 1070 (m), 997 (s), 844 (m), 744 (m) cm^{-1}.

LRMS (ES$^+$): *m/z* 232.3 ([M+Na]$^+$, 100%, 233.3, 10%).

4.5.7 Preparation of *N*, *N*'-dipropyl succinamide (49)



M.W. = 219.3 g Mol^{-1}
$C_{11}H_9NO_4$
48

M.W. = 228.3 g Mol^{-1}
$C_{12}H_{24}N_2O_2$
49

48 (0.25 g, 1.1 mmol) was added portion-wise to butylamine (20 mL). The mixture was then stirred at room temperature overnight. The reaction mixture was then concentrated *in vacuo* and the resulting solid was crystallised (toluene) to give **49** as a white solid (0.23 g, 1.0 mmol, 88%), (R_f = 0.71 (20% MeOH/80% CH_2Cl_2), (m.p. 189 °C (toluene), (Lit.[212] 187 °C)).

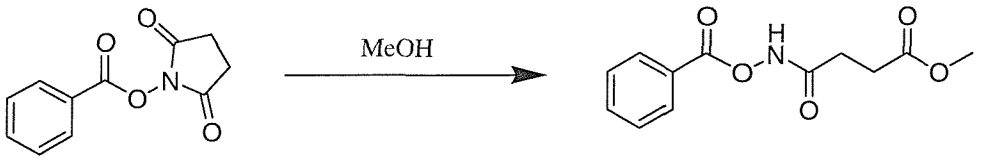
^{1}H NMR (300MHz, $CDCl_3$): δ 3.19 (dt, 4H, *J* = 8.4, 7.0 Hz, NHC**H**$_2$), 2.51 (s, 4H, COC**H**$_2$), 1.51 - 1.25 (m, 8H, *n*-C*H*$_2$), 0.90 (t, 6H, *J* = 7.1 Hz, *n*-C**H**$_3$) ppm.

^{13}C NMR + DEPT (75MHz, $CDCl_3$): δ 172.3 (<u>C</u>ONH), 39.3, 32.0, 31.6, 20.0, 19.8, (<u>C</u>H_2), 13.7 (*n*-<u>C</u>H_3) ppm.

IR (neat) ν_{max}: 3297 (m, CONH), 2956 (m), 2930 (m), 2873 (m), 1632 (s, CONH), 1547 (s), 1464 (m), 1423 (m), 1338 (m), 1213 (m), 1157 (w), 725 (m) cm^{-1}.

LMRS (ES^+): *m/z* 251.3 ($[M+Na]^+$, 100%, 252.3 15%).

4.5.8 Preparation of *N*-benzoyloxy succiamic acid methyl ester (50)



M.W. = 219.3 g Mol^{-1}
$C_{11}H_9NO_4$
48

M.W. = 251.2 g Mol^{-1}
$C_{12}H_{13}NO_5$
50

48 (0.50 g, 2.3 mmol) was stirred in MeOH (10 mL) for 1 week. The reaction mixture was then concentrated *in vacuo*. Purification of the residue by column chromatography (CH_2Cl_2, Silica 60) gave a white solid which was crystallised (CH_2Cl_2/petroleum ether 40-60 °C) to give **50** as white needle-like crystals (0.19g, 0.7 mmol, 30%), (R_f = 0.48 (10% MeOH/90% CH_2Cl_2), (m.p. 93°C (CH_2Cl_2/petroleum ether 40-60 °C)).

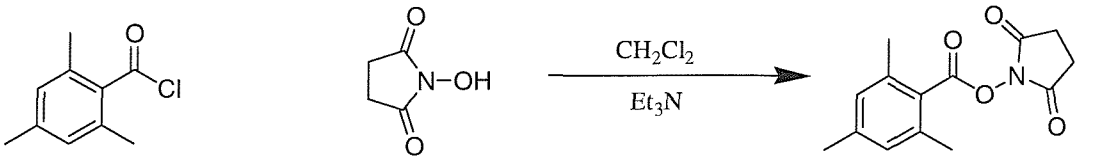
^{1}H NMR (300MHz, $CDCl_3$): δ 8.09 (dd, 1H, *J* = 8.4, 1.3 Hz, Ar<u>H</u>), 7.62 (tt, 2H, *J* = 8.8, 1.3 Hz, Ar<u>H</u>), 7.52 (tt, 2H, *J* = 8.8, 1.3 Hz, Ar<u>H</u>), 3.72 (s, 3H, OC<u>H</u>$_3$), 2.76 (t, 2H, *J* = 7.1 Hz, CH_2CONHC<u>H</u>$_2$), 2.66 (t, 2H, *J* = 7.1 Hz, NHCONHC<u>H</u>$_2$) ppm.

^{13}C NMR + DEPT (75MHz, $CDCl_3$): δ 173.3, 170.1, 164.8 (<u>C</u>O) 134.3, 130.0, 128.7 (<u>Ar</u>H), 126.6 (<u>Ar</u>), 52.1 (O<u>C</u>H_3), 28.8, 27.9 (CO<u>C</u>H_2) ppm.

IR (neat) ν_{max}: 1769 (m), 1728 (s, C=O), 1660 (s, C=O), 1600 (w), 1515 (w), 1441 (m), 1338 (m), 1235 (s), 1178 (s), 1081 (s), 1058 (m), 993 (s), 853 (w) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{12}H_{13}NO_5$ 274.0686 $(M+Na)^+$, found 274.0684.

4.5.9 Preparation of 2, 4, 6-trimethyl-benzoic acid 2, 5-dioxo-pyrrolidin-1-yl ester (51)



M.W. = 182.7 g Mol^{-1} M.W. = 115.1 g Mol^{-1} M.W. = 261.3 g Mol^{-1}

$C_{10}H_{11}OCl$ C_4H_5NO $C_{14}H_{15}NO_4$

40 **51**

N-Hydroxysuccinimide (**40**) (2.74 g, 23.8 mmol) was added portion-wise to a solution of 2,4,6-trimethylbenzoyl chloride (4.58 g, 25.0 mmol, 3.0 mL) and Et_3N (3.60 g, 35.6 mmol, 5.0 mL) in CH_2Cl_2 (50 mL). The reaction mixture was stirred for 3 hours, during which time a white precipitate formed. H_2O (10 mL) was added slowly. The organic phase was then washed with H_2O (4 x 50 mL), dried (anhydrous $MgSO_4$) and concentrated *in vacuo* to give a white solid which was crystallised (CH_2Cl_2/petroleum ether 40-60 °C) to give **51** as white crystals. (4.50 g, 17.3 mmol, 69%), (R_f = 0.34 (CH_2Cl_2), (m.p. 114 °C (CH_2Cl_2/petroleum ether 40-60 °C)).

^{1}H NMR (300MHz, $CDCl_3$): δ 6.93 (s, 2H, Ar**H**), 2.89 (s, 4H, COC**H**$_2$), 2.47 (s, 6H, ArC**H**$_3$), 2.32 (s, 3H, ArC**H**$_3$) ppm.

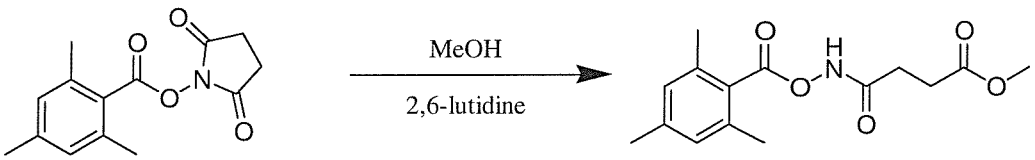
^{13}C NMR + DEPT (75MHz, $CDCl_3$): δ 169.2 (Ar**C**O), 165.0 (CH_2**C**O), 141.4, 137.2, 125.1 (**Ar**), 128.7 (**Ar**H), 25.8 (CO**C**H_2), 21.2, 19.9 (Ar**C**H_3) ppm.

IR (neat) ν_{max}: 1806 (w), 1775 (m, C=O), 1746 (s, C=O), 1608 (w), 1356 (m), 1224 (m), 1224 (s), 1153 (m), 1058 (s), 996 (s), 980 (s), 943 (m), 850 (m), 813 (m), 748 (w) cm^{-1}.

LRMS (ES^+): *m/z* 283.3 ($[M+Na]^+$, 100%, 283.3, 10%).

Anal. Calcd. for $C_{14}H_{15}NO_4$: C, 64.36; H, 5.79; N, 5.36. Found: C, 64.43; H, 5.79; N, 5.38.

4.5.10 Preparation of *N*-2, 4, 6-trimethyl-benzoyloxy succiamic acid methyl ester (52)



M.W. = 261.3 g Mol^{-1}
$C_{14}H_{15}NO_4$
51

M.W. = 293.3 g Mol^{-1}
$C_{15}H_{19}O_5N$
52

51 (0.50 g, 1.9 mmol) was stirred in MeOH and 2,6-lutidine for 7 days. The reaction mixture was then concentrated *in vacuo*. Purification of the residue by column chromatography (99% CH_2Cl_2/1% EtOAc, Silica 60) followed by crystallisation (toluene/petroleum ether 80-100°C) gave **52** as a white solid (0.24 g, 0.8 mmol, 42%), (R_f = 0.66 (EtOAc), (m.p. 96 °C (toluene/petroleum ether 80-100 °C)).

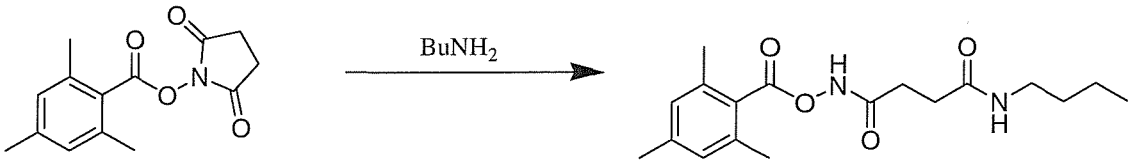
^{1}H NMR (300MHz, $CDCl_3$): δ 6.88 (s, 2H, Ar**H**), 3.73 (s, 3H, OC**H**$_3$), 2.76 (t, 2H, *J* = 6.0 Hz, CONC**H**$_2$), 2.66 (t, 2H, *J* = 6.0 Hz, COC**H**$_2$), 2.38 (s, 6H, ArC**H**$_3$), 2.30 (s, 3H, ArC**H**$_3$) ppm.

^{13}C NMR + DEPT (75MHz, $CDCl_3$): δ 173.2, 167.9 (**C**O), 140.8, 136.7, 126.6 (**Ar**), 128.6 (**Ar**H), 52.1 (O**C**H$_3$), 28.8, 27.9 (CO**C**H$_2$), 21.2, 19.9 (Ar**C**H$_3$) ppm.

IR (neat) ν_{max}: 1768 (m, C=O), 1731 (s, C=O), 1668 (s, C=O), 1611 (w), 1441 (w), 1339 (w), 1227 (w), 1197 (w), 1162 (w), 1077 (w), 1049 (w), 1017 (w), 989 (s), 954 (w), 776 (w) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{15}H_{19}O_5N$ 316.1155 $(M+Na)^+$, found 316.1150.

4.5.11 Preparation of *N*-butyl-*N*'-(2, 4, 6-trimethyl-benzoyloxy)-succinamide (53)



M.W. = 261.3 g Mol^{-1}
$C_{14}H_{15}NO_4$
51

M.W. = 334.4 g Mol^{-1}
$C_{18}H_{26}N_2O_4$
53

51 (0.64 g, 2.4 mmol) was stirred in butylamine (25 mL) for 1 hour. The reaction mixture was then concentrated *in vacuo*. Purification of the residue by column chromatography (80% EtOAc/20% petroleum ether 40-60°C, Silica 60) followed by crystallisation (CH_2Cl_2/petroleum ether 40-60°C) gave **53** as a white solid (0.52 g, 1.6 mmol, 72%), (R_f = 0.52 (EtOAc), (m.p. 153 °C (CH_2Cl_2/petroleum ether 40-60 °C)).

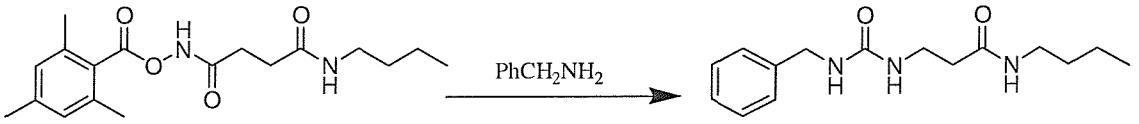
^{1}H NMR (300 MHz, $CDCl_3$): δ 6.87 (s, 2H, Ar**H**), 2.25 (dt, 2H, *J* = 7.0, 5.7 Hz, CONHC**H**$_2$), 2.72, 2.63 (t, 2H, *J* = 6.8 Hz, COC**H**$_2$), 2.42 (s, 6H, ArC**H**$_3$), 2.29 (s, 3H, ArC**H**$_3$), 1.53-1.27 (m, 4H, *n*-C**H**$_2$), 0.90 (t, 3H, *J* = 7.3 Hz, *n*-C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (75 MHz, $CDCl_3$): δ 172.2 (**C**ONH), 170.6, 167.7 (**C**O), 140.5, 136.6, 127.1 (**Ar**), 128.5 (**Ar**H), 39.6 (NH**C**H$_2$), 31.4, 31.2, 28.8, 20.0 (**C**H$_2$), 21.2, 19.9 (Ar**C**H$_3$) 13.7 (*n*-**C**H$_3$) ppm.

IR (neat) ν_{max}: 1770 (s, C=O), 1668 (w), 1638 (s, CONH), 1612 (w), 1556 (w), 1504 (w), 1421 (w), 1338 (w), 1229 (m), 1158 (m), 1044 (w), 1035 (w), 1013 (w), 986 (m), 950 (w), 846 (m) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{18}H_{26}N_2O_4$ 357.1785 $(M+Na)^+$, found 357.1785.

4.5.12 Preparation of 3-(3-benzyl-ureido)-*N*-butyl-propionamide (54)



M.W. = 334.4 g Mol^{-1}
$C_{18}H_{26}N_2O_4$
53

M.W. = 277.4 g Mol^{-1}
$C_{15}H_{23}N_3O_2$
54

53 (0.16 g, 0.47 mmol) was stirred in benzylamine (5 mL) for 5 days. The benzylamine was then removed by distillation under vacuum. Purification of the resulting solid by column chromatography (70% EtOAc/30% petroleum ether 40-60 °C) followed by crystallisation (CH_2Cl_2) gave **54** as a white solid (93 mg, 0.34 mmol, 72%), (R_f = 0.36 (80% EtOAc/20% petroleum ether 40-60 °C)), (m.p. 185-187 °C (CH_2Cl_2)).

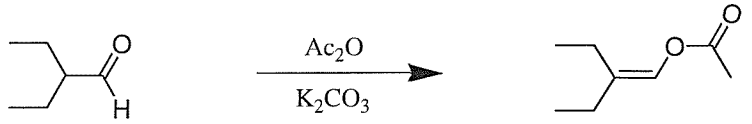
^{1}H NMR (400 MHz, CD_3OD): δ 7.14-7.11 (m, 5H, Ar**H**), 4.21 (s, 2H, ArCH_2), 3.30 (t, 2H, *J* = 6.5 Hz, NHCONHC**H**$_2$), 3.07 (t, 2H, *J* = 7.0 Hz, CONHC**H**$_2$), 2.28 (t, 2H, *J* = 6.5 Hz, C**H**$_2$CO), 2.29-1.21 (m, 4H, *n*-C**H**$_2$), 0.84 (t, 3H, *J* = 7.3 Hz, *n*-C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, CD_3OD): δ 174.9 (CH_2**C**O), 161.9 (NH**C**ONH), 142.2 (**Ar**CH_2), 130.4, 129.1, 128.9 (**Ar**H), 49.7 (Ar**C**H_2), 45.1, 40.5, 38.0, 32.9, 21.5 (**C**H_2), 14.5 (*n*-**C**H_3) ppm.

IR (neat) ν_{max}: 2249 (m), 3279 (m), 3087 (w), 3031 (w), 2953 (w), 2926 (w), 2869 (w), 1625 (s, NHCONH), 1554 (s), 1452 (m), 1332 (m), 1227 (s), 1153 (m), 1104 (m), 1026 (m), 968 (m), 728 (s) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{15}H_{23}N_3O_2$ 300.1682 $(M+Na)^+$, found 300.1679.

4.6.1 Preparation of acetic acid 2-ethyl-but-1-enyl ester (59)



M.W. = 100.2 g Mol^{-1}
$C_6H_{12}O$
58

M.W. = 143.2 g Mol^{-1}
$C_8H_{14}O_2$
59

Using a procedure by Barbier *er al*[213], 2-ethyl butyraldehyde (**53**) (40.70 g, 0.41 mol, 50.0 mL) was refluxed in acetic anhydride (62.20 g, 0.61 mol, 57.6 mL) with K_2CO_3 (7.10 g, 51.2 mmol) for 2 hours. The reaction mixture was then left to cool then diluted with Et_2O (100 mL). It was then washed with sat. $NaHCO_3$ solution (200 mL) and then H_2O (2 x 200 mL). It was then dried (anhydrous $MgSO_4$) and concentrated *in vacuo* to give a liquid from which the product was distilled to give **59** as a colourless liquid (31.3 g, 0.22 mol, 53%), (b.p 169 °C (760 mmHg), Lit.[213] 165 °C (760 mmHg)), (R_f = 0.68 (20% Et_2O/80% Petroleum Ether 40-60 °C).

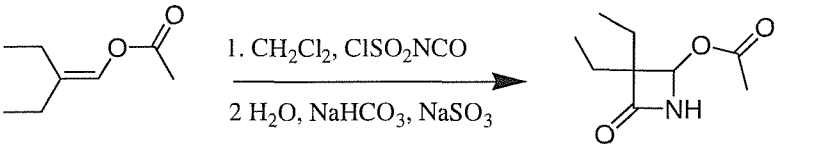
^{1}H NMR (400 MHz, $CDCl_3$): δ 6.89 (s, 1H, C**H**OCO), 2.18 (q, 2H, *J* = 7.5 Hz, C**H**$_2$CH$_3$), 2.16 (s, 3H, COC**H**$_3$), 2.06 (q, 2H, *J* = 7.5 Hz, C**H**$_2$CH$_3$), 1.05 (t, 3H, *J* = 7.5 Hz, CH$_2$C**H**$_3$), 1.02 (t, 3H, *J* = 7.5 Hz, CH$_2$C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 168.7 (**C**OCH$_3$) 129.6 (**C**HOCO), 24.8 (**C**H$_2$CH$_3$), 21.5 (CO**C**H$_3$), 12.9, 12.7 (CH$_2$**C**H$_3$) ppm.

IR (neat) ν_{max}: 2967 (w), 2937 (w), 1748 (s, C=O), 1680 (w), 1463 (w), 1370 (m), 1219 (s), 1117 (s), 1070 (m), 1049 (m), 951 (w), 903 (w), 831 (m), 638 (w), 601 (w) cm^{-1}.

Unable obtain LRMS.

4.6.2 Preparation of acetic acid 3, 3-diethyl-4-oxo-azetidin-2-yl ester (60)



M.W. = 143.2 g Mol^{-1}
$C_8H_{14}O_2$
59

M.W. = 185.2 g Mol^{-1}
$C_9H_{15}NO_3$
60

Using a procedure by Shah *et al*[214], **59** (10.00 g, 69.8 mmol), was stirred at -10 °C in CH_2Cl_2 (50 mL). Chlorosulfonyl isocyanate (12.0 g, 84.7 mmol, 7.4 mL) was added drop-wise. The reaction mixture was then left to stir at room temperature overnight. It was then diluted with Et_2O (100 mL) and cooled to 0 °C and added drop-wise to ice cold H_2O (200 mL) which contained Na_2SO_3 (5.00 g, 40.0 mmol) and $NaHCO_3$ (15.00 g, 0.18 mol). The solution was then stirred for 2 hours. The organic layer and aqueous layer were then separated. The aqueous layer was further washed with Et_2O (100 mL). The combined organic fractions were washed with H_2O (100 mL) followed by brine (100 mL) and then dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. The resulting yellow oil was diluted with hexane and left in the freezer overnight. The resulting solid was then collected by filtration and washed with cold hexane to give **60** in the form of a yellow solid (2.21 g, 11.8 mmol, 16%), (m.p. 28 °C), (R_f = 0.44 (50% Et_2O/50% petroleum ether 40-60 °C).

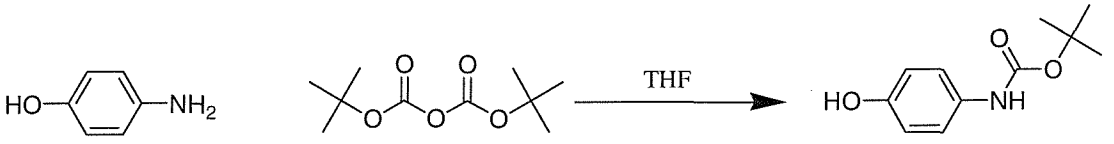
^{1}H NMR (400 MHz, $CDCl_3$)[214]: δ 5.61 (s, 1H, C**H**OCO), 2.13 (s, 3H, COC**H**$_3$), 1.86-1.66 (m, 4H, C**H**$_2$CH$_3$), 1.03 (t, 3H, J = 7.5 Hz, CH$_2$C**H**$_3$), 1.00 (t, 3H, J = 7.5 Hz, CH$_2$C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 172.5 (**C**O), 171.4 (**C**O), 80.2 (**C**HCOC), 64.6 (**C**(CH$_2$CH$_3$)$_2$), 25.2 (**C**H$_2$CH$_3$), 21.3 (CO**C**H$_3$), 9.1, 8.8 (CH$_2$**C**H$_3$) ppm.

IR (neat) ν_{max}: 2970 (w), 2941 (w), 2883 (w), 1736 (s, C=O), 1460 (w), 1363 (m), 1225 (s), 1029 (s), 897 (w), 605 (w) cm^{-1}.

LRMS (ES$^+$): *m/z* 208.1 ([M+Na]$^+$, 100%, 209.1, 10%).

4.6.3 Preparation of (4-hydroxy-phenyl)-carbamic acid *tert*-butyl ester (61)



M.W. = 109.1 g Mol^{-1}
C_6H_7NO

M.W. = 218.3 g Mol^{-1}
$C_{10}H_{18}O_5$

M.W. = 209.2 g Mol^{-1}
$C_{11}H_{15}NO_3$
61

4-Aminophenol (5.75 g, 52. 6 mmol) was stirred in THF (50 mL) at 0 °C. Di-*tert*-butoxy dicarbonate (12.70 g, 57.9 mmol) was dissolved in THF (30 mL) and added drop-wise over 1 hour. The reaction mixture was left stirring at room temperature overnight and then concentrated *in vacuo*. The resulting solid was dissolved in EtOAc (100 mL) and washed with 2M HCl (100mL) followed by H_2O (2 x 100 mL). The organic phase was dried (anhydrous Na_2SO_4) and concentrated *in vacuo*. The residue was crystalised (toluene) to give **61** as a white solid (7.89 g, 37.7 mmol, 72%), (m.p. 139 °C (toluene), Lit.[215] 144-145 °C), (R_f = 0.72 (10% MeOH/90% CH_2Cl_2)).

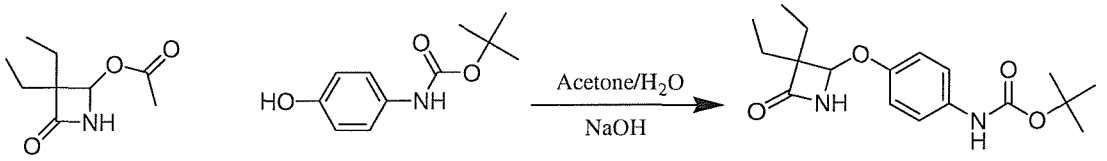
^{1}H NMR (300 MHz, d_6-DMSO): δ 7.20 (d, 2H, *J* = 8.6 Hz, Ar**H**), 6.65 (d, 2H, *J* = 8.6 Hz, Ar**H**), 1.45 (s, 9H, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT 135 (75 MHz, d_6-DMSO): δ 153.0 (NH**C**O), 152.5 (**Ar**OH), 130.0 (**Ar**NHCO), 120.0, 115.0 (**Ar**H) 78.2 (**C**(CH$_3$)$_3$), 26.7 (C(**C**H$_3$)$_3$) ppm.

IR (neat) ν_{max}: 3360 (w), 1696 (m, C=O), 1526 (m), 1514 (m), 1436 (w), 1368 (w), 1311 (w), 1229 (m), 1163 (s), 1058 (m), 829 (m), 803 (w), 762 (w), 734 (w), 629 (m) cm^{-1}.

LRMS (ES$^+$): *m/z* 232.1 ([M+Na]$^+$, 100%, 233.1, 10%).

4.6.4 Preparation of [4-(3, 3-diethyl-4-oxo-azetidin-2-yloxy)-phenyl]-carbamic acid *tert*-butyl ester (62)



M.W. = 185.2 g Mol^{-1} — $C_9H_{15}NO_3$ — **60**

M.W. = 209.2 g Mol^{-1} — $C_{11}H_{15}NO_3$ — **61**

M.W. = 334.4 g Mol^{-1} — $C_{18}H_{26}N_2O_4$ — **62**

61 (0.95 g, 4.5 mmol) was dissolved in acetone (10 mL). A solution of NaOH (0.18 g, 4.5 mmol) in H_2O (10 mL) was added and the reaction mixture was stirred for 10 minutes. **60** (0.80, 4.3 mmol) was dissolved in acetone (10 mL) and added to the reaction mixture which was then stirred for 30 minutes, partitioned between Et_2O (50 mL) and washed with H_2O (2 x 50 mL) and then brine (50 mL). The organic layer was then dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. Purification of the residue by column chromatography (30% Et_2O/70% petroleum ether 40-60°C,

silica 60) gave **62** as a white solid (0.79 g, 2.4 mmol, 55%), (m.p. 43 °C), (R_f = 0.26 (50% Et_2O/50% petroleum ether 40-60°C).

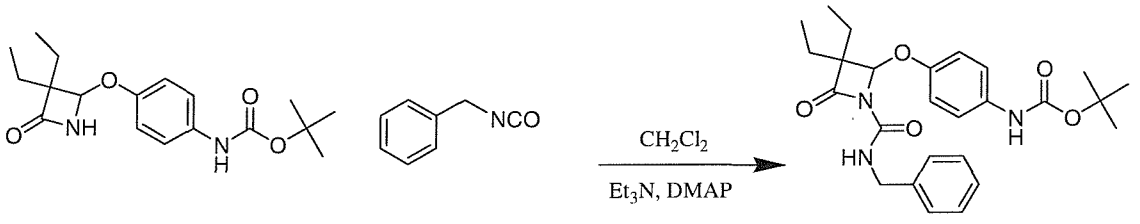
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.16, 6.66 (d, 2H, *J* = 8.5 Hz, Ar**H**), 5.14 (s, 1H, C**H**OAr), 1.67-1.37 (m, 4H, C**H**$_2$CH$_3$), 1.38 (s, 3H, C(C**H**$_3$)$_3$), 0.92 (t, 3H, *J* = 7.5 Hz, CH_2C**H**$_3$), 0.85 (t, 3H, *J* = 7.5 Hz, CH_2C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 173.0 (**C**O), 153.4 (**C**OC(CH$_3$)$_3$), 152.7 (**Ar**O), 133.6 (**Ar**NH), 121.0, 117.1 (**Ar**H), 84.5 (**C**HOAr), 80.9 (**C**(CH$_3$)$_3$) 65.1 (**C**(CH$_2$CH$_3$)$_2$), 28.8 (C(**C**H$_3$)$_3$) 21.1, 20.9 (**C**H$_2$CH$_3$), 9.3, 9.1 (CH_2**C**H$_3$) ppm.

IR (neat) ν_{max}: 3327 (w), 2968 (w), 2936 (w), 1737 (s, C=O), 1714 (s, C=O), 1532 (w), 1510 (w), 1366(w), 1298 (w), 1214 (m), 1157 (m), 1083 (m), 965 (m), 901 (w), 827 (m), 777 (w), 739 (w), 671 (w) cm^{-1}.

HRMS (ES$^+$): Calcd. for $C_{18}H_{26}N_2O_4$ 335.1965 $(M+H)^+$, found 335.1975.

4.6.5 Preparation of [4-(1-benzylcarbamoyl-3, 3-diethyl-4-oxo-azetidin-2-yloxy)-phenyl]-carbamic acid *tert*-butyl ester (63)



M.W. = 334.4 g Mol^{-1}
$C_{18}H_{26}N_2O_4$
62

M.W. = 133.2 g Mol^{-1}
C_7H_5NO

M.W. = 467.6 g Mol^{-1}
$C_{26}H_{33}N_3O_5$
63

62 (0.42 g, 1.3 mmol) was dissolved in CH_2Cl_2 (5 mL) with DMAP (5.0 mg) and Et_3N (0.13 g, 1.3 mmol, 0.18 mL). Benzyl isocyanate (0.50 g, 3.8 mmol, 0.47 mL) was added and the reaction mixture was stirred overnight. It was then diluted with

Et_2O (50 mL) and the organic phase was washed with 10% citric acid solution (50 mL) then H_2O (2 x 50 mL). The organic phase was then dried (anhydrous Na_2SO_4) and concentrated *in vacuo*. Purification of the residue by column chromatography (30% Et_2O/70% petroleum ether 40-60°C, silica 60) gave **63** as a white solid (0.40 g, 0.9 mmol, 68%), (m.p. 130 °C), (R_f = 0.44 (50% Et_2O/50% hexane).

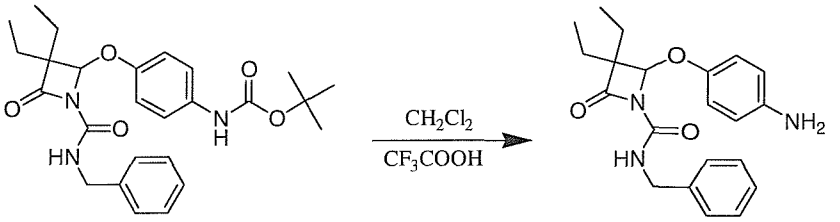
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.45-7.36 (m, 6H, Ar**H**), 7.27 (d, 2H, *J* = 8.5 Hz, Ar**H**), 7.02 (t, 1H, *J* = 8.5 Hz, CON**H**), 5.68 (s, 1H, C**H**OAr), 4.59 (d, 1H, *J* = 8.5 Hz, ArC**H**$_2$), 2.12-2.05 (m, 1H, C**H**$_2$CH$_3$), 1.96-1.72 (m, 3H, C**H**$_2$CH$_3$), 1.61 (s, 3H, C(C**H**$_3$)$_3$), 1.17 (t, 3H, *J* = 7.5 Hz, CH$_2$C**H**$_3$), 1.07 (t, 3H, *J* = 7.5 Hz, CH$_2$C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 173.6 ((Et)$_2$C**C**O), 154.7 (N**C**OCH), 154.4 (NH**C**OO), 151.5, 139.0, 135.2 (**Ar**), 130.2, 129.1, 128.6, 120.6, 119.0 (Ar**H**), 88.9 (**C**HOAr), 81.8 (**C**(CH$_3$)$_3$), 65.1 (**C**(CH$_2$CH$_3$)$_2$), 45.1 (Ar**C**H$_2$NH), 29.7 (C(**C**H$_3$)$_3$), 25.2, 22.5 (**C**H$_2$CH$_3$), 10.2, 9.9 (CH$_2$**C**H$_3$) ppm.

IR (neat) ν_{max}: 1755 (s, C=O), 1713 (m, C=O), 1694 (s, C=O), 1541 (w), 1508 (w), 1366 (m), 1338 (m), 1252 (w), 1150 (s), 1076 (w), 1052 (m), 1026 (w), 835 (w), 700 (w), cm^{-1}.

HRMS (ES$^+$): Calcd. for $C_{26}H_{33}N_3O_5$ 490.2312 $(M+Na)^+$, found 490.2310.

4.6.6 Preparation of 2-(4-amino-phenoxy)-3, 3-diethyl-4-oxo-azetidine-1-carboxylic acid benzylamide (64)



M.W. = 467.6 g Mol^{-1}
$C_{26}H_{33}N_3O_5$
63

M.W. = 367.4 g Mol^{-1}
$C_{21}H_{25}N_3O_3$
64

63 (0.25 g, 0.5 mmol) was dissolved in CH_2Cl_2 (5 mL). Trifluoroacetic acid (1 mL) was then added and the reaction mixture was stirred for 1 hour. Sat. $NaHCO_3$ solution (50 mL) was slowly added and the solution was stirred for another hour. CH_2Cl_2 (50 mL) was then added and the organic layer was washed with H_2O (2 x 50 mL), dried (anhydrous Na_2SO_4) and concentrated *in vacuo*. Purification of the residue by column chromatography (50% Et_2O/50% petroleum ether 40-60°C, silica 60) gave **64** as a colourless oil (0.17 g, 0.5 mmol, 87%), (R_f = 0.38 (Et_2O).

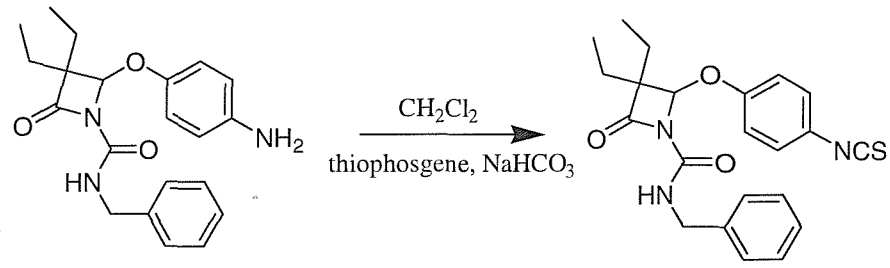
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.26-7.16 (m, 4H, Ar**H**), 6.97 (d, 2H, *J* = 8.5 Hz, Ar**H**), 6.86 (t, 1H, *J* = 8.5 Hz, CON**H**), 6.53 (d, 2H, *J* = 8.5 Hz, Ar**H**), 5.41 (s, 1H, C**H**OAr), 4.39 (d, 1H, *J* = 8.5 Hz, ArC**H**$_2$), 1.96-1.84 (m, 1H, C**H**$_2$CH$_3$), 1.77-1.57 (m, 3H, C**H**$_2$CH$_3$), 0.98 (t, 3H, *J* = 7.5 Hz, CH$_2$C**H**$_3$), 0.87 (t, 3H, *J* = 7.5 Hz, CH$_2$C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 172.4 ((Et)$_2$C**C**O), 150.6 (N**C**OCH), 150.3, 142.3, 137.8 (**Ar**), 128.7, 127.6, 127.5, 119.7, 116.2 (**Ar**H), 88.9 (**C**HOAr), 64.2 (**C**(CH$_2$CH$_3$)$_2$), 43.7 (Ar**C**H$_2$NH), 23.8, 21.2 (**C**H$_2$CH$_3$), 8.7, 8.5 (CH$_2$**C**H$_3$) ppm.

IR (neat) ν_{max}: 3360 (m), 2968 (m), 2880 (m), 1763 (s, C=O), 1699 (s, C=O), 1625 (w), 1533 (w), 1506 (s), 1455 (w), 1366 (w), 1264 (w), 1211 (s), 1121 (w), 1081 (m), 1061 (m), 1028 (w), 871 (w), 825 (m), 754 (w), 732 (w), 698 (m) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{21}H_{25}N_3O_3$ 367.1969 $(M+H)^+$, found 367.1967.

4.6.7 Preparation of 3, 3-diethyl-2-(4-isothiocyanato-phenoxy)-4-oxo-azetidine-1-carboxylic acid benzylamide (65)



M.W. = 367.4 g Mol^{-1}
$C_{21}H_{25}N_3O_3$
64

M.W. = 409.2 g Mol^{-1}
$C_{22}H_{23}N_3O_3S$
65

Thiophosgene (0.13 g, 1.1 mmol, 0.08 mL), and Na_2CO_3 (0.53 g, 5.0 mmol) were stirred in CH_2Cl_2 (5 mL) at -10 °C. **64** (0.20 g, 0.54 mmol) in CH_2Cl_2 (20 mL) was then added drop-wise over 1 hour. The reaction mixture was left to warm to room temperature and stirred for 2 hours. It was then washed with H_2O (3 x 20 mL), dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. Purification of the residue by column chromatography (30% Et_2O/70% petroleum ether 40-60°C, silica 60) gave **65** as an off white solid (0.19 g, 0.5 mmol, 85%), (m.p. 76 °C), (R_f = 0.48 (50% Et_2O/50% petroleum ether 40-60°C).

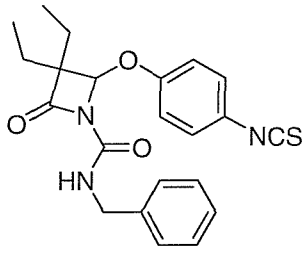
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.26-7.09 (m, 9H, Ar**H**), 6.82 (t, 1H, *J* = 8.5 Hz, CON**H**), 5.54 (s, 1H, C**H**OAr), 4.40 (d, 1H, *J* = 8.2 Hz, ArC**H**$_2$), 1.92-1.84 (m, 1H, C**H**$_2$CH$_3$), 1.77-1.67 (m, 3H, C**H**$_2$CH$_3$), 0.97 (t, 3H, *J* = 7.5 Hz, CH$_2$C**H**$_3$), 0.90 (t, 3H, *J* = 7.5 Hz, CH$_2$C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 172.4 ($(Et)_2C\underline{C}O$), 150.6 ($N\underline{C}OCH$), 156.7, 150.5, 137.8. 135.4 (**Ar**), 129.2, 128.0, 127.9, 127.4, 119.2 (**Ar**), 87.0 ($\underline{C}HOAr$), 64.7 ($\underline{C}(CH_2CH_3)_2$), 44.2 ($Ar\underline{C}H_2NH$), 24.3, 21.6 ($\underline{C}H_2CH_3$), 9.2, 8.9 ($CH_2\underline{C}H_3$) ppm.

IR (neat) ν_{max}: 3340 (w), 2974 (w), 2939 (w), 2879 (w), 2185 (w, N=C=S), 1080 (m), 1765 (s), 1702 (m), 1686 (s), 1601 (w), 1522 (m), 1500 (s), 1454 (w), 1323 (m), 1295 (m), 1226 (s), 1170 (m), 1127 (m), 1077 (s), 1061 (m), 932 (w), 915 (w), 827 (m), 608 (m) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{22}H_{23}N_3O_3S$ 432.1352 $(M+Na)^+$, found 432.1356.

4.6.8 Preparation of 5.0 [EDA]-128-[3, 3-diethyl-2-oxo-4-(4-thioureido-phenoxy)-azetidine-1-carboxylic acid benzylamide] (66)



M.W. = 409.2 g Mol^{-1}

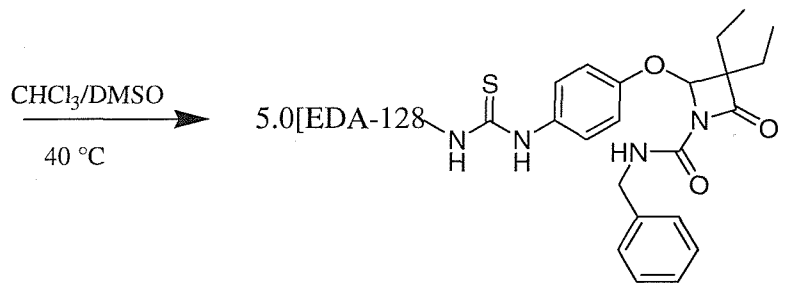
$C_{22}H_{23}N_3O_3S$

65

5.0[EDA]-128-amine

M.W. = 28225.1 g Mol^{-1}

$C_{1262}H_{2528}N_{506}O_{252}$



M.W. = 81241.3 g Mol^{-1}

$C_{4078}H_{5472}N_{890}O_{636}S_{128}$

66

5.0 [EDA]-128-amine (79.8 mg, 2.76 x 10^{-6} mol) was dissolved in DMSO (3 mL). **65** (0.17 g, 0.41 mmol) was dissolved in CH_2Cl_2 (3 mL) and the solution was added to the reaction mixture which was then stirred at 40 °C overnight. The reaction mixture was then diluted with Et_2O (100 mL). The resulting precipitate was filtered and washed with Et_2O to give a **66** as a yellow solid (0.11 g, 1.35 x 10^{-6} mol, 48%).

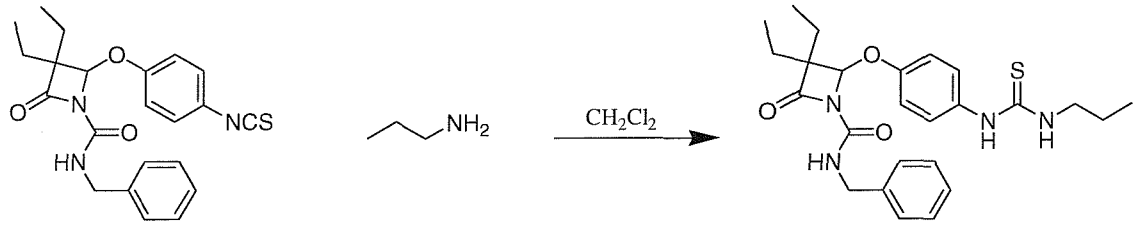
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.45-6.89 (m, Ar**H**), 5.58 (s, C**H**OAr), 4.40-1.70 (m), 1.13-0.90 (m) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 171.0 (**C**O), 153.6 (N**C**OCH), 149.2, 136.6, 125.5 (**Ar**), 129.1, 127.8, 127.7, 127.4, 119.0 (**Ar**H), 87.1 (**C**HOAr), 66.2 (*n*-**C**H_2), 64.8 (**C**$(CH_2CH_3)_2$), 44.1 (Ar**C**H_2NH), 24.2, 21.6 (**C**H_2CH_3), 9.2, 9.0 (CH_2**C**H_3) ppm.

IR (neat) ν_{max}: 2965 (w), 1769 (m, C=O), 1704 (w), 1644 (m), 1531 (s), 1505 (s), 1455 (m), 1317 (m), 1261 (m), 1213 (s, NHCS), 1079 (s, C=S), 910 (w), 872 (w), 727 (m), 697 (m) cm^{-1}.

Anal. Calcd. for $C_{4078}H_{5472}N_{890}O_{636}S_{128}$: C, 60.29; H, 6.79; N, 15.34; S, 5.05. Found: C, 58.51; H, 6.58; N, 14.26; S, 5.33.

4.6.9 Preparation of 3,3-diethyl-2-oxo-4-[4-(3-propyl-thioureido)-phenoxy]-azetidine-1-carboxylic acid benzylamide (67)



M.W. = 409.2 g Mol^{-1}	M.W. = 59.1 g Mol^{-1}	M.W. = 468.6 g Mol^{-1}
$C_{22}H_{23}N_3O_3S$	C_3H_9N	$C_{25}H_{32}N_4O_3S$
65		**67**

65 (0.21 g, 0.5 mmol) and propylamine (40 mg, 0.5 mmol, 21 μL) were stirred together in CH_2Cl_2 (5 mL) for 2 hours and the mixture was then concentrated *in vacuo*. The residue was purified by column chromatography (70% Et_2O/30% petroleum ether 40-60 °C, silica 60) to give **67** as an off-white solid. (0.16 g, 0.33 mmol, 66%), (m.p. 38 °C), (R_f = 0.33 (80% Et_2O/20% petroleum ether 40-60 °C)).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.29-7.19 (m, 7H, Ar**H**), 7.10 (d, 2H, *J* = 8.8 Hz, Ar**H**), 6.87 (t, 1H, *J* = 8.5 Hz, CON**H**), 5.58 (s, 1H, C**H**OAr), 4.40 (d, 1H, *J* = 8.2

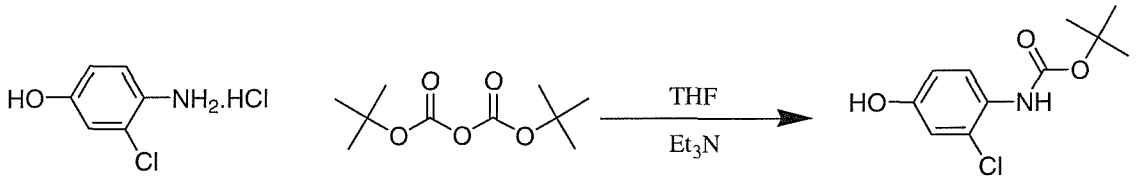
Hz, ArC$\underline{H}_2$), 3.49 (dt, 2H, J = 7.2, 6.0 Hz, CSNHC$\underline{H}_2$), 1.94-1.88 (m, 1H, C$\underline{H}_2$CH$_3$), 1.78-1.72 (m, 3H, C$\underline{H}_2$CH$_3$), 1.54 – 1.48 (m, 2H, CSNHCH$_2$C$\underline{H}_2$), 0.97 (t, 3H, J = 7.5 Hz, CH$_2$C$\underline{H}_3$), 0.90 (t, 3H, J = 7.5 Hz, CH$_2$C$\underline{H}_3$), 0.83 (t, 3H, J = 7.2 Hz, CH$_2$C$\underline{H}_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 181.5 ($\underline{C}$S), 172.3 ((Et)$_2$C$\underline{C}$O), 157.0 (N$\underline{C}$OCH), 150.5, 137.9, 131.4, ($\underline{Ar}$), 129.2, 128.1, 128.0, 127.9, 119.6 ($\underline{Ar}$), 87.1 ($\underline{C}$HOAr), 64.9 ($\underline{C}$(CH$_2$CH$_3$)$_2$), 47.6 (CSNH$\underline{C}$H$_2$), 44.2 (Ar$\underline{C}$H$_2$NH), 24.3, 22.7, 21.6, ($\underline{C}$H$_2$CH$_3$), 11.7, 9.2, 8.9 (CH$_2$$\underline{C}H_3$) ppm.

IR (neat) ν_{max}: 3307 (w), 2964 (w), 2930 (w), 2874 (w), 1767 (s, C=O), 1698 (s, C=O), 1531 (s), 1504 (s), 1455 (m), 1296 (s, CSNH), 1214 (s), 1169 (m), 1144 (m), 1078 (s), 1013 (m), 872 (m), 730 (m), 698 (s) cm^{-1}.

HRMS (ES$^+$): Calcd. for $C_{25}H_{32}N_4O_3S$ 469.2268 $(M+H)^+$, found 469.2269.

4.6.10 Preparation of (2-chloro-4-hydroxy-phenyl)-carbamic acid *tert*-butyl ester (128)



M.W. = 180.0 g Mol^{-1}
$C_6H_7Cl_2NO$

M.W. = 218.3 g Mol^{-1}
$C_{10}H_{18}O_5$

M.W. = 243.7 g Mol^{-1}
$C_{11}H_{14}ClNO_3$
128

4-Amino-3-chlorophenol (2.00 g, 11.1 mmol) was stirred in THF (50 mL) with Et_3N (1.23 g, 11.1 mmol, 1.6 mL) at 0 °C. Di-*tert*-butoxy dicarbonate (2.66 g, 12.2 mmol) was dissolved in THF (30 mL) and the solution was added drop-wise over 1 hour. The reaction mixture was left stirring at room temperature overnight then concentrated *in vacuo*. The resulting oil was partitioned between Et_2O (50 mL) and 2M HCl (50 mL). The organic phase was washed further with H_2O (2 x 50 ml), dried

(anhydrous Na_2SO_4) and concentrated *in vacuo*. The resulting solid was crystallised (toluene) to give **128** as an off-white solid (1.37 g, 5.6 mmol, 51%), (m.p. 116 °C (toluene)), (R_f = 0.53 (CH_2Cl_2)).

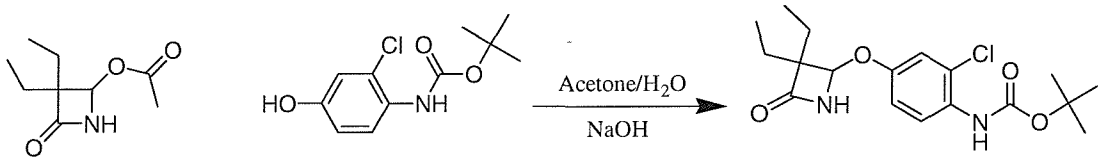
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.30 (d, 1H, *J* = 8.8 Hz, Ar**H**), 6.93 (d, 1H, *J* = 2.8 Hz, Ar**H**), 6.79 (dd, *J* = 8.8, 2.8 Hz, 1H, Ar**H**), 1.54 (s, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 157.8 (**C**O), 156.1 (**Ar**OH), 131.9 (**Ar**), 130.1 (**Ar**Cl), 128.9, 117.9, 116.7 (**Ar**H), 81.0 (**C**(CH$_3$)$_3$), 30.4 (C(**C**H$_3$)$_3$) ppm.

IR (neat) ν_{max}: 3300 (w), 2973 (w), 1684 (s, C=O), 1609 (w), 1505 (s), 1429 (w), 1391 (w), 1369 (w), 1275 (m), 1251 (w), 1158 (s), 1069 (w), 921 (w), 858 (m), 814 (w), 767 (m, C-Cl), cm^{-1}.

HRMS (ES$^+$): Calcd. for $C_{11}H_{14}ClNO_3$ 266.0554 $(M+H)^+$, found 266.0553.

4.6.11 Preparation of [2-chloro-4-(3, 3-diethyl-4-oxo-azetidin-2-yloxy)-phenyl]-carbamic acid *tert*-butyl ester (129)



M.W. = 185.2 g Mol^{-1}	M.W. = 243.7 g Mol^{-1}	M.W. = 368.9 g Mol^{-1}
$C_9H_{15}NO_3$	$C_{11}H_{14}ClNO_3$	$C_{18}H_{25}ClN_2O_4$
60	**128**	**129**

128 (0.72 g, 3.1 mmol) was dissolved in acetone (10 mL). A solution of NaOH (0.12 g, 3.0 mmol) in H_2O (10 mL) was added and the reaction mixture was stirred for 10 minutes. **60** (0.52 g, 2.8 mmol) was dissolved in acetone (10 mL) and added to the reaction mixture which was then stirred for 30 minutes. The reaction mixture was

then partitioned between Et_2O (50 mL) and washed with H_2O (2 x 50 mL). The organic phase was then washed with brine (50 mL) then dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. Purification of the residue by column chromatography (20% Et_2O/80% petroleum ether 40-60°C, silica 60) gave **129** as an oil (0.88 g, 2.4 mmol, 86%), (R_f = 0.17 (50% Et_2O/50% petroleum ether 40-60°C).

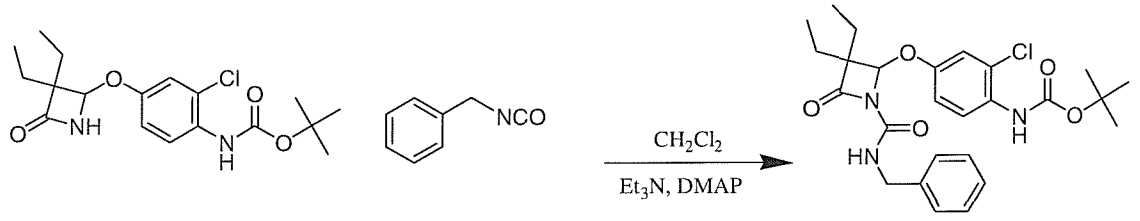
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.02 (d, 1H, *J* = 8.8 Hz, Ar**H**), 6.90 (d, 1H, *J* = 2.8 Hz, Ar**H**), 6.74 (dd, 1H, *J* = 8.8, 2.8 Hz, Ar**H**), 5.24 (s, 1H, C**H**OAr), 1.94-1.85 (m, 1H, C**H**$_2$CH$_3$), 1.78-1.70 (m, 3H, C**H**$_2$CH$_3$), 1.50 (s, 3H, C(C**H**$_3$)$_3$), 1.03 (t, 3H, *J* = 7.5 Hz, CH$_2$C**H**$_3$), 1.00 (t, 3H, *J* = 7.5 Hz, CH$_2$C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 172.9 (**C**O), 153.0 (**C**OC(CH$_3$)$_3$), 152.4 (**Ar**O), 130.6 (**Ar**NH), 123.6 (**Ar**Cl), 121.9 117.6, 115.7 (**Ar**H), 84.5 (**C**HOAr), 81.5 (**C**(CH$_3$)$_3$) 65.3 (**C**(CH$_2$CH$_3$)$_2$), 28.7 (C(**C**H$_3$)$_3$) 24.2, 22.0 (**C**H$_2$CH$_3$), 9.3, 9.0 (CH$_2$**C**H$_3$) ppm.

IR (neat) ν_{max}: 3266 (w), 2971 (w), 2936 (w), 1763 (s, C=O), 1732 (s, C=O), 1514 (s), 1548 (w), 1367 (m), 1252 (m), 1204 (m), 1151 (s), 1057 (s), 1021 (m), 910 (w), 766 (w, C-Cl) cm^{-1}.

HRMS (ES$^+$): Calcd. for $C_{18}H_{25}ClN_2O_4$ 391.1395 $(M+Na)^+$, found 391.1399.

4.6.12 Preparation of [4-(1-benzylcarbamoyl-3, 3-diethyl-4-oxo-azetidin-2-yloxy)-2-chloro-phenyl]-carbamic acid *tert*-butyl ester (68)



M.W. = 368.8 g Mol^{-1}	M.W. = 133.2 g Mol^{-1}	M.W. = 502.0 g Mol^{-1}
$C_{18}H_{25}ClN_2O_4$	C_7H_5NO	$C_{26}H_{32}ClN_3O_5$
129		**68**

129 (0.33 g, 0.9 mmol) was dissolved in CH_2Cl_2 (5 mL) together with DMAP (5.0 mg) and Et_3N (0.27 g, 2.7 mmol, 0.37 mL). Benzyl isocyanate (0.36 g, 2.7 mmol, 0.33 mL) was added and the reaction mixture was stirred overnight. It was then diluted with Et_2O (50 mL) and the organic layer was washed with 10% citric acid solution (50 mL) then H_2O (2 x 50 mL) and finally brine (50 mL). The organic phase was then dried (anhydrous Na_2SO_4) and concentrated *in vacuo*. Purification of the residue by column chromatography (30% Et_2O/70% petroleum ether 40-60°C, silica 60) gave **68** as a white solid (0.37 g, 0.74 mmol, 83%), (m.p. 137 °C), (R_f = 0.50 (50% Et_2O/50% hexane).

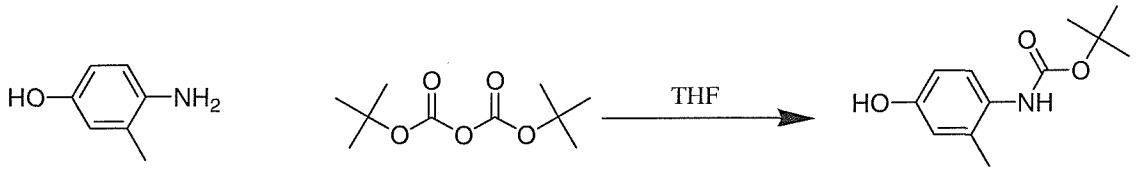
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.97 (d, 1H, *J* = 8.8 Hz, Ar**H**), 7.28-7.18 (m, 6H, Ar**H**), 7.03 (dd, 1H, *J* = 8.8, 2.8 Hz, Ar**H**), 6.82 (t, 1H, *J* = 8.5 Hz, CON**H**), 5.48 (s, 1H, C**H**OAr), 4.41 (d, 1H, *J* = 8.5 Hz, ArC**H**$_2$), 1.92-1.86 (m, 1H, C**H**$_2$CH$_3$), 1.73-1.66 (m, 3H, C**H**$_2$CH$_3$), 1.45 (s, 3H, C(C**H**$_3$)$_3$), 0.97 (t, 3H, *J* = 7.5 Hz, CH$_2$C**H**$_3$), 0.89 (t, 3H, *J* = 7.5 Hz, CH$_2$C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 170.9 ((Et)$_2$C**C**O), 154.7 (N**C**OCH), 154.4 (NH**C**OO), 149.0, 136.6, 127.7 (**Ar**), 129.9, 126.6, 121.4, 119.4, 117.7 (**Ar**H), 116.3 (**Ar**Cl), 87.8 (**C**HOAr), 80.0 (**C**(CH$_3$)$_3$), 63.4 (**C**(CH$_2$CH$_3$)$_2$), 42.8 (ArCH$_2$NH), 28.7 (C(**C**H$_3$)$_3$), 22.8, 20.1 (**C**H$_2$CH$_3$), 7.8, 7.5 (CH$_2$**C**H$_3$) ppm.

IR (neat) ν_{max}: 3421 (w), 3364 (w), 2983 (w), 2943 (w), 1760 (s, C=O), 1722 (s, C=O), 1701 (s, C=O), 1578 (s), 1519 (s), 1390 (s), 1364 (m), 1306 (m), 1146 (s), 1064 (m), 1053 (m), 916 (m), 768 (m, C-Cl), 701 (m), 584 (m), 559 (m) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{26}H_{32}ClN_3O_5$ 524.1923 $(M+Na)^+$, found 524.1915.

4.6.13 Preparation of (2-methyl-4-hydroxy-phenyl)-carbamic acid *tert*-butyl ester (130)



M.W. = 123.2 g Mol^{-1}
C_7H_9NO

M.W. = 218.3 g Mol^{-1}
$C_{10}H_{18}O_5$

M.W. = 223.3 g Mol^{-1}
$C_{12}H_{17}NO_3$
130

4-Amino-3-methylphenol (3.00 g, 24.3 mmol) was dissolved in THF (50 mL). Di-*tert*-butoxy dicarbonate (6.37 g, 29.2 mmol) was dissolved in THF (30 mL) and the solution was added to the reaction mixture. The reaction mixture was left stirring at room temperature overnight and then concentrated *in vacuo*. The resulting oil was partitioned between Et_2O (50 mL) and 2M HCl (50 mL). The organic phase was washed further with H_2O (2 x 50 ml), dried (anhydrous Na_2SO_4) and concentrated *in vacuo*. The resulting solid was crystallised (toluene/petroleum ether 40-60 °C) to give **130** as an off-white solid (2.78 g, 12.4 mmol, 51%), (m.p. 126 °C (toluene/petroleum ether 40-60 °C)), (R_f = 0.71 (EtOAc)).

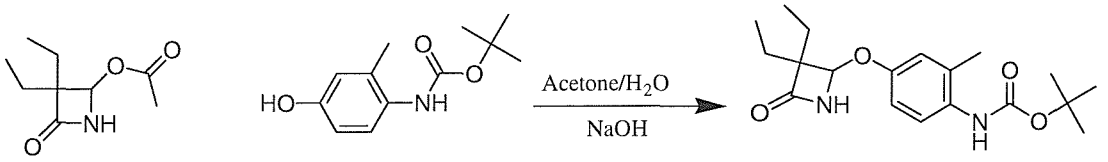
^{1}H NMR (300 MHz, d_6-DMSO): δ 6.96 (d, 1H, *J* = 8.4 Hz, Ar**H**), 6.57 (d, 1H, *J* = 2.6 Hz, Ar**H**), 6.51 (dd, *J* = 8.4, 2.6 Hz, 1H, Ar**H**), 2.09 (s, 3H, OC**H**$_3$), 1.43 (s, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT 135 (75 MHz, d_6-DMSO): δ 154.7 (<u>C</u>O), 154.1, 134.3, 127.9 (<u>Ar</u>), 127.1, 116.4, 112.5 (<u>Ar</u>H), 78.0 (<u>C</u>(CH_3)$_3$), 28.2 (C(<u>C</u>H_3)$_3$), 17.8 (O<u>C</u>H_3) ppm.

IR (neat) ν_{max}: 3303 (m), 2978 (w), 2931 (w), 1678 (s, C=O), 1611 (w), 1506 (s), 1456 (m), 1391 (m), 1369 (m), 1277 (s), 1250 (s), 1224 (s), 1156 (s), 1111 (s), 1056 (s), 1026 (m), 1003 (m), 955 (w), 902 (m), 859 (m), 810 (s), 733 (m), 703 (s) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{12}H_{17}NO_3$ 246.1101 $(M+Na)^+$, found 246.1098.

4.6.14 Preparation of [4-(3, 3-diethyl-4-oxo-azetidin-2-yloxy)-2-methyl-phenyl]-carbamic acid *tert*-butyl ester (131)



M.W. = 185.2 g Mol^{-1}	M.W. = 223.3 g Mol^{-1}	M.W. = 348.4 g Mol^{-1}
$C_9H_{15}NO_3$	$C_{12}H_{15}NO_3$	$C_{19}H_{28}N_2O_4$
60	**130**	**131**

130 (0.66 g, 3.0 mmol) was dissolved in acetone (10 mL). A solution of NaOH (0.12 g, 3.0 mmol) in H_2O (10 mL) was added and the reaction mixture was stirred for 10 minutes. **60** (0.50 g, 2.7 mmol) was dissolved in acetone (10 mL) and this solution was added to the reaction mixture which was then stirred for 30 minutes. Et_2O (50 mL) was added and the organic phase was washed with H_2O (2 x 50 mL) and then brine (50 mL). The organic layer was then dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. Purification of the residue by column chromatography (20% Et_2O/80% petroleum ether 40-60 °C, silica 60) gave **131** as an oil (0.72 g, 2.1 mmol, 70%), (m.p. 36 °C), (R_f = 0.56 (Et_2O)).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.47 (d, 1H, *J* = 6.4 Hz, Ar<u>H</u>), 6.61 (d, 1H, *J* = 2.8 Hz, Ar<u>H</u>), 6.48 (dd, 1H, *J* = 8.8, 2.8 Hz, Ar<u>H</u>), 5.19 (s, 1H, C<u>H</u>OAr), 2.15 (s, 3H,

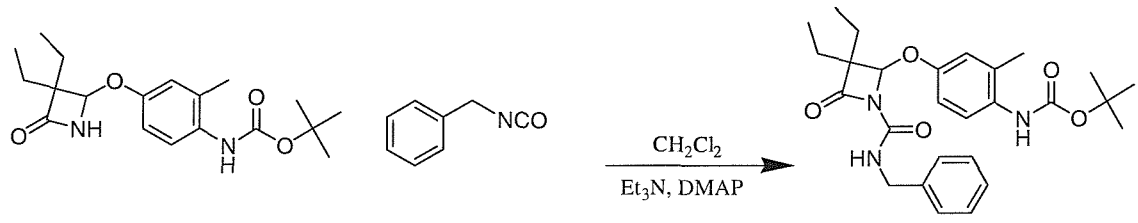
ArC**H**$_3$), 1.88—1.83 (m, 1H, C**H**$_2$CH$_3$), 1.73-1.66 (m, 3H, C**H**$_2$CH$_3$), 1.43 (s, 3H, C(C**H**$_3$)$_3$), 0.98 (t, 3H, *J* = 7.5 Hz, CH$_2$C**H**$_3$), 0.95 (t, 3H, *J* = 7.5 Hz, CH$_2$C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 173.2 (**C**O), 153.9 (**C**OC(CH$_3$)$_3$), 153.5 (**Ar**O), 131.3 (**Ar**NH), 128.7 (**Ar**CH$_3$), 118.8, 117.5, 114.2 (**Ar**H), 84.3 (**C**HOAr), 80.9 (**C**(CH$_3$)$_3$) 65.0 (**C**(CH$_2$CH$_3$)$_2$), 28.7 (C(**C**H$_3$)$_3$) 24.2, 22.0 (**C**H$_2$CH$_3$), 18.4 (Ar**C**H$_3$), 9.3, 9.0 (CH$_2$**C**H$_3$) ppm.

IR (neat) ν_{max}: 3285 (w), 2972 (w), 2932 (w), 1755 (m, C=O), 1691 (m, C=O), 1610 (w), 1508 (s), 1455 (w), 1366 (m), 1217 (m), 1154 (s), 1079 (m), 1048 (s), 1024 (s), 976 (m), 900 (w), 765 (w) cm^{-1}.

HRMS (ES$^+$): Calcd. for $C_{19}H_{28}N_2O_4$ 371.1941 $(M+Na)^+$, found 371.1945.

4.6.15 Preparation of [4-(1-benzylcarbamoyl-3, 3-diethyl-4-oxo-azetidin-2-yloxy)-2-methyl-phenyl]-carbamic acid *tert*-butyl ester (69)



M.W. = 348.4 g Mol^{-1}
$C_{19}H_{28}N_2O_4$
131

M.W. = 133.2 g Mol^{-1}
C_7H_5NO

M.W. = 481.6 g Mol^{-1}
$C_{27}H_{35}N_3O_5$
69

131 (0.69 g, 2.0 mmol) was dissolved in CH_2Cl_2 (5 mL) together with DMAP (5.0 mg) and Et_3N (0.2 g, 2.0 mmol, 0.25 mL). Benzyl isocyanate (0.79 g, 5.9 mmol, 0.70 mL) was added and the reaction mixture was stirred overnight. It was then diluted with Et_2O (50 mL) and the organic layer was washed with 10% citric acid solution (50 mL), then with H_2O (2 x 50 mL) and finally with brine (50 mL). The organic phase was then dried (anhydrous Na_2SO_4) and concentrated *in vacuo*.

Purification of the residue by column chromatography (20% Et_2O/80% petroleum ether 40-60°C, silica 60) gave **69** as a white solid (0.56 g, 1.16 mmol, 59%), (m.p. 48 °C), (R_f = 0.71 (Et_2O).

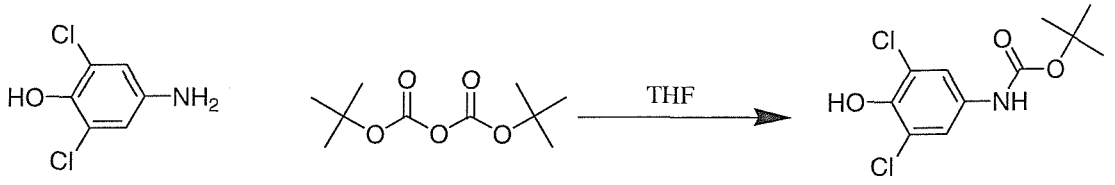
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.61 (d, 1H, *J* = 8.1 Hz, Ar**H**), 7.36-7.26 (m, 3H, Ar**H**), 7.08 (d, 1H, *J* = 2.7 Hz, Ar**H**), 7.03 (dd, 1H, *J* = 8.8, 2.7 Hz, Ar**H**), 6.91 (t, 1H, *J* = 5.8 Hz, CON**H**), 5.60 (s, 1H, C**H**OAr), 4.49 (d, 2H, *J* = 5.8 Hz, ArC$\underline{H}_2$), 2.24 (s, 3H, ArC$\underline{H}_3$), 2.00-1.93 (m, 1H, C$\underline{H}_2$CH$_3$), 1.81-1.75 (m, 3H, C$\underline{H}_2$CH$_3$), 1.45 (s, 3H, C(C$\underline{H}_3$)$_3$), 1.05 (t, 3H, *J* = 7.5 Hz, CH$_2$C$\underline{H}_3$), 0.98 (t, 3H, *J* = 7.5 Hz, CH$_2$C$\underline{H}_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 172.6 ((Et)$_2$C**C**O), 154.4 (N**C**OCH), 153.8 (NH**C**OO), 150.5, 138.1, 132.0 (**Ar**), 131.1 (**Ar**CH$_3$), 129.1, 128.1, 128.0, 123.9, 120.3, 116.2 (**Ar**H), 87.6 (**C**HOAr), 80.7 (**C**(CH$_3$)$_3$), 64.8 (**C**(CH$_2$CH$_3$)$_2$), 44.1 (Ar**C**H$_2$NH), 28.8 (C(**C**H$_3$)$_3$), 24.3, 21.6 (**C**H$_2$CH$_3$), 18.4 (Ar**C**H$_3$), 9.2, 8.9 (CH$_2$**C**H$_3$) ppm.

IR (neat) ν_{max}: 3360 (w), 2972 (w), 1767 (s, C=O), 1698 (s, C=O), 1506 (s), 1455 (m), 1365 (m), 1297 (m), 1240 (m), 1211 (m), 1155 (s), 1119 (s), 1081 (s), 1047 (s), 1023 (s), 898 (w), 734 (m), 698 (m) cm^{-1}.

HRMS (ES$^+$): Calcd. for $C_{27}H_{35}N_3O_5$ 504.2469 (M+Na)$^+$, found 504.2477.

4.6.16 Preparation of (3, 5-dichloro-4-hydroxy-phenyl)-carbamic acid *tert*-butyl ester (132)



M.W. = 178.0 g Mol^{-1}
$C_6H_5Cl_2NO$

M.W. = 218.3 g Mol^{-1}
$C_{10}H_{18}O_5$

M.W. = 278.1 g Mol^{-1}
$C_{11}H_{13}Cl_2NO_3$
132

4-Amino-2,6-dichlorophenol (2.00 g, 11.2 mmol) was stirred in THF (50 mL) at 0 °C. Di-*tert*-butoxy dicarbonate (3.67 g, 16.8 mmol) was dissolved in THF (30 mL) and this solution was added drop-wise over 1 hour. The reaction mixture was left stirring at room temperature for two days and then concentrated *in vacuo*. Purification by column chromatography (CH_2Cl_2, silica 60) followed by recrystallisation (toluene) gave **132** as a white solid (2.52 g, 9.1 mmol, 81%), (m.p. 143 °C (toluene)), (R_f = 0.65 (CH_2Cl_2)).

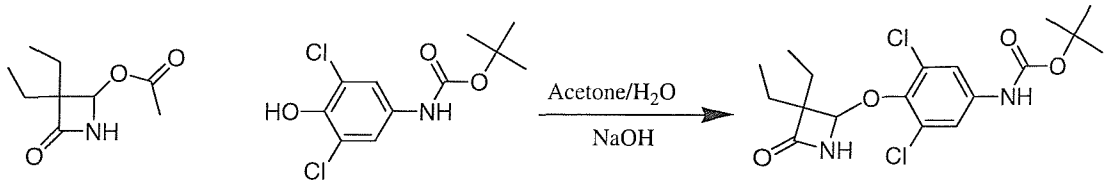
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.56 (s, Ar**H**), 1.57 (s, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 151.3 (**C**O), 143.5 (**Ar**OH), 131.4 (**Ar**), 121.1 (**Ar**Cl), 116.7 (**Ar**H), 78.2 (**C**(CH_3)$_3$), 26.7 (C(**C**H_3)$_3$) ppm.

IR (neat) ν_{max}: 2270 (s), 1673 (s, C=O), 1583 (s), 1519 (s), 1486 (s), 1462 (s), 1399 (m), 1368 (m), 1270 (s), 1249 (s), 1164 (w), 1143 (m), 1065 (w), 875 (w), 853 (w), 755 (m, C-Cl) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{11}H_{13}Cl_2NO_3$ 300.0165 $(M+Na)^+$, found 300.0165.

4.6.17 Preparation of [3, 5-dichloro-4-(3,3-diethyl-4-oxo-azetidin-2-yloxy)-phenyl]-carbamic acid *tert*-butyl ester (70)



M.W. = 185.2 g Mol^{-1} M.W. = 209.2 g Mol^{-1} M.W. = 403.3 g Mol^{-1}

$C_9H_{15}NO_3$ $C_{11}H_{13}Cl_2NO_3$ $C_{18}H_{24}Cl_2N_2O_4$

60 **132** **70**

132 (1.25 g, 4.3 mmol) was dissolved in acetone (10 mL). A solution of NaOH (0.18 g, 4.5 mmol) in H_2O (10 mL) was added and the reaction mixture was stirred for 10 minutes. **60** (0.76, 4.1 mmol) was dissolved in acetone (10 mL) and the resulting solution was added to the reaction mixture which was then stirred for 30 minutes. Et_2O (50 mL) was added and the organic phase was then washed with H_2O (2 x 50 mL) and finally brine (50 mL). The organic layer was dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. Purification of the residue by column chromatography (30% Et_2O/70% petroleum ether 40-60 °C, silica 60) gave **70** as a white solid (0.79 g, 2.4 mmol, 55%), (m.p. 43 °C), (R_f = 0.26 (50% Et_2O/50% petroleum ether 40-60°C).

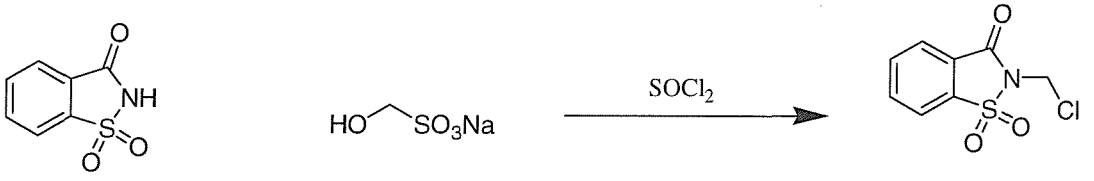
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.45 (s, 2H, Ar**H**), 5.12 (s, 1H, C**H**OAr), 2.20-2.11 (m, 1H, C**H**$_2$CH$_3$), 1.99-1.74 (m, 3H, C**H**$_2$CH$_3$), 1.54 (s, 3H, C(C**H**$_3$)$_3$), 1.19 (t, 3H, *J* = 7.5 Hz, CH$_2$C**H**$_3$), 1.02 (t, 3H, *J* = 7.5 Hz, CH$_2$C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 172.7 (**C**O), 152.8 (**C**OC(CH$_3$)$_3$), 145.6 (**Ar**O), 133.6 (**Ar**NH), 129.5 (**Ar**Cl), 119.2 (**Ar**H), 89.3 (**C**HOAr), 81.9 (**C**(CH$_3$)$_3$) 65.8 (**C**(CH$_2$CH$_3$)$_2$), 26.6 (C(**C**H$_3$)$_3$) 24.3, 22.4 (**C**H$_2$CH$_3$), 9.5, 9.0 (CH$_2$**C**H$_3$) ppm.

IR (neat) ν_{max}: 2970 (w), 2359 (w), 1759 (m, C=O), 1729 (w), 1703 (m), 1582 (s), 1519 (s), 1471 (s), 1390 (w), 1367 (m), 1267 (s), 1217 (m), 1152 (s), 1019 (s), 966 (w), 872 (w), 805 (w), 768 (w, C-Cl) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{18}H_{24}Cl_2N_2O_4$ 403.1185 $(M+H)^+$, found 403.1195

4.7.1 Preparation of 2-chloromethyl-1, 1-dioxo-1, 2-dihydro-1, λ^6-benzo[*d*]isothiazol-3-one (74)



M.W. = 183.2 g Mol^{-1}
$C_7H_5NO_3S$

M.W. = 134.1 g Mol^{-1}
CH_3NO_4SNa

M.W. = 231.6 g Mol^{-1}
$C_8H_6NO_3SCl$
74

Using a procedure similar to the one described by Shu *et al*[188], saccharine (10.00 g, 54.6 mmol) and formaldehyde sodium bisulphite (35.00 g, 0.27 Mol) were gently refluxed together in $SOCl_2$ (80 mL) overnight. The reaction mixture was concentrated *in vacuo* and sat. $NaHCO_3$ solution (100 mL) was slowly added to the residue. The product was extracted with CH_2Cl_2 (200 mL). The organic phase was washed with sat. $NaHCO_3$ solution (2 x 100 mL) then H_2O (2 x 100 mL), dried (anhydrous Na_2SO_4) then concentrated *in vacuo* to give a white solid which was crystallised (toluene) to give **74** as white needles (9.60 g, 44.1 mmol, 81%), (m.p. 141-142 °C (toluene), Lit.[215] 140-142 °C), (R_f = 0.63 (60% EtOAc/40% petroleum ether 40-60 °C)).

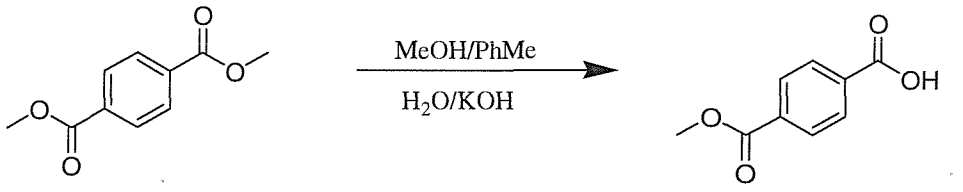
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.16 (d, *J* = 8.2 Hz, 1H, Ar**H**), 8.02 - 7.19 (m, 3H, Ar**H**), 5.62 (s, 2H, NC**H**$_2$Cl) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 158.4 (<u>C</u>O), 138.5, 127.3 (<u>Ar</u>), 136.5, 135.6, 126.6, 122.2 (<u>Ar</u>H), 46.2 (N<u>C</u>H_2Cl) ppm.

IR (neat) ν_{max}: 1746 (s, C=O), 1593 (w), 1464 (w), 1343 (s, SO_2-N), 1322 (m), 1251 (s), 1186 (s, SO_2-N), 750 (m, C-Cl), 702 (w) cm^{-1}.

Unable to obtain LRMS.

4.7.2 Preparation of terephalic acid mono-methyl ester (67)



M.W. = 194.2 g Mol^{-1}
$C_{10}H_{10}O_4$
75

M.W. = 180.2 g Mol^{-1}
$C_9H_8O_4$
76

Dimethyl terephalate (**75**) (25.60 g, 0.13 Mol) was fully dissolved in toluene (500 mL) and MeOH (100 mL). A solution of KOH (7.40 g, 0.13 Mol) in MeOH (100 mL) and H_2O (5 mL) was added. A white solid immediately started to precipitate and the reaction mixture was left to stir overnight. The white precipitate was filtered off and washed with toluene. It was then dissolved in H_2O and any remaining solid was removed by filtration. 2M HCl (100 ml) was added to the filtrate. A white solid immediately precipitated which was filtered, washed with H_2O then re-dissolved in EtOAc. The solution was dried (anhydrous Na_2SO_4) and concentrated *in vacuo* to give **76** as a white solid (19.90 g, 0.11 Mol, 84%), (m.p. 221-223 °C, Lit.[216] 221-223 °C), (R_f = 0.59 (20% MeOH/80% CH_2Cl_2)).

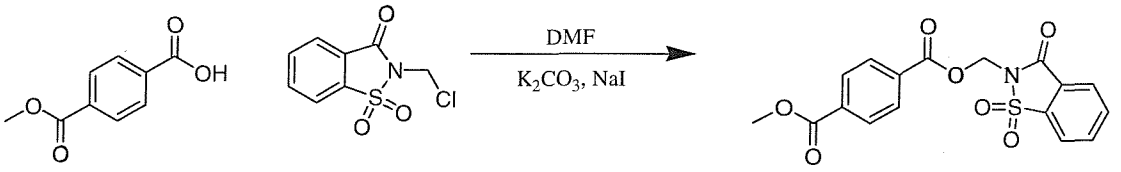
^{1}H NMR (300 MHz, d_6-DMSO): δ 8.04 (s, 4H, Ar<u>H</u>), 3.87 (s, 3H, OC<u>H</u>$_3$) ppm.

^{13}C NMR + DEPT 135 (75 MHz, d_6-DMSO): δ 166.6 (Ar<u>C</u>OOH), 165.5 (Ar<u>C</u>OOCH$_3$), 134.8, 133.1 (<u>Ar</u>), 129.5, 129.3 (<u>Ar</u>H), 52.5 (O<u>C</u>H$_3$) ppm.

IR (neat) ν_{max}: 3200-2200 (br, COOH), 1884 (s, C=O), 1719 (s, C=O), 1573 (w), 1508 (w), 1426 (m), 1409 (m), 1274 (s), 1129 (m), 1104 (s), 1019 (m), 1104 (s), 1019 (m), 938 (m), 876 (m), 797 (m), 727 (s), 694 (m), 553 (w) cm^{-1}.

LRMS (ES$^-$): *m/z* 179.2 ([M-H]$^-$, 100%, 180.2, 10%).

4.7.3 Preparation of terephthalic acid 1-methyl ester 4-(1,1,3-trioxo-1,3-dihydro-1λ^6-benzo[*d*]isothiazol-2-ylmethyl) ester (77)



M.W. = 180.2 g Mol^{-1}
$C_9H_8O_4$
76

M.W. = 231.6 g Mol^{-1}
$C_8H_6NO_3SCl$
74

M.W. = 375.5 g Mol^{-1}
$C_{17}H_{13}NO_7S$
77

76 (1.40 g, 7.8 mmol), **74** (1.78, 7.7 mmol), K_2CO_3 (5.38 g, 39.0 mmol) and NaI (0.10 g, 0.7 mmol) were stirred in DMF (20 mL) overnight. The reaction mixture was then partitioned between H_2O (50 mL) and EtOAc (50mL). The organic phase was washed with H_2O (2 x 50 mL), dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. The residue was crystallised (EtOH) to give **77** as a white solid (1.65 g, 4.4 mmol, 57%), (m.p. 148 °C (EtOH)), (R_f = 0.63 CH_2Cl_2).

^{1}H NMR (400 MHz, d_6-DMSO): δ 8.08-8.00 (m, 4H, Ar<u>H</u>), 7.91-7.79 (m, 4H, Ar<u>H</u>), 6.03 (s, 2H, NC<u>H</u>$_2$O), 3.86 (s, 3H, OC<u>H</u>$_3$) ppm.

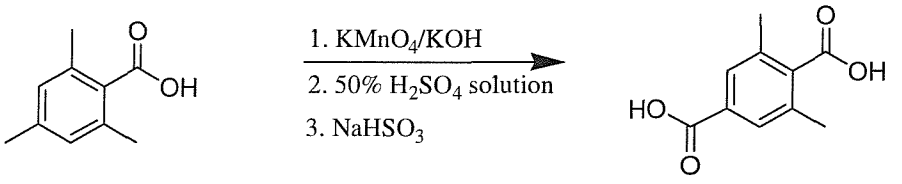
^{13}C NMR + DEPT 135 (100 MHz, d_6-DMSO): δ 166.5, 164.9, 158.6 (<u>C</u>O), 138.2, 135.0, 132.9, 126.9 (<u>Ar</u>), 136.0, 135.1, 130.4, 130.0, 126.3, 121.7 (<u>Ar</u>H), 62.1 (COO<u>C</u>H$_2$), 52.9 (COO<u>C</u>H$_3$) ppm.

IR (neat) ν_{max}: 1749 (m, C=O), 1716 (s, C=O), 1464 (w), 1437 (w), 1336 (s, SO_2-N), 1277 (s), 1247 (s), 1189 (s, SO_2-N), 1114 (s), 1061 (w), 1018 (m), 979 (m), 901 (w), 877 (w), 753 (m), 726 (s), 688 (m), 588 (m) cm^{-1}.

LRMS (ES$^+$): *m/z* 408.5 ([M+Na]$^+$, 100%, 409.5, 10%).

Anal. Calcd. for $C_{17}H_{13}NO_7S$: C, 54.40; H, 3.49; N, 3.73. Found: C, 53.89; H, 3.39; N, 4.06.

4.7.4 Preparation of 2, 6-dimethyl terephthalic acid (79)



M.W. = 164.2 g Mol^{-1}
$C_{10}H_{12}O_2$
78

M.W. = 194.2 g Mol^{-1}
$C_{10}H_{10}O_4$
79

Following a procedure by Noyes and Hufferd[217], KOH (2.45 g, 61.3 mmol) was dissolved in H_2O (150 mL). 2,4,6-trimethylbenzoic acid (**78**) (3.35 g, 20.4 mmol) was added and the mixture was stirred until complete dissolution had occurred. $KMnO_4$ (9.68 g, 61.2 mmol) was added portion-wise over 1 hour and the reaction mixture was then stirred at room temperature for a further 3 hours. The solution was then heated until all the $KMnO_4$ was destroyed and then left to cool. 50% H_2SO_4 solution (30 mL) was added slowly, $NaHSO_3$ (14.70g, 0.14 mol) was then slowly added portion-wise. The reaction mixture was then heated and filtered hot. The resulting solid was dissolved in ammonia 880 solution (10 mL). This solution was

then shaken with Et_2O 30 (mL) and clarified by filtration. 50% H_2SO_4 was added slowly drop-wise until precipitation ceased. H_2O (150 mL) was added and the solution was stirred for 10 minutes. The solution was filtered hot and washed with H_2O. The remaining solid was heated in Et_2O, filtered and crystallised (MeOH/H_2O) to give **79** as a white solid (1.55 g, 8.0 mmol, 40%), (m.p 303 °C (MeOH/H_2O), Lit.[218] 297-298 °C (EtOH)), (R_f = 0.69 (50% MeOH/50% CH_2Cl_2)).

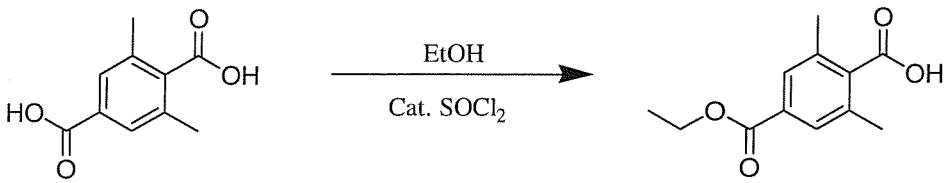
^{1}H NMR (300 MHz, d_6-DMSO): δ 7.65 (s, 2H, Ar**H**), 2.31 (s, 6H, ArC**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (75 MHz, d_6-DMSO): δ 170.2, 166.8 (Ar**C**O), 139.4, 133.8, 130.7, 127.9 (**Ar**), 128.2 (**Ar**H), 19.0 (**Ar**CH_3) ppm.

IR (neat) ν_{max}: 3272-2258 (br, COOH), 1682 (s, C=O), 1572 (w), 1432 (m), 1384 (w), 1433 (m), 1285 (m), 1240 (m), 1144 (m), 1092 (m), 1038 (w), 912 (m), 892 (m), 781 (m), 710 (w) cm^{-1}.

LRMS (ES^-): *m/z* 193 ($[M-H]^-$, 100%, 194, 10%).

4.7.5 Preparation of 2, 6-dimethyl terephthalic acid monoethyl ester (80)



M.W. = 194.2 g Mol^{-1}
$C_{10}H_{10}O_4$
79

M.W. = 222.2 g Mol^{-1}
$C_{12}H_{14}O_4$
80

$SOCl_2$ (4 drops) was added to EtOH (10 mL). **79** (0.61 g, 3.1 mmol) was added and the reaction mixture was refluxed for 5 hours then concentrated *in vacuo*. Ice (2 g) was added and the resulting solid collected by filtration. The solid was crystallised

(EtOH/H_2O) to give **80** as white needles (0.44 g, 2.0 mmol, 64%), (m.p. 153 °C (EtOH/H_2O), Lit.[219] 157-158 °C), (R_f = 0.27 (10% MeOH/90% CH_2Cl_2)).

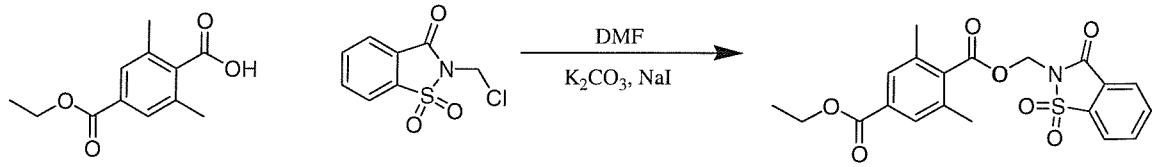
^{1}H NMR (300 MHz, d_6-DMSO): δ 7.65 (s, 2H, Ar**H**), 4.30 (q, 2H, *J* = 7.1 Hz, COOC**H**$_2$), 2.32 (s, 6H, Ar(C**H**$_3$)$_2$), 1.31 (t, 3H, *J* = 7.1 Hz, $COOCH_2C\mathbf{H}_3$) ppm.

^{13}C NMR + DEPT 135 (75 MHz, d_6-DMSO): δ 170.0, (Ar**C**OOH), 165.3 (Ar**C**OOCH$_2$), 139.7, 134.0, 127.9 (**Ar**), 129.8 (**Ar**H), 60.8 (ArCOO**C**H$_2$), 19.0 (**Ar**CH$_3$) 14.1 ($COOCH_2\mathbf{C}H_3$) ppm.

IR (neat) ν_{max}: 1712 (w), 1684 (s, C=O), 1232 (w), 1365 (w), 1284 (s), 1210 (s), 1134 (s), 1091 (w), 1033 (m), 896 (m), 759 (m) cm^{-1}.

LRMS (ES$^-$): *m/z* 221 ([M-H]$^-$, 100%, 222, 10%).

4.7.6 Preparation of 2, 6-dimethyl-terephthalic acid 4-ethyl ester 1-(1, 1, 3-trioxo-1, 3-dihydro-1λ^6-benzo[*d*]isothiazol-2-ylmethyl) ester (81)



M.W. = 222.2 g Mol^{-1} — $C_{12}H_{14}O_4$ — **80**

M.W. = 231.6 g Mol^{-1} — $C_8H_6NO_3SCl$ — **74**

M.W. = 417.4 g Mol^{-1} — $C_{20}H_{19}NO_7S$ — **81**

80 (1.35 g, 6.1 mmol), **74** (1.35, 6.2 mmol), K_2CO_3 (4.28 g, 31.0 mmol) and NaI (0.10 g, 0.7 mmol) were stirred in DMF (20 mL) overnight. The reaction mixture was then partitioned between H_2O (50 mL) and EtOAc (50mL). The organic phase was washed with H_2O (2 x 50 mL), dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. The resulting oil was triturated (Et_2O/petroleum ether 40-60°C) to give a

white solid which was then crystallised (EtOH) to give **81** as a white solid (1.27 g, 3.0 mmol, 49%), (m.p 109 °C (EtOH)), (R_f = 0.80 (10% MeOH/90% CH_2Cl_2).

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.97 (d, 2H, *J* = 6.6 Hz, Ar**H**), 7.93-7.85 (m, 6H, Ar**H**), 7.70 (s, 2H, Ar**H**), 6.08, (s, 2H. NC**H**$_2$O), 4.36 (q, 2H, *J* = 7.1 Hz, COOC**H**$_2$), 2.40 (s, 6H, Ar(C**H**$_3$)$_2$), 1.39 (t, 3H, *J* = 7.1 Hz, COOCH$_2$C**H**$_3$) ppm.

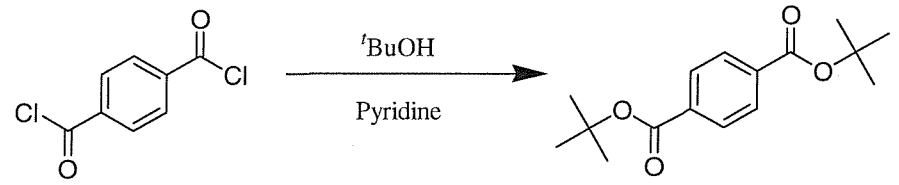
^{13}C NMR + DEPT 135 (75 MHz, $CDCl_3$): δ 167.9, 166.0 (Ar**C**O), 158.3 (N**C**O), 137.8, 135.9, 131.5, 126.4 (**Ar**), 135.7, 134.7, 128.7, 125.8, 121.3 (**Ar**H), 61.3 (COO**C**H$_2$N), 61.2 (COO**C**H$_2$), 19.7 (Ar(**C**H$_3$)$_3$), 14.3 (COOCH$_2$**C**H$_3$) ppm.

IR (neat) ν_{max}: 1759 (s, C=O), 1703 (s, C=O), 1592 (w), 1341 (m, SO_2-O), 1316 (m), 1229 (s), 1193 (s, SO_2-N), 1112 (m), 1041 (s), 964 (m), 868 (w), 750 (s), 689 (m), 586 (m) cm^{-1}.

LRMS (ES^+): *m/z* 440.0 ($[M+Na]^+$, 100%, 441.0, 10%).

Anal. Calcd. for $C_{20}H_{19}NO_7S$: C, 57.55; H, 4.59; N, 3.35. Found: C, 57.20; H, 4.58; N, 3.30.

4.7.7 Preparation of terephthalic acid di-*tert*-butyl ester (83)



M.W. = 203.2 g Mol^{-1}
$C_8H_4O_2Cl_2$
82

M.W. = 278.3 g Mol^{-1}
$C_{16}H_{22}O_4$
83

t-BuOH (20 mL) was added to a solution of terephthaloyl dichloride (**82**) (5.00 g, 24.6 mmol) in pyridine (20 mL). The reaction mixture was heated gently for 5

minutes and then stirred for 3 hours. The reaction mixture was then concentrated *in vacuo* and then partitioned between sat. $NaHCO_3$ solution (200 mL) and Et_2O (250 mL). The organic phase was washed twice more with sat. $NaHCO_3$ solution (200 mL). The organic phase was then clarified by filtration, dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. The resulting solid was crystallised (MeOH) to give **83** as white crystals (3.26 g, 11.7 mmol, 48%), (m.p. 117 °C (MeOH), Lit.[220] 116-117 °C), (R_f = 0.58 (CH_2Cl_2)).

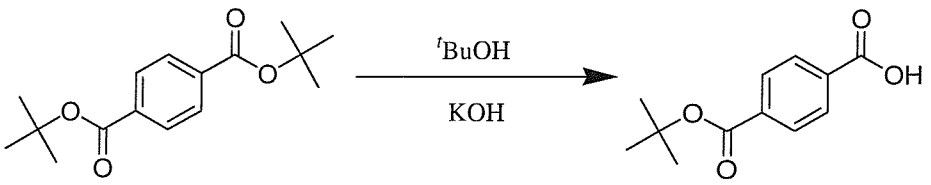
^{1}H NMR (300 MHz, $CDCl_3$): δ 8.02 (s, 4H, Ar**H**), 1.62 (s, 18H, (C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT 135 (75 MHz, $CDCl_3$): δ 165.0 (**C**O), 135.4 (**Ar**), 129.2 (Ar**H**), 81.6 (**C**(CH$_3$)$_3$), 28.1 (C(**C**H$_3$)$_3$) ppm.

IR (neat) ν_{max}: 2970 (w), 1704 (s, C=O), 1458 (w), 1391 (w), 1366 (m), 1280 (s), 1252 (s), 1163 (s), 1118 (s), 1103 (s), 1013 (m), 874 (w), 842 (s), 729 (s) cm^{-1}.

Unable to obtain LRMS.

4.7.8 Preparation of terephthalic acid mono-*tert*-butyl ester (84)



M.W. = 278.3 g Mol^{-1}
$C_{16}H_{22}O_4$
83

M.W. = 222.2 g Mol^{-1}
$C_{12}H_{14}O_4$
84

83 (1.50 g, 5.4 mmol) was heated at 50 °C in ^{t}BuOH (10.0 mL) in the presence of powdered KOH (0.16 g, 2.8 mmol) for 3 hours. The reaction mixture was then concentrated *in vacuo* and partitioned between H_2O (100 mL) and Et_2O (100 mL).

The aqueous phase was then washed with Et_2O (2 x 100 mL). Any remaining Et_2O in the aqueous phase was removed *in vacuo*. The aqueous phase was then clarified by filtration. 2M HCl (100 mL) was then added and the resulting precipitate was filtered and washed with H_2O. The residue was dissolved in Et_2O and the resulting solution was dried (anhydrous $MgSO_4$), filtered and concentrated *in vacuo*. The residue was then crystallised (MeOH) to give **84** as a white solid (0.66 g, 3.0 mmol, 53%), (m.p. 203 °C (MeOH), Lit.[221] 200-201 °C), (R_f = 0.29 (CH_2Cl_2)).

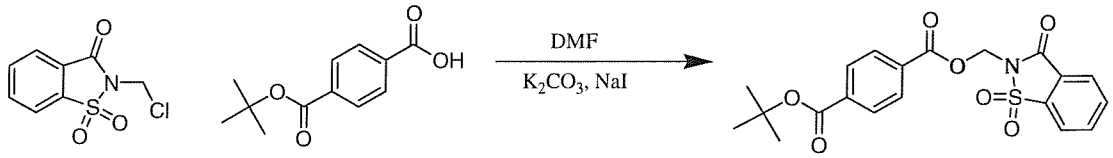
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.15 (d, 2H, *J* = 8.0 Hz, Ar**H**), 8.10 (d, 2H, *J* = 8.0 Hz, Ar**H**), 1.67 (s, 9H, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 164.9 (**C**OOH), 162.6 (**C**OC(CH$_3$)$_3$), 133.2, (**Ar**COOH), 132.8 (**Ar**COC(CH$_3$)$_3$), 127.7 (**Ar**HCCOC(CH$_3$)$_3$), 127.4 (**Ar**HCCOOH), 79.7 (O**C**(CH$_3$)$_3$), 26.0 (C(**C**H$_3$)$_3$) ppm.

IR (neat) ν_{max}: 2972 -2359 (br, COOH), 1709 (m, C=O), 1682 (s, C=O), 1574 (w), 1506 (w), 1426 (w), 1367 (w), 1281 (s), 1253 (s), 1164 (s), 1130 (s), 1105 (s), 1015 (w), 943 (m), 879 (m), 847 (m), 804 (m), 730 (s) cm^{-1}.

LRMS (ES$^-$): *m/z* 220.9 ([M-H]$^-$, 100%, 221.9 10%).

4.7.9 Preparation of terephthalic acid 1-*tert*-butyl ester 4-(1, 1, 3-trioxo-1, 3-dihydro-1λ^6-benzo[*d*]isothiazol-2-ylm ethyl) ester (85)



M.W. = 231.6 g Mol^{-1}
$C_8H_6NO_3SCl$
84

M.W. = 222.2 g Mol^{-1}
$C_{12}H_{14}O_4$
74

M.W. = 417.4 g Mol^{-1}
$C_{20}H_{19}NO_7S$
85

84 (0.47 g, 2.1 mmol), **74** (0.47 g, 2.1 mmol) and K_2CO_3 (0.33 g, 2.4 mmol) were stirred in DMF (20 mL) with NaI (0.10 g, 0.7 mmol) overnight. The reaction mixture was then partitioned between H_2O (50 mL) and EtOAc (50mL). The organic phase was washed with H_2O (2 x 50 mL), dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. The resulting solid was crystallised (EtOH) to give **85** as a white solid (0.36 g, 0.9 mmol, 40%), (m.p. 99 °C (EtOH)), (R_f = 0.42 (CH_2Cl_2)).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.91 (d, 2H, *J* = 7.0 Hz, Ar**H**), 7.89-7.78 (m 6H, Ar**H**), 6.03 (s, 2H, NC**H**$_2$O), 1.52 (s, 9H, C(C**H**$_3$)$_3$) ppm.

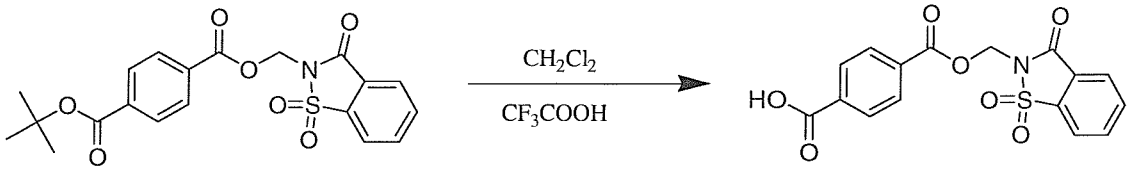
^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 163.4, 163.9 (Ar**C**O), 158.0 (N**C**O), 136.7, 135.8, 132.0, 125.6 (**Ar**), 136.5, 135.5, 129.6, 129.4, 125.7, 121.8 (**Ar**H), 81.8 (**C**(CH$_3$)$_3$), 61.6 (COO**C**H$_2$N), 27.6 (C(**C**H$_3$)$_3$) ppm.

IR (neat) ν_{max}: 1745 (m, C=O), 1711 (s, C=O), 1454 (w), 1338 (m, SO_2-N), 1289 (m), 1240 (s), 1181 (s, SO_2-N), 1094 (s), 1013 (m), 970 (s), 875 (s), 842 (m), 794 (w), 724 (s) cm^{-1}.

LRMS (ES^+): *m/z* 440.0 ($[M+Na]^+$, 100%, 441.0, 10%).

Anal. Calcd. for $C_{20}H_{19}NO_7S$: C, 57.55; H, 4.59; N, 3.35. Found: C, 57.39; H, 4.32; N, 2.98.

4.7.10 Preparation of terephthalic acid mono-(1, 1, 3-trioxo-1, 3-dihydro-1λ^6-benzo[*d*]isothiazol-2-ylm ethyl) ester (86)



M.W. = 417.4 g Mol^{-1}
$C_{20}H_{19}NO_7S$
85

M.W. = 361.3 g Mol^{-1}
$C_{16}H_{11}NO_7S$
86

Using a procedure by Hlasta *et al*[222], **85** (0.20 g, 0.48 mmol) was stirred in CH_2Cl_2 (7.5 mL) and trifluoroacetic acid (2.5 mL) under nitrogen for 2 hours. The reaction mixture was then concentrated *in vacuo* and the resulting solid was heated briefly in Et_2O (10 mL). The suspension was then filtered and washed with Et_2O (5 mL) to give **86** as a white solid (0.14 g, 0.4 mmol, 81%), (m.p. 229-231 °C), (R_f = 0.22 (10% MeOH/90% CH_2Cl_2)).

^{1}H NMR (400 MHz, d_6-DMSO): δ 8.35 (d, 2H, *J* = 10.0 Hz, Ar**H**), 8.20-8.01 (m, 6H, Ar**H**), 6.09 (s, 2H, NC**H**$_2$O) ppm.

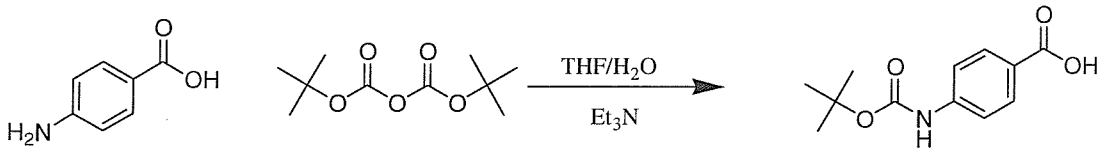
^{13}C NMR + DEPT 135 (100 MHz, d_6-DMSO): δ 166.4 (Ar**C**OOH), 163.9 (Ar**C**OOCH$_2$), 158.0 (N**C**O), 136.7, 135.4 132.0, 125.6 (**Ar**), 136.5, 135.5, 129.7, 125.7, 121.8 (**Ar**H), 61.5 (COO**C**H$_2$N) ppm.

IR (neat) ν_{max}: 1750 (m, C=O), 1723 (s, C=O), 1693, 1431 (w), 1333 (m, SO_2-N), 1276 (s), 1243 (s), 1191 (s, SO_2-N), 1104 (m), 1061 (m), 1016 (m), 976 (m), 924 (w), 879 (m), 804 (w), 750 (m) cm^{-1}.

LRMS (ES$^-$): *m/z* 359.7 ([M-H]$^-$, 100%, 360.8, 10%).

Anal. Calcd. for $C_{16}H_{11}NO_7S$: C, 53.19; H, 3.07; N, 3.87. Found: C, 52.85; H, 3.03; N, 3.66.

4.7.11 Preparation of 4-(*tert*-butoxycarbonylamino)-benzoic acid (89)



M.W. = 137.1 g Mol^{-1} M.W. = 218.3 g Mol^{-1} M.W. = 237.1 g Mol^{-1}

$C_7H_7NO_2$ $C_{10}H_{18}O_5$ $C_{12}H_{15}NO_4$

88 **89**

4-Aminobenzoic acid (**88**) (8.40 g, 61.2 mmol) was stirred in THF (50 mL) and H_2O (50 mL) at 0 °C with Et_3N (7.56 g, 74.7 mmol, 10.5 mL). Di-*tert*-butoxy dicarbonate (14.85, 68.0 mmol) was dissolved in THF (50 mL) and this solution was added drop-wise over 1 hour. The reaction mixture was left stirring overnight then concentrated *in vacuo*. 2M HCl (30 mL) was added drop-wise to the residue. The resulting solid was filtered and washed with H_2O. The solid was dissolved in EtOAc (100 mL), the solution was dried (anhydrous $MgSO_4$) then concentrated *in vacuo*. The residue was then crystallised (EtOAc/hexane) to give **89** as white crystals (10.90 g, 45.9 mmol, 75%), (m.p. 190 °C (EtOAc/hexane), Lit.[223] 190-191 °C (MeOH/H_2O)), (R_f = 0.61 (20% MeOH/80% CH_2Cl_2)).

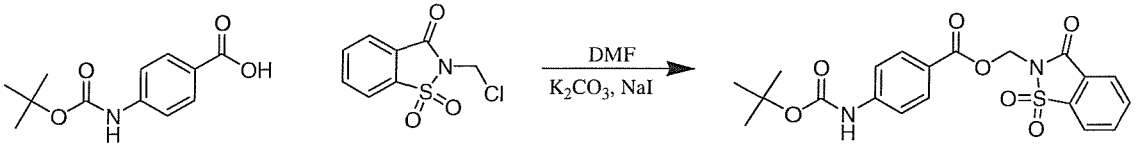
^{1}H NMR (400 MHz, d_6-DMSO): δ 7.96 (d, 2H, *J* = 8.5 Hz, Ar**H**), 7.67 (d, 2H, *J* = 8.5 Hz, Ar**H**), 1.59 (s, 9H, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, d_6-DMSO): δ 165.1 (NH**C**OOC), 150.6 (**C**OOH), 141.9, 122.1 (**Ar**), 128.5, 115.3 (**Ar**H), 77.7 (**C**(CH$_3$)$_3$), 26.1 (C(C**H**$_3$)$_3$) ppm.

IR (neat) ν_{max}: 3354 (m), 2982 (w), 2930 (w), 1683 (s, C=O), 1611 (w), 1578 (w), 1505 (m), 1427 (w), 1386 (w), 1360 (w), 1162 (m), 1120 (w), 1050 (m), 1030 (m), 939 (m), 860 (m), 838 (w), 755 (m), 696 (w) cm^{-1}.

LRMS (ES^-): *m/z* 236 ($[M-H]^-$, 100%, 237, 10%).

4.7.12 Preparation of 4-(*tert*-butoxycarbonylamino)-benzoic acid 1, 1, 3-trioxo-1,3-dihydro-1λ^6-benzo[*d*]isothiazol-2-ylmethyl ester (90)



M.W. = 237.1 g Mol^{-1}
$C_{12}H_{15}NO_4$
89

M.W. = 231.6 g Mol^{-1}
$C_8H_6ClNO_3S$
74

M.W. = 432.4 g Mol^{-1}
$C_{20}H_{20}N_2O_7S$
90

89 (1.08 g, 4.6 mmol), **74** (0.93 g, 4.3 mmol) and K_2CO_3 (3.13 g, 22.7 mmol) were stirred in DMF (20 mL) with NaI (0.10 g, 0.7 mmol) overnight. The reaction mixture was then partitioned between H_2O (50 mL) and EtOAc (50mL). The organic phase was washed with H_2O (2 x 50 mL), dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. The resulting solid was crystallised (EtOH) to give **90** as a white powder (1.48 g, 3.4 mmol, 79%), (R_f = 0.48 (CH_2Cl_2), (m.p. 140-141 °C (EtOH)).

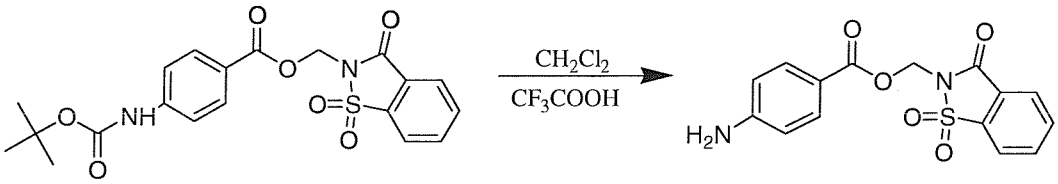
^{1}H NMR (300 MHz, d_6-DMSO): δ 8.37 (d, 1H, *J* = 7.0 Hz, Ar**H**), 8.20-8.03 (m, 3H, Ar**H**), 7.89 (d, 2H, *J* = 7.0 Hz, Ar**H**), 7.70 (d, 2H, *J* = 7.0 Hz, Ar**H**), 6.02 (s, 2H, NC$\underline{H}_2$O), 1.47 (s, 9H, C(C$\underline{H}_3$)$_3$) ppm.

^{13}C NMR + DEPT 135 (75 MHz, d_6-DMSO): δ 164.5, 158.5 (Ar$\underline{C}$O), 152.9 (N$\underline{C}$O), 145.3, 137.3, 126.1, 121.9 (**Ar**), 137.0, 135.9, 131.1, 126.1, 122.2, 117.9 (**Ar**H), 80.3 (**C**(CH$_3$)$_3$), 61.5 (COO$\underline{C}$H$_2$N), 28.5 (C(**C**H$_3$)$_3$) ppm.

IR (neat) ν_{max}: 3353 (w), 1727 (m, C=O), 1698 (s, C=O), 1605 (m), 1528 (m), 1414 (w), 1345 (m, SO_2-N), 1320 (w), 1231 (m), 1157 (s, SO_2-N), 1127 (w), 1060 (m), 972 (w), 856 (w), 748 (m), 675 (m), 584 (m) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{20}H_{20}N_2O_7S$ 455.0883 $(M+Na)^+$, found 455.0885.

4.7.13 Preparation of 4-aminobenzoic acid 1,1,3-trioxo-1,3-dihydro-1λ^6-benzo[*d*]isothiazol-2-ylmethyl ester (91)



M.W. = 432.4 g Mol^{-1}
$C_{20}H_{20}N_2O_7S$
90

M.W. = 332.3 g Mol^{-1}
$C_{15}H_{12}N_2O_5S$
91

90 (2.00 g, 4.6 mmol) was dissolved in CH_2Cl_2 (5 mL) and trifluoroacetic acid (5 mL) was added drop-wise. The solution was stirred for 1 hour and then concentrated *in vacuo*. Sat. $NaHCO_3$ solution (30 mL) was added slowly. The resulting solid was filtered and washed thoroughly with Et_2O to give **91** as a white solid (1.40 g, 4.2 mmol, 91%), (m.p. 169 °C), (R_f = 0.78 (20% MeOH/80% CH_2Cl_2)).

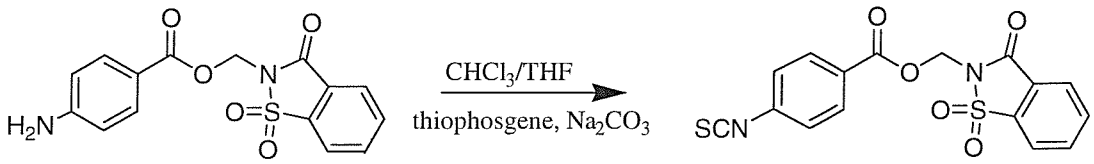
^{1}H NMR (300 MHz, d_6-DMSO): δ 8.34 (d, 1H, *J* = 7.0 Hz, Ar**H**), 8.19-8.00 (m, 3H, Ar**H**), 7.64 (d, 2H, *J* = 7.0 Hz, Ar**H**), 6.58 (d, 2H, *J* = 7.0 Hz, Ar**H**), 5.97, (s, 2H, NC**H**$_2$O) ppm.

^{13}C NMR + DEPT 135 (100 MHz, d_6-DMSO): δ 164.9, 158.6 (Ar**C**O), 154.4 (N**C**O), 137.3, 114.8 (**Ar**), 136.9, 135.9, 132.1, 126.1, 122.2, 113.3 (**Ar**H), 61.1 (COO**C**H$_2$N) ppm.

IR (neat) ν_{max}: 3452 (w), 3387 (w), 3363 (w), 3224-2386 (br, NH_2), 1727 (m, C=O), 1699 (m, C=O), 1600 (s, NH_2), 1355 (m, SO_2-N), 1246 (s), 1198 (s, SO_2-N), 1071 (s), 1058 (s), 977 (m), 848 (m), 750 (s), 699 (m), 678 (m), 583 (s) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{15}H_{12}N_2O_5S$ 333.05396 $(M+H)^+$, found 333.05388.

4.7.14 Preparation of 4-isothiocyanato-benzoic acid 1,1,3-trioxo-1,3-dihydro-1λ⁶-benzo[*d*]isothiazol-2-ylmethyl ester (92)



M.W. = 332.3 g Mol^{-1}
$C_{15}H_{12}N_2O_5S$
91

M.W. = 374.4 g Mol^{-1}
$C_{16}H_{10}N_2O_5S_2$
92

Thiophosgene (1.05 g, 9.0 mmol, 0.7 mL) and Na_2CO_3 (2.00 g, 1.9 mmol) were stirred in $CHCl_3$ (20 mL) at 0 °C. **91** (1.50 g, 4.5 mmol) was dissolved in THF (10 mL) and $CHCl_3$ (20 mL) and this solution added drop-wise over 2 hours. The reaction mixture was left to warm to room temperature overnight. The organic phase was washed with H_2O (3 x 50 mL), dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. Purification of the residue by column chromatography (50% CH_2Cl_2/50% petroleum ether 40-60 °C, silica 60) gave **92** as an off-white solid (1.10 g, 2.9 mmol, 65%), (m.p. 158 °C), (R_f = 0.64 (CH_2Cl_2)).

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.07 (d, 1H, *J* = 7.0 Hz, Ar**H**), 8.00-7.77 (m, 5H, Ar**H**), 7.17 (d, 2H, *J* = 7.0 Hz, Ar**H**), 6.01 (s, 2H, NC**H**$_2$O) ppm.

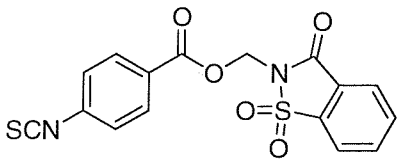
^{13}C NMR + DEPT 135 (75 MHz, $CDCl_3$): δ 164.9 (Ar**C**O), 158.2 (N**C**O), 138.3, 137.7, 136.4, 127.3, 126.5 (**Ar**), 135.6, 134.7, 131.6, 125.8, 125.7, 121.3 (**Ar**H), 61.7 (COO**C**H$_2$N) ppm.

IR (neat) ν_{max}: 3100 (w), 2972 (w), 2050 (s, N=C=S), 1747 (s, C=O), 1728 (s, C=O), 1595 (s), 1463 (w), 1339 (m, SO_2-N), 1237 (m), 1184 (m, SO_2-N), 1145 (m), 1061 (m), 972 (w), 856 (m), 767 (m), 693 (m), 582 (s) cm^{-1}.

LRMS (ES^+): *m/z* 397 ($[M+Na]^+$, 100%, 398, 10%).

Anal. Calcd. for $C_{16}H_{10}N_2O_5S_2$: C, 51.33; H, 2.69; N, 7.48. Found: C, 51.04; H, 2.67; N, 7.27.

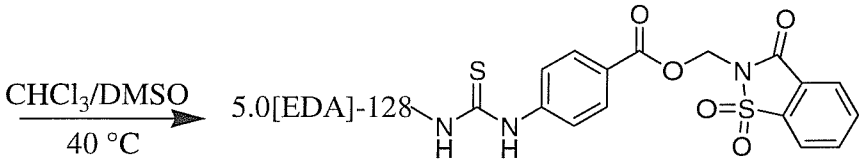
4.7.15 Preparation of 5.0 [EDA]-128-[4-thioureido-benzoic acid 1,1,3-trioxo-1,3-dihydro-1λ^6-benzo[*d*]isothiazol-2-ylmethyl ester] (93)



5.0[EDA]-128-amine

M.W. = 374.4 g Mol^{-1}
$C_{16}H_{10}N_2O_5S_2$
92

M.W. = 28225.1 g Mol^{-1}
$C_{1262}H_{2528}N_{506}O_{252}$



M.W. = 76745.6 g Mol^{-1}
$C_{3310}H_{3808}N_{762}O_{892}S_{256}$
93

5.0 [EDA]-128-amine (81.0 mg, 2.8 x 10^{-6} mol) was dissolved in DMSO (10 mL). A solution of **92** (0.19 g, 0.5 mmol) in $CHCl_3$ (10 mL) was added to the reaction mixture which was then stirred at 40 °C overnight. $CHCl_3$ (50 mL) and H_2O (100mL) were then added and the reaction mixture was shaken; **93** formed as a brown solid from the solution. The solid was collected and washed with $CHCl_3$. The shaking was repeated continually until the organic phase turned clear to give a final yield of (0.12 g, 1.56 x 10^{-6} mol, 56%).

^{1}H NMR (400 MHz, d_6-DMSO): δ 8.40–7.71 (m, Ar**H**), 6.13 (s, NC**H**$_2$O), 4.01-2.31 (m, *n*-C**H**$_2$) ppm.

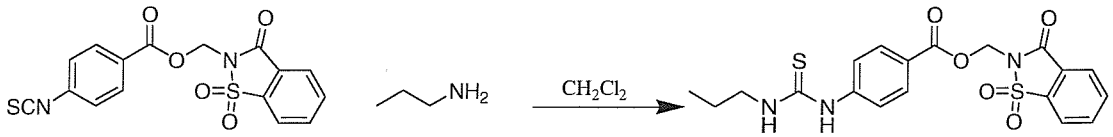
^{13}C NMR + DEPT 135 (100 MHz, d_6-DMSO): δ 180.6, 180.2, 172.1, 171.5 (**C**O), 164.3, 158.3 (N**C**O), 164.3, 137.0, 135.7, 121.2 (**Ar**) 136.9, 135.9, 130.7, 126.1, 122.2 (**Ar**H), 61.4 (N**C**H$_2$O), 52.4, 49.7, 43.8, 39.1, 37.0, 33.4 (**C**H$_2$) ppm.

IR (neat) ν_{max}: 3533-2662 (br), 2362 (m), 2243 (m), 1744 (m, C=O), 1716 (m, C=O), 1634 (m), 1541 (m), 1509 (m), 1338 (m, SO_2-N), 1239 (s), 1187 (s, SO_2-N), 1071 (s, C=S), 970 (m), 749 (m), 676 (m), 584 (m) cm^{-1}.

Unable to obtain LRMS or Maldi mass spec.

Anal. Calcd for $C_{3310}H_{3808}N_{762}O_{892}S_{256}$: C, 51.80; H, 5.00; N, 13.90; S, 10.69 Found: C, 50.56; H, 4.59; N, 11.97; S. 10.02.

4.7.16 Preparation of 4-(3-propyl-thioureido)-benzoic acid 1,1,3-trioxo-1,3-dihydro-1λ^6-benzo[*d*]isothiazol-2-ylmethylester (95)



M.W. = 374.4 g Mol^{-1}
$C_{16}H_{10}N_2O_5S_2$
92

M.W. = 59.1 g Mol^{-1}
C_3H_9N

M.W. = 433.5 g Mol^{-1}
$C_{19}H_{19}N_3O_5S_2$
95

92 (0.10 g, 0.27 mmol) and propylamine (17.0 mg, 0.28 mmol, 23 μL) were stirred together in CH_2Cl_2 (10 mL) for 2 hours. The reaction mixture was then concentrated *in vacuo*. Purification of the residue by column chromatography (50% Et_2O/50% petroleum ether 40-60 °C, silica 60) gave **95** as a white solid (76 mg, 0.18 mmol, 66%), (m.p. 153 °C), (R_f = 0.53 (Et_2O)).

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.36 (d, 1H, *J* = 7.0 Hz, Ar**H**), 8.19 (d, 1H, *J* = 7.0 Hz, Ar**H**), 8.10-8.02 (m, 2H, Ar**H**), 7.88 (d, 2H, *J* = 7.0 Hz, Ar**H**), 7.68 (d, 2H, *J* = 7.0 Hz, Ar**H**), 6.04 (s, 2H, NC**H**$_2$O), 3.42 (m, 2H, NHC**H**$_2$), 1.59-1.52 (m, 2H, *n*-C**H**$_2$), 0.89 (t, 3H, *J* = 7.6 Hz, *n*-C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 179.7 (**C**S), 163.7 (Ar**C**O), 157.7 (N**C**O), 144.7, 135.1, 125.3, 120.3 (**Ar**), 137.0, 136.0, 130.7, 126.1, 122.2, 121.1 (**Ar**H), 61.6 (COO**C**H_2N), 46.1, 21.7 (*n*-C**H**$_2$), 11.8 (*n*-C**H**$_3$) ppm.

IR (neat) ν_{max}: 1784 (m, C=O), 1703 (s, C=O), 1593 (m), 1512 (m), 1472 (m), 1372 (m), 1336 (s, SO_2-N), 1293 (m, CSNH), 1253 (s), 1167 (m, SO_2-N), 1107 (s), 934 (m), 717 (m), 689 (m), 597 (s) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{19}H_{19}N_3O_5S_2$ 456.0658 $(M+Na)^+$, found 456.0663.

4.7.17 Preparation of 3-(*tert*-butoxycarbonylamino)-benzoic acid (133)

M.W. = 137.1 g Mol^{-1} M.W. = 218.3 g Mol^{-1} M.W. = 237.1 g Mol^{-1}

$C_7H_7NO_2$ $C_{10}H_{18}O_5$ $C_{12}H_{15}NO_4$

133

3-Aminobenzoic acid (4.20 g, 30.6 mmol) was stirred in THF (50 mL) and H_2O (50 mL) at 0 °C in the presence of Et_3N (3.95 g, 39.1 mmol, 5.0 mL). Di-*tert*-butoxy dicarbonate (7.50, 34.4 mmol) was dissolved in THF (50 mL) and the resulting solution was added drop-wise over 1 hour. The reaction mixture was left overnight and then concentrated *in vacuo*. 2M HCl (50 mL) was added drop-wise to the residue. The resulting solid was filtered off and washed with H_2O. The solid was then dissolved in EtOAc (100 mL), dried (anhydrous $MgSO_4$) and then concentrated *in vacuo*. The residue was then heated in $CHCl_3$ (100 mL), cooled, filtered and washed with $CHCl_3$ to give **133** as a white solid (5.95 g, 25.1 mmol, 82%), (m.p. 191 °C, Lit.[224] 189-190 °C), (R_f = 0.56 (EtOAc)).

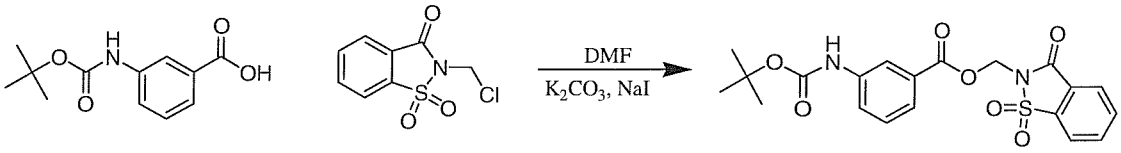
^{1}H NMR (300 MHz, d_6-DMSO): δ 8.16 (s, 1H, Ar**H**), 7.62 (d, 1H, J = 8.0 Hz, Ar**H**), 7.54 (d, 2H, J = 8.0 Hz, Ar**H**), 7.35 (t, 1H, J = 8.0 Hz, Ar**H**), 1.47 (s, 9H, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT 135 (75 MHz, d_6-DMSO): δ 167.2 (NH**C**OOC), 152.7 (**C**OOH), 139.7, 131.3 (**Ar**), 128.7, 122.9, 122.2, 118.8 (**Ar**H), 79.3 (**C**(CH$_3$)$_3$), 28.0 (C(C**H**$_3$)$_3$) ppm.

IR (neat) ν_{max}: 3352 (w), 3001 (w), 1690 (s, C=O), 1593 (m), 1526 (s), 1479 (s), 1450 (m), 1417 (w), 1307 (m), 1289 (s), 1240 (s), 1154 (s), 1057 (w), 945 (w), 774 (w), 745 (s), 632 (m) cm^{-1}.

LRMS (ES$^-$): *m/z* 236 ([M-H]$^-$, 100%, 237, 10%).

4.7.18 Preparation of 3-*tert*-butoxycarbonylamino-benzoic acid 1,1,3-trioxo-1,3-dihydro-1 λ^6-benzo[*d*]isothiazol-2-ylmethyl ester (96)



M.W. = 237.1 g Mol^{-1}
$C_{12}H_{15}NO_4$
133

M.W. = 231.6 g Mol^{-1}
$C_8H_6ClNO_3S$
74

M.W. = 432.4 g Mol^{-1}
$C_{20}H_{20}N_2O_7S$
96

133 (1.08 g, 4.6 mmol), **74** (0.93 g, 4.3 mmol) and K_2CO_3 (3.13 g, 22.7 mmol) were stirred in DMF (20 mL) with NaI (0.10 g, 0.7 mmol) overnight. The reaction mixture was then partitioned between H_2O (50 mL) and EtOAc (50mL). The organic layer was washed with H_2O (2 x 50 mL), dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. The resulting solid was crystallised (EtOH) to give **96** as a white powder. (1.56 g, 3.6 mmol, 84%), (m.p. 155 °C (EtOH)), (R_f = 0.81 (CH_2Cl_2)).

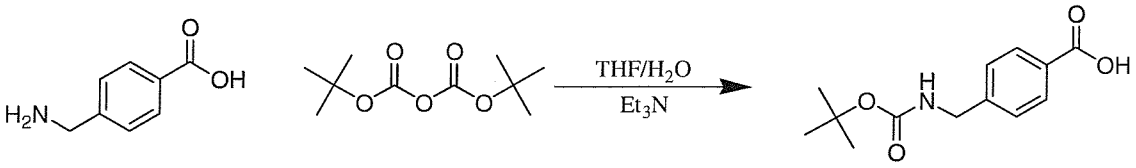
^{1}H NMR (400 MHz, d_6-DMSO): δ 8.35 (d, 1H, J = 7.0 Hz, Ar**H**), 8.20-8.03 (m, 4H, Ar**H**), 7.89 (d, 1H, J = 7.0 Hz, Ar**H**), 7.56 (d, 1H, J = 8.0 Hz, Ar**H**), 7.40 (t, 1H, J = 8.0 Hz, Ar**H**) 6.06 (s, 2H, NC**H**$_2$O), 1.46 (s, 9H, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, d_6-DMSO): δ 165.1, 158.7 (Ar**C**O), 153.4 (N**C**O), 140.1, 137.5, 126.3, 121.9 (**Ar**), 137.0, 136.0, 129.7, 126.2, 123.8, 123.5, 122.3, 119.2 (**Ar**H), 80.1 (**C**(CH$_3$)$_3$), 61.9 (COO**C**H$_2$N), 28.7 (C(**C**H$_3$)$_3$) ppm.

IR (neat) ν_{max}: 3352 (w), 3001 (w), 1690 (s, C=O), 1593 (m), 1526 (s), 1450 (m), 1417 (w), 1307 (m, SO_2-N), 1289 (s), 1240 (s), 1154 (s, SO_2-N), 1057 (m), 945 (w), 863 (m), 774 (m), 745 (s), 679 (w), 632 (m) cm^{-1}.

HRMS (ES$^+$): Calcd. for $C_{20}H_{20}N_2O_7S$ 455.0883 (M+Na$^+$), found 455.0873.

4.7.19 Preparation of 4-(*tert*-butoxycarbonylamino-methyl)-benzoic acid (134)



M.W. = 151.8 g Mol^{-1}
$C_8H_9NO_2$

M.W. = 218.3 g Mol^{-1}
$C_{10}H_{18}O_5$

M.W. = 251.1 g Mol^{-1}
$C_{13}H_{17}NO_4$
134

4-Aminomethylbenzoic acid (10.00 g, 65.9 mmol) was stirred in THF (50 mL) and H_2O (50 mL) at 0 °C with Et_3N (8.80 g, 86.9 mmol, 12.2 mL). Di-*tert*-butoxy dicarbonate (17.30 g, 79.0 mmol) was dissolved in THF (50 mL) and added drop-wise over 1 hour. After 3 hours the reaction mixture was concentrated *in vacuo*. 2M HCl (30 mL) was added drop-wise to the residue, the resulting solid was filtered and washed with H_2O. The solid was dissolved in EtOAc (100 mL), the solution was dried (anhydrous $MgSO_4$) and then concentrated *in vacuo*. The solid residue was crystallised (EtOAc/hexane) to give **134** as white crystals (15.50 g, 61.6 mmol,

78%), (m.p. 166 °C (EtOAc/hexane), Lit.[225] 164-166 °C (EtOAc/hexane)), (R_f = 0.70 (20% MeOH/80% CH_2Cl_2)).

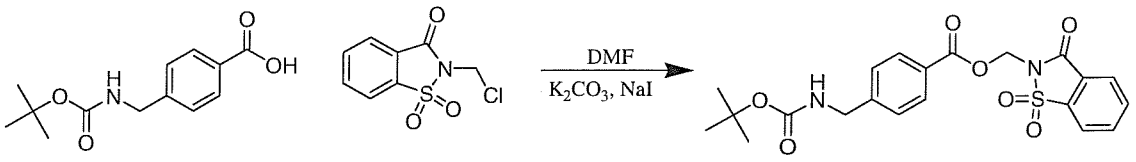
^{1}H NMR (300 MHz, d_6-DMSO): δ 7.89 (d, 2H, *J* = 8.4 Hz, Ar**H**), 7.33 (d, 2H, *J* = 8.4 Hz, Ar**H**), 4.19 (d, 2H, *J* = 6.2 Hz, ArC**H**$_2$), 1.39 (s, 9H, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT 135 (75 MHz, d_6-DMSO): δ 167.2 (NH**C**OOC), 155.8 (**C**OOH), 145.3, 129.2 (**Ar**), 129.3, 126.8 (**Ar**H), 77.9 (**C**(CH$_3$)$_3$), 43.2 (Ar**C**H$_2$), 28.2 (C(C**H**$_3$)$_3$) ppm.

IR (neat) ν_{max}: 3355 (w), 2981-2555 (br, COOH), 1683 (s, C=O), 1611 (w), 1501 (w), 1427 (w), 1292 (w), 1240 (m), 1160 (w), 938 (w), 876 (w), 838 (w), 696 (w), 601 (w), 540 (m) cm^{-1}.

LRMS (ES$^-$): *m/z* 250 ([M-H]$^-$, 100%, 251, 10%).

4.7.20 Preparation of 4-(*tert*-Butoxycarbonylamino-methyl)-benzoic acid 1,1,3-trioxo-1,3-dihydro-1λ^6-benzo[*d*]isothiazol-2-ylmethyl ester (97)



M.W. = 251.3 g Mol^{-1}	M.W. = 231.6 g Mol^{-1}	M.W. = 446.3 g Mol^{-1}
$C_{13}H_{17}NO_4$	$C_8H_6ClNO_3S$	$C_{21}H_{22}N_2O_7S$
134	**74**	**97**

134 (4.00 g, 15.9 mmol), **74** (3.30 g, 15.1 mmol) and K_2CO_3 (11.00 g, 79.5 mmol) were stirred in DMF (20 mL) with NaI (0.10 g, 0.7 mmol) overnight. The reaction mixture was then partitioned between H_2O (50 mL) and EtOAc (50mL). The organic layer was washed with H_2O (2 x 50 mL), dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. The resulting solid was crystallised (EtOH) to give **97** as white needles (0.51 g, 1.1 mmol, 7%), (m.p. 133 °C (EtOH)), (R_f = 0.44 (CH_2Cl_2)).

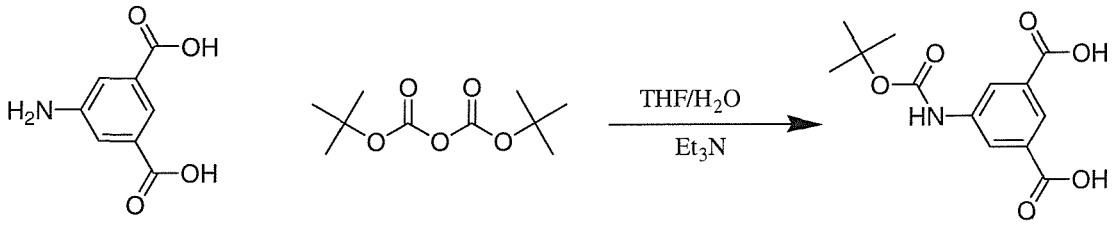
^{1}H NMR (300 MHz, $CDCl_3$): δ 7.91 (d, 2H, *J* = 7.0 Hz, Ar**H**), 7.88-7.76 (m, 6H, Ar**H**), 6.00 (s, 2H, NC**H**$_2$O), 4.27 (d, 2H, *J* = 5.7 Hz, ArC**H**$_2$), 1.37 (s, 9H, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT 135 (75 MHz, $CDCl_3$): δ 165.0, 158.2 (Ar**C**O), 155.9 (N**C**O), 136.7, 135.8, 132.0, 125.6 (**Ar**), 135.6, 134.6, 130.4, 127.2, 125.8, 121.2 (**Ar**H), 79.8 (**C**(CH$_3$)$_3$), 61.5 (COO**C**H$_2$N), 44.3 (Ar**C**H$_2$), 28.4 (C(**C**H$_3$)$_3$) ppm.

IR (neat) ν_{max}: 3334 (w), 1742 (s, C=O), 1721 (s, C=O), 1678 (s, C=O), 1507 (m), 1458 (w), 1335 (m, SO_2-N), 1245 (s), 1189 (m, SO_2-N), 1094 (w), 1055 (w), 960 (w), 855 (m), 750 (s), 675 (m), 583 (s) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{21}H_{22}N_2O_7S$ 469.1040 $(M+Na)^+$, found 469.1038.

4.7.21 Preparation of 5-*tert*-butoxycarbonylamino-isophthalic acid (135)



M.W. = 181.2 g Mol^{-1}
$C_8H_7NO_4$

M.W. = 218.3 g Mol^{-1}
$C_{10}H_{18}O_5$

M.W. = 281.3 g Mol^{-1}
$C_{13}H_{15}NO_6$
135

5-Aminoisopthalic acid (3.00 g, 16.5 mmol) and Et_3N (3.66 g, 36.3 mmol, 4.64 mL) were stirred in THF:H_2O (2:1, 30 mL). Di-*tert*-butoxy dicarbonate (4.34 g, 19.9 mmol) was added and the reaction mixture was stirred overnight and then concentrated *in vacuo*. 2M HCl (100 mL) was added to the residue and the resulting solid was collected by filtration, washed with H_2O and crystallised (EtOAc) to give

135 as a white solid (3.30 g, 11.7 mmol, 71%), (m.p. 187 °C (decomposition), Lit.[226] 185 °C (decomposition)), (R_f = 0.48 (20% MeOH/80% EtOAc)).

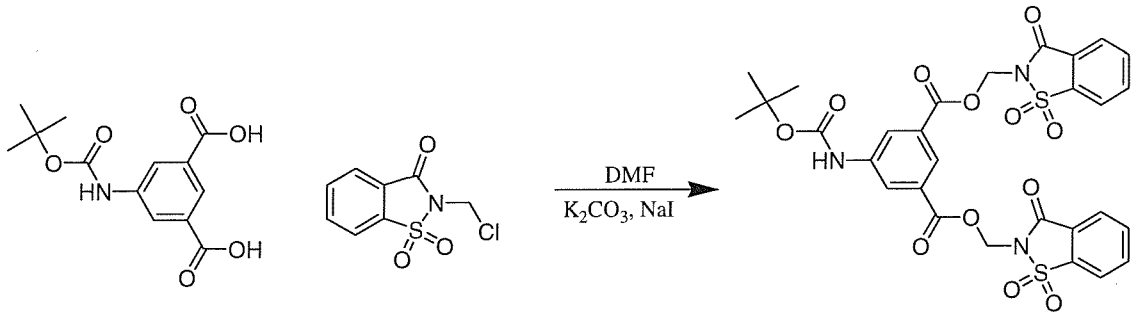
^{1}H NMR (400 MHz, d_6-DMSO): δ 8.32 (s, 2H, Ar**H**), 8.10 (s, 1H, Ar**H**), 1.49 (s, 9H, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, d_6-DMSO): δ 168.5, 154.7 (**C**O), 142.1, 133.6 (**Ar**), 125.4, 124.4 (**Ar**H), 81.6 (**C**(CH$_3$)$_3$), 29.9 (C(**C**H$_3$)$_3$) ppm.

IR (neat) ν_{max}: 3343 (w), 2994 (w), 2824 (w), 2574 (w), 1690 (s, C=O), 1605 (m), 1525 (m), 1439 (m), 1408 (m), 1330 (w), 1272 (s), 1237 (s), 1159 (s), 945 (m), 906 (m), 865 (m), 752 (s), 695 (s), 663 (m), 620 (m) cm^{-1}.

Unable to obtain LRMS.

4.7.22 Preparation of 5-*tert*-butoxycarbonylamino-isophthalic acid bis-(1, 1, 3-trioxo-1, 3-dihydro-1 λ^6-benzo[*d*]isothiazol-2-ylmethyl) ester (98)



M.W. = 281.3 g Mol^{-1}
$C_{13}H_{15}NO_6$
135

M.W. = 231.6 g Mol^{-1}
$C_8H_6ClNO_3S$
74

M.W. = 671.6 g Mol^{-1}
$C_{29}H_{25}N_3O_{12}S_2$
98

135 (0.20 g, 0.7 mmol) and **74** (0.30 g, 1.39 mmol) were stirred in DMF (20 mL) in the presence of K_2CO_3 (0.59 g, 4.27 mmol) and NaI (0.10 g, 0.7 mmol) overnight. The reaction mixture was then diluted with EtOAc (50 mL) and the organic phase was washed with sat. $NaHCO_3$ solution (50 mL) then H_2O (50 mL), dried

(anhydrous Na_2SO_4) and concentrated *in vacuo*. The resulting solid was crystallised (EtOH) to give **98** as a white solid (0.27 g, 4.0 mmol, 56%), (m.p. 201 °C (EtOH)), (R_f = 0.66 (EtOAc)).

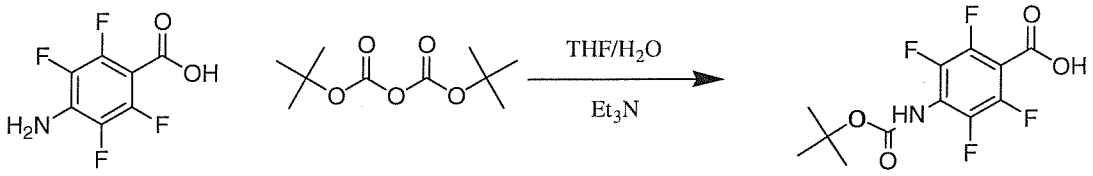
^{1}H NMR (400 MHz, d_6-DMSO): δ 8.49 (d, 1H, *J* = 1.0 Hz, Ar**H**), 8.46 (d, 1H, *J* = 7.6 Hz, Ar**H**), 8.29 (d, 1H, *J* = 7.6 Hz, Ar**H**), 8.24-8.13 (m, 4H, Ar**H**), 6.18 (s, 2H, NC**H**$_2$O), 1.57 (s, 9H, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, d_6-DMSO): δ 164.1, 158.5, 153.1 (**C**O), 141.3, 137.2, 130.2, 126.0 (**Ar**), 137.0, 136.0, 126.2, 123.9, 123.8, 122.2 (**Ar**H), 80.5 (**C**(CH$_3$)$_3$), 62.0 (COO**C**H$_2$N), 28.4 (C(**C**H$_3$)$_3$) ppm.

IR (neat) ν_{max}: 3352 (w), 1739 (s, C=O), 1700 (m), 1606 (w), 1532 (m), 1342 (m, SO_2-N), 1208 (m), 1155 (m, SO_2-N), 1101 (m), 1073 (m), 988 (s), 877 (s), 864 (w), 785 (m), 745 (s), 675 (s), 581 (s), 567 (s) cm^{-1}.

Anal. Calcd. for $C_{29}H_{25}N_3O_{12}S_2$: C, 51.86; H, 3.75; N, 6.25. Found: C, 51.45; H, 3.72; N, 6.22.

4.7.23 Preparation of 4-*tert*-butoxycarbonylamino-2, 3, 5, 6-tetrafluoro-benzoic acid (100)



M.W. = 209.1 g Mol^{-1}
$C_7H_3F_4NO_2$

M.W. = 218.3 g Mol^{-1}
$C_{10}H_{18}O_5$

M.W. = 309.1 g Mol^{-1}
$C_{12}H_{11}F_4NO_4$
100

4-Amino-2,3,5,6-benzoic acid (1.85 g, 8.8 mmol) was stirred in THF (40 mL) and H_2O (20 mL) at 0 °C with Et_3N (0.98 g, 9.7 mmol, 1.4 mL). Di-*tert*-butoxy dicarbonate (4.82 g, 22.1 mmol) was dissolved in THF (30 mL) and this solution was added drop-wise over 1 hour. The reaction mixture was left stirring at room

temperature for 5 days and then concentrated *in vacuo*. 10% Citric acid solution (100 mL) was then added to the residue and the resulting solid was filtered off and washed with H_2O. The solid was then dissolved in EtOAc (100 mL), dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. The residue was then crystallised (toluene) to give **100** as white crystals (1.12 g, 3.6 mmol, 40%), (m.p. 171 °C (toluene)), (R_f = 0.13 (20% MeOH/80% CH_2Cl_2)).

^{1}H NMR (300 MHz, d_6-DMSO): δ 6.64 (s, 1H, ArN**H**), 1.46 (s, 9H, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT 135 (75 MHz, d_6-DMSO): δ 161.3 (Ar**C**OOH), 146.1 (NH**C**O), 145.7 (dd, J = 3.2, 0.1 Hz, **Ar**F), 135.2 (dd, J = 3.2, 0.1 Hz, **Ar**F), 131.8 (t, J = 0.2 Hz, **Ar**), 96.1 (t, J = 0.2 Hz, **Ar**), 85.5 (**C**(CH$_3$)$_3$), 26.8 (C(**C**H$_3$)$_3$) ppm.

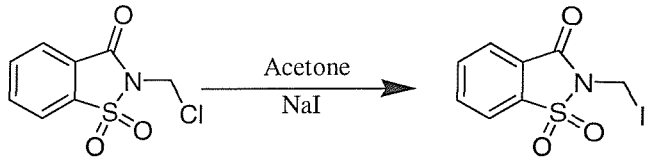
^{19}F NMR (282 MHz, d_6-DMSO): -142.6 (d, J = 0.06 Hz, Ar**F**), -162.3 (d, J = 0.06 Hz, Ar**F**) ppm.

IR (neat) ν_{max}: 3396 (w), 1826 (m), 1692 (m, C=O), 1660 (m, C=O), 1641 (w), 1597 (w), 1501 (m, C-F), 1414 (w), 1307 (w), 1254 (w), 1071 (m), 939 (m), 833 (w), 718 (m) cm^{-1}.

Unable to obtain LRMS

Anal. Calcd. for $C_{12}H_{11}O_4NF_4$: C, 46.61; H, 3.59; N, 4.53. Found: C, 46.14; H, 3.71; N, 4.40.

4.7.24 Preparation of 2-iodomethyl-1,1-dioxo-1,2-dihydro-1λ^6-benzo[*d*]isothiazol-3-one (101)



M.W. = 231.6 g Mol^{-1}	M.W. = 323.1 g Mol^{-1}
$C_8H_6ClNO_3S$	$C_8H_6INO_3S$
74	**101**

74 (2.00 g, 8.6 mmol) and NaI (1.90 g, 12.6 mmol) were stirred together in acetone (40 mL) overnight. The reaction mixture was concentrated *in vacuo* and the resulting solid was dissolved in CH_2Cl_2 (50 mL) and washed with H_2O (2 x 50 mL). The organic phase was dried (anhydrous $MgSO_4$) and concentrated *in vacuo* to produce a solid which was crystallised (toluene/petroleum ether 40-60 °C) to give **101** as a yellow solid (2.20 g, 6.8 mmol, 79%), (m.p. 145 °C (toluene/petroleum ether 40-60 °C)), Lit.[227] 146 °C), (R_f = 0.66 (CH_2Cl_2)).

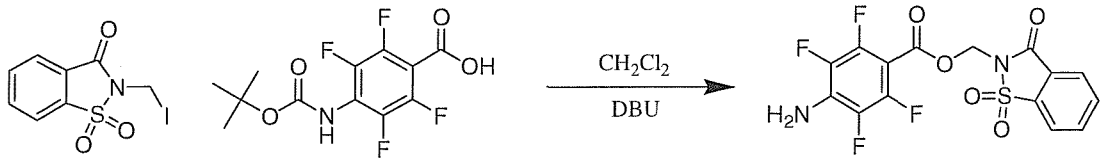
^{1}H NMR (300 MHz, $CDCl_3$): δ 8.10 (dd, *J* = 7.8, 1.4 Hz, 1H, Ar**H**), 7.94 - 7.87 (m, 3H, Ar**H**), 5.43 (s, 2H, NC**H**$_2$I) ppm.

^{13}C NMR + DEPT (75 MHz, $CDCl_3$): δ 158.4 (**C**O), 138.9, 127.8 (**Ar**), 136.5, 135.7, 126.6, 122.2 (**Ar**H), 46.4 (N**C**H$_2$I) ppm.

IR (neat) υ_{max}: 1739 (m, CO), 1460 (w), 1338 (m, SO_2-N), 1297 (m), 1184 (m, SO_2-N), 783 (w) cm^{-1}.

Unable to obtain LRMS

4.7.25 Preparation of 4-amino-2,3,5,6-tetrafluoro-benzoic acid 1,1,3-trioxo-1,3-dihydro-1λ^6-benzo[*d*]isothiazol-2-ylmethyl ester (102)



M.W. = 323.1 g Mol^{-1}
$C_8H_6IO_3NS$
101

M.W. = 293.2 g Mol^{-1}
$C_{12}H_{11}F_4NO_3$,
100

M.W. = 404.3 g Mol^{-1}
$C_{15}H_8F_4N_2O_5S$
102

Following a procedure by Groutas *et al*[228], **101** (0.40 g, 1.2 mmol), DBU (0.19 g, 0.19 mL, 1.3 mmol) and **100** (0.38 g, 1.3 mmol) were stirred in CH_2Cl_2 (40 mL) overnight. The resulting solution was washed with 10% citric acid solution (70 mL) and H_2O (2 x 70 mL), dried (anhydrous $MgSO_4$), and concentrated *in vacuo*. The resulting solid was crystallised (EtOH) to give **102** as an off-white solid (0.19 g, 0.5 mmol, 40%), (m.p. 224 °C (EtOH)), (R_f = 0.39 (CH_2Cl_2)).

^{1}H NMR (400 MHz, d_6-DMSO): δ 8.30 (d, *J* = 7.5, 1H, Ar**H**), 8.05 - 8.22 (m, 3H, Ar**H**), 6.11 (s, 2H, NC**H**$_2$O) ppm.

^{13}C NMR + DEPT (100 MHz, d_6-DMSO): δ 159.4, 158.5 (**C**O), 146.1 (dd, *J* = 2.5, 0.1 Hz, **Ar**F), 137.3 (dd, *J* = 2.5, 0.1 Hz, **Ar**F), 145.4, 136.8 (**Ar**), 134.5, 93.4 (t, *J* = 0.2 Hz, **Ar**), 137.1, 136.0, 126.2, 122.3 (**Ar**H) 61.5 (**C**H$_2$) ppm.

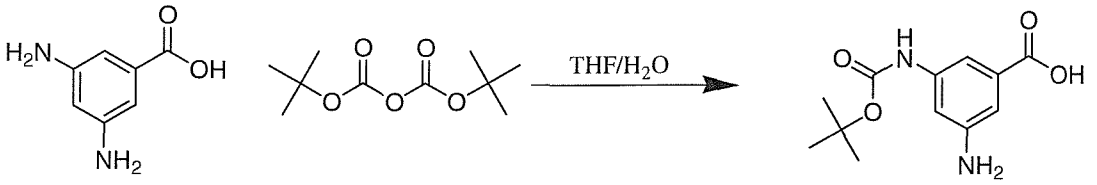
^{19}F NMR (282 MHz, d_6-DMSO): -140.9 (d, *J* = 0.06 Hz, Ar**F**), -161.9 (d, *J* = 0.06 Hz, Ar**F**) ppm.

IR (neat) υ_{max}: 3334 (w), 1741 (m), 1716 (m, C=O), 1651 (m,C=O), 1532 (w), 1504 (m), 1411 (w), 1339 (m, SO_2-N), 1292 (m), 1145 (w, SO_2-N), 1115 (m), 985 (m), 792 (w), 685 (m) cm^{-1}.

Unable to obtain LRMS.

Anal. Calcd. for $C_{15}H_8F_4O_5N_2S$: C, 44.56; H, 1.99; N, 6.93. Found: C, 44.56; H, 2.04; N, 6.77.

4.7.26 Preparation of 3-amino-5-*tert*-butoxycarbonylamino-benzoic acid (104)



M.W. = 152.2 g Mol^{-1}
$C_7H_8N_2O_2$
103

M.W. = 218.3 g Mol^{-1}
$C_{10}H_{18}O_5$

M.W. = 252.2 g Mol^{-1}
$C_{12}H_{16}N_2O_4$
104

3,5-Diaminobenzoic acid (**103**) (5.00 g, 32.9 mmol) was dissolved in THF: H_2O (2:1, 100 mL) and stirred at 0 °C. Di-*tert*-butoxy dicarbonate (7.17 g, 32.8 mmol) was dissolved in THF (50 mL) and the solution was added drop-wise over 1 hour. The reaction mixture was stirred overnight and then concentrated *in vacuo*. The residue was partitioned between 2M HCl (100 mL) and Et_2O (100 mL), the aqueous phase was washed further with Et_2O (100 mL). $NaHCO_3$ was added to the aqueous phase until CO_2 evolution ceased. The product was extracted with EtOAc (2 x 100 mL), the organic phase was then washed with 10% citric acid solution (100 mL) then H_2O (100 mL), dried (anhydrous Na_2SO_4) and concentrated *in vacuo* to give **104** as an off-white solid. (2.52 g, 10.0 mmol, 30%), (m.p. 172 °C), (R_f = 0.28 (10% MeOH/90% CH_2Cl_2)).

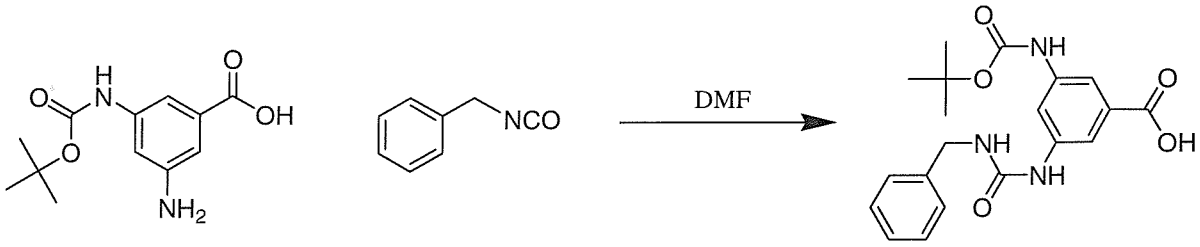
^{1}H NMR (300 MHz, d_6-DMSO): δ 7.28, 7.02, 6.86 (t, 1H, *J* = 1.8 Hz, Ar**H**), 1.51 (s, 9H, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT 135 (75 MHz, d_6-DMSO): δ 167.8 (<u>C</u>OOH), 152.7 (NH<u>C</u>O), 150.0 (<u>Ar</u>NH_2), 140.1 (<u>Ar</u>NHCO), 131.4 (<u>Ar</u>COOH), 109.1, 107.6, 107.3 (<u>Ar</u>H), 78.8 (<u>C</u>$(CH_3)_3$), 28.1 (C(<u>C</u>$H_3)_3$) ppm.

IR (neat) ν_{max}: 3294 (w), 1697 (s, C=O), 1601 (m), 1562 (m, NH_2), 1432 (w), 1366 (w), 1278 (w), 1243 (w), 1218 (m), 1164 (s), 1164 (m), 1067 (w), 839 (w), 696 (m) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{12}H_{16}N_2O_4$ 275.1002 $(M+Na)^+$, found 275.1008.

4.7.27 Preparation of 3-(3-benzyl-ureido)-5-*tert*-butoxycarbonylamino-benzoic acid (105)



M.W. = 252.3 g Mol^{-1} M.W. = 133.2 g Mol^{-1} M.W. = 385.4 g Mol^{-1}

$C_{12}H_{16}N_2O_4$ C_7H_5NO $C_{20}H_{23}N_3O_5$

104 **105**

104 (0.50 g, 2.0 mmols) and benzyl isocyanate (0.52 g, 4.0 mmols, 0.50 mL) were stirred together in DMF (10 mL) for 2 hours. The reaction mixture was diluted with $CHCl_3$ (50 mL) followed by petroleum ether 40-60 °C (200 mL). The precipitate was then collected by filtration to give **105** as an off-white solid (0.37 g, 1.0 mmol, 48%), (m.p. 180 °C), (R_f = 0.43 (20% MeOH/80% CH_2Cl_2)).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.89 (s, 1H, Ar<u>H</u>), 7.83 (s, 1H, Ar<u>H</u>), 7.75 (s, 1H, Ar<u>H</u>), 7.35-7.46 (m, 5H, Ar<u>H</u>), 6.68 (t, *J* = 5.8 Hz, 1H, CON<u>H</u>CH_2), 4.41 (d, *J* = 5.8 Hz, 2H, C<u>H</u>$_2$Ar), 1.58 (s, 9H, C(C<u>H</u>$_3)_3$) ppm.

^{13}C NMR + DEPT (100 MHz, $CDCl_3$): δ 168.0, 155.7, 153.4 (**C**O), 141.5, 140.9, 140.6, 132.1 (**Ar**) 128.9, 127.7, 127.3, 133.2, 112.9, 112.0 (**Ar**H), 79.8 (**C**$(CH_3)_3$), 43.4 (**C**H_2Ar), 28.7 (C(**C**$H_3)_3$) ppm.

IR (neat) υ_{max}: 3301 (w), 2978 (w), 1687 (s, C=O), 1647 (m), 1557 (m), 1532 (m), 1455 (m), 1393 (w), 1366 (w), 1240 (s), 1159 (s), 1071 (w), 884 (w), 722 (w), 697 (w) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{20}H_{23}N_3O_5$ 408.1530 $(M+Na)^+$, found 408.1539.

4.7.28 Preparation of 3-*tert*-Butoxycarbonylamino-5-(3-ethyl-ureido)-benzoic acid (106)

M.W. = 252.2 g Mol^{-1}
$C_{12}H_{16}N_2O_4$
104

M.W. = 71.1 g Mol^{-1}
C_3H_5NO

M.W. = 323.3 g Mol^{-1}
$C_{15}H_{21}N_3O_5$
106

104 was dissolved in THF (10 mL). Ethyl isocyanate (0.19 g, 2.7 mmol, 0.24 mL) was added and the reaction mixture was stirred for 4 hours. The solution was then concentrated *in vacuo*. The residue was heated in toluene, cooled, filtered and the solid washed with petroleum ether 40-60 °C to give **106** as a white solid (0.62 g, 1.9 mmol, 87%), (m.p. 173 °C (decomposition)), (R_f = 0.32 (50% MeOH/50% EtOAc)).

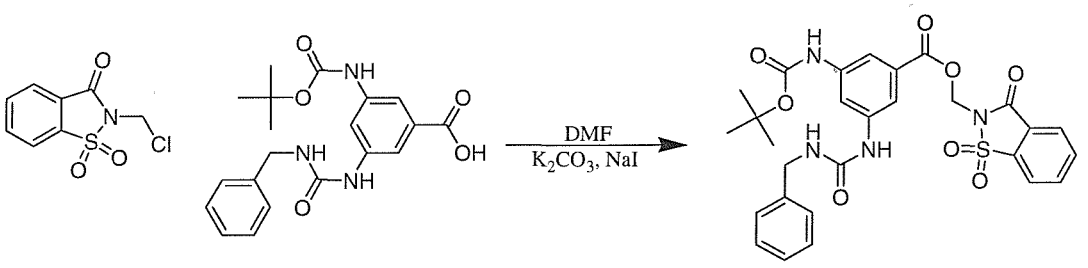
^{1}H NMR (400 MHz, d_6-DMSO): δ 7.87, 7.79, 7.73 (s, 1H, Ar**H**), 3.21 (m, 2H, NHC**H**$_2$CH$_3$), 1.58 (s, 9H, OC(C**H**$_3)_3$), 1.16 (t, 3H, J = 7.2 Hz, NHCH$_2$C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, d_6-DMSO): δ 167.9, 155.4, 153.2 (<u>C</u>O), 141.5, 140.4, 131.9 (<u>Ar</u>), 113.0, 112.5, 111.8 (Ar<u>H</u>), 79.6 (<u>C</u>$(CH_3)_3$), 34.4 (NH<u>C</u>H_2CH_3), 28.6 (C(<u>C</u>$H_3)_3$), 15.9 ($NHCH_2$<u>C</u>H_3) ppm.

IR (neat) ν_{max}: 3310 (w), 2975 (w), 1702 (s, C=O), 1605 (s), 1562 (s), 1538 (s), 1448 (m), 1432 (m), 1366 (m), 1281 (m), 1234 (s), 1162 (s), 1077 (w), 884 (m), 772 (m), 716 (m), 676 (m) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{13}H_{17}N_1O_5$ 346.1373 $(M+Na)^+$, found 346.1369.

4.7.29 Preparation of 3-(3-benzyl-ureido)-5-*tert*-butoxycarbonylamino-benzoic acid 1,1,3-trioxo-1,3-dihydro-1λ^6-benzo[*d*]isothiazol-2-ylmethyl ester (107)



M.W. = 231.6 g Mol^{-1}
$C_8H_6NO_3SCl$
74

M.W. = 385.4 g Mol^{-1}
$C_{20}H_{23}N_3O_5$
105

M.W. = 580.6 g Mol^{-1}
$C_{28}H_{28}N_4O_8S$
107

74 (0.10 g, 0.5 mmol), **105** (0.20 g, 0.5 mmol), K_2CO_3 (0.36 g, 2.4 mmol) and NaI (0.10 g, 0.7 mmol) were stirred in DMF (10 mL) overnight. The reaction mixture was diluted with EtOAc (20 mL) and the organic phase was washed with sat. $NaHCO_3$ solution (20 mL) then H_2O (2 x 20 mL), dried (anhydrous Na_2SO_4) and concentrated *in vacuo*. Purification of the residue by column chromatography (CH_2Cl_2, silica 60) gave **107** in the form of a white solid (0.13 g, 0.2 mmol, 43%), (m.p. 128 °C), (R_f = 0.56 (10% MeOH/90% CH_2Cl_2)).

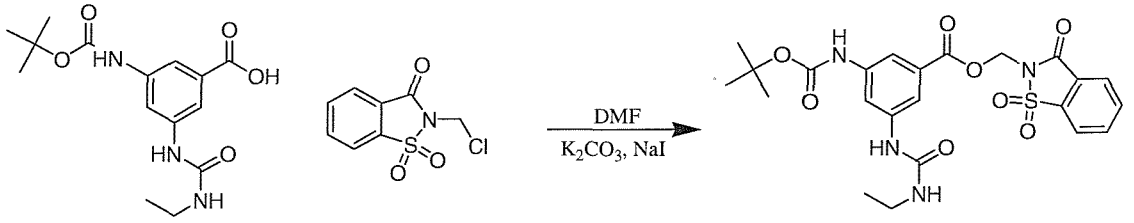
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.17 (d, 1H, *J* = 7.5 Hz, Ar**H**), 8.00-7.92 (m, 3H, Ar**H**), 7.68 (s, 1H, Ar**H**), 7.52 (s, 1H, Ar**H**), 7.38-7.27 (m, 5H, Ar**H**), 6.82 (s, 1H, Ar**H**), 6.10 (s, 2H, NC**H**$_2$O), 4.46 (s, 2H, NHC**H**$_2$Ar), 1.56 (s, 9H, C(C**H**$_3$)$_3$) ppm.

C^{13} + DEPT 135 NMR (100 MHz, $CDCl_3$): δ 165.1, 158.6, 155.4, 153.1 (**C**O), 162.7, 137.1, 136.0, 128.7, 127.5, 127.1, 126.1. 122.3, 112.8, 112.6 (**Ar**H), 141.7, 140.8, 140.7, 137.3, 129.5, 126.0 (**Ar**), 79.8 (**C**(CH$_3$)$_3$), 61.7 (N**C**H$_2$O), 43.2 (**C**H$_2$Ar), 28.5 (C(**C**H$_3$)$_3$) ppm.

IR (neat) υ_{max}: 2982 (w), 1731 (m, C=O), 1651 (w), 1607 (w), 1538 (m), 1495 (w), 1453 (m), 1344 (m, SO_2-N), 1192 (w), 1150 (m, SO_2-N), 1096 (m), 979 (s), 870 (m), 747 (m), 583 (s) cm^{-1}.

HRMS (ES$^+$): Calcd. for $C_{28}H_{28}N_4O_8S$ 603.1520 $(M+Na)^+$, found 603.1505.

4.7.30 Preparation of 3-*tert*-Butoxycarbonylamino-5-(3-ethyl-ureido)-benzoic acid 1,1,3-trioxo-1,3-dihydro-1λ^6-benzo[*d*]isothiazol-2-ylmethyl ester (108)



M.W. = 323.3 g Mol^{-1} — $C_{15}H_{21}N_3O_5$ — **106**

M.W. = 231.6 g Mol^{-1} — $C_8H_6NO_3SCl$ — **74**

M.W. = 518.5 g Mol^{-1} — $C_{23}H_{26}N_4O_8S$ — **108**

106 (0.30 g, 0.93 mmol), **74** (0.19 g, 0.88 mmol) and K_2CO_3 (0.64 g, 4.6 mmol) were stirred in DMF (5 mL) with NaI (0.10 g, 0.7 mmol) overnight. The reaction mixture was then partitioned between H_2O (50 mL) and EtOAc (50mL). The organic phase was washed with H_2O (2 x 50 mL), dried (anhydrous $MgSO_4$) and concentrated *in vacuo* to give **108** as a white powder (0.31 g, 0.60 mmol, 64%), (m.p. 111 °C), (R_f = 0.62 (EtOAc)).

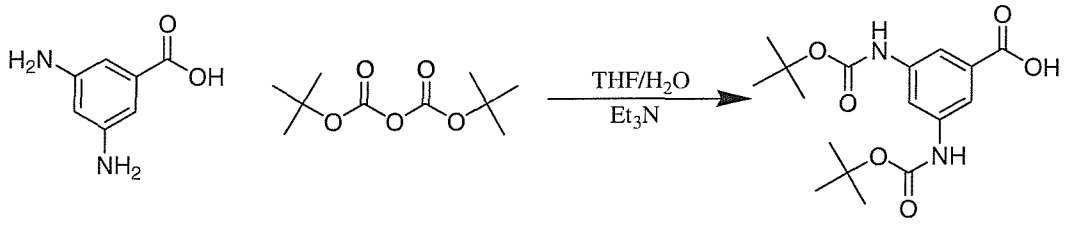
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.01 (d, 1H, J = 7.3 Hz, Ar**H**), 7.84 (m, 3H, Ar**H**), 7.52, (s, 1H, Ar**H**), 7.34 (s, 1H, Ar**H**), 7.26 (s, 1H, Ar**H**), 5.94 (s, 2H, NC**H**$_2$O), 3.12 (q, 2H, J = 6.8 Hz, NHC**H**$_2$CH$_3$), 1.38 (s, 9H, C(C**H**$_3$)$_3$), 0.99 (t, 2H, J = 7.2 Hz, NHCH$_2$C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 163.8, 157.1, 154.5, 151.7 (Ar**C**O), 139.1, 138.2, 133.5, 124.6, 124.2 (**Ar**), 136.0, 135.0, 126.2, 121.6, 115.7, 115.4, 114.7 (**Ar**H), 79.6 (**C**(CH$_3$)$_3$), 60.3 (COO**C**H$_2$N), 33.9 (NH**C**H$_2$CH$_3$), 27.1 (C(**C**H$_3$)$_3$), 15.6 (NHCH$_2$**C**H$_3$) ppm.

IR (neat) υ_{max}: 1732 (m, C=O), 1659 (m, C=O), 1609 (m), 1548 (m), 1344 (m, SO_2-N), 1214 (s), 1195 (s, SO_2-N), 1155 (s), 1099 (m), 987 (m), 876 (m), 750 (m), 677 (m), 584 (s) cm^{-1}.

HRMS (ES$^+$): Calcd. for $C_{23}H_{26}N_4O_8S$ 541.1364 $(M+Na)^+$, found 541.1367.

4.7.31 Preparation of 3,5-bis-*tert*-butoxycarbonylamino-benzoic acid (136)



M.W. = 152.2 g Mol^{-1} M.W. = 218.3 g Mol^{-1} MW = 352.4 g Mol^{-1}

$C_7H_8N_2O_2$ $C_{10}H_{18}O_5$ $C_{17}H_{24}N_2O_6$

103 **136**

3,5-Diaminobenzoic acid (**103**) (1.00 g, 5.7 mmol) was dissolved in H_2O (50 mL) with Et_3N (0.73 g, 7.2 mmol, 0.9 mL). Di-*tert*-butoxy dicarbonate (3.00 g, 13.8 mmol) was dissolved in THF (50 mL) and added drop-wise over an hour. The reaction mixture was stirred overnight and then concentrated *in vacuo*. 2M HCl (70

mL) was added to the residue. The resulting solid was dissolved in Et_2O (50 mL) and washed in H_2O (2 x 50 mL), dried (anhydrous $MgSO_4$), concentrated *in vacuo* then crystallised (Et_2O/petroleum ether 40-60 °C) to give **136** as a white solid (1.57 g, 4.5 mmol, 79%), (m.p. 167 °C (Et_2O/petroleum ether 40-60 °C)), (R_f = 0.45 (EtOAc)).

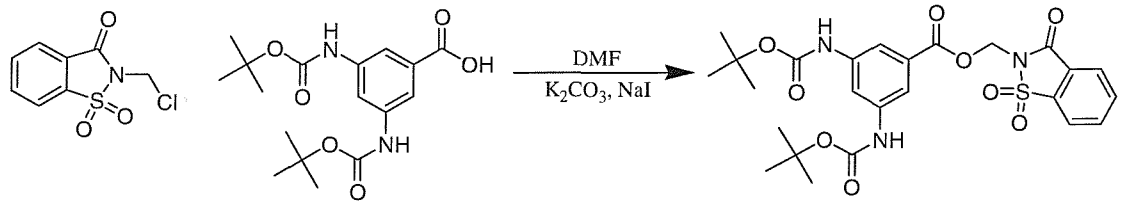
^{1}H NMR (400 MHz, d_6-DMSO): δ 7.86 (s, 1H, Ar**H**), 7.70 (s, 2H, Ar**H**), 1.47 (s, 18H, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT (100 MHz, d_6-DMSO): δ 167.7, 153.2 (**C**O), 140.4, 131.8 (**Ar**), 113.7, 112.8 (**Ar**H), 79.6 (**C**(CH$_3$)$_3$), 28.6 (C(**C**H$_3$)$_3$) ppm.

IR (neat) υ_{max}: 3010 (w), 1693 (s), 1607 (m, C=O), 1532 (m), 1454 (w), 1392 (w), 1367 (w), 1152 (s), 866 (w), 772 (w), 726 (w), 674 (w) cm^{-1}.

HRMS (ES$^+$): Calcd. for $C_{17}H_{24}N_2O_6$ 375.1515 $(M+Na)^+$, found 375.1519.

4.7.32 Preparation of 3,5-bis-*tert*-butoxycarbonylamino-benzoic acid 1,1,3-trioxo-1,3-dihydro-1λ^6-benzo[*d*]isothiazol-2-ylmethyl ester (109)



M.W. = 231.6 g Mol^{-1}
$C_8H_6ClNO_3S$
74

M.W. = 352.4 g Mol^{-1}
$C_{17}H_{24}N_2O_6$
136

M.W. = 547.6 g Mol^{-1}
$C_{25}H_{29}SN_3O_9$
109

136 (0.50 g, 2.2 mmol), **74** (0.78 g, 2.2 mmol), K_2CO_3 (0.33 g, 2.2 mol) and NaI (0.10 g, 0.7 mmol) were stirred together in DMF (10 mL) overnight. The reaction mixture was then partitioned between EtOAc (40 mL) and washed with H_2O (3 x 40 mL). The organic phase was dried (anhydrous $MgSO_4$) and the solution concentrated

in vacuo. The resulting residue was purified by column chromatography (CH_2Cl_2, silica 60) to give **109** as a white solid (0.59 g, 1.1 mmol, 49%), (m.p. 179 °C), (R_f = 0.80 (10% MeOH/90% CH_2Cl_2)).

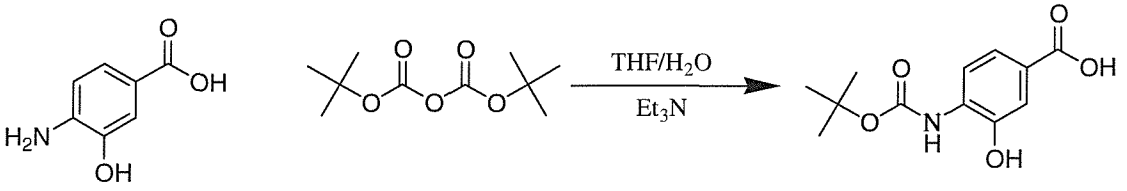
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.98 (d, J = 7.3 Hz, 1H, Ar**H**), 7.92 – 7.84 (m, 5H, Ar**H**), 7.58 (d, 2H, J = 1.9 Hz, Ar**H**), 6.05 (s, 2H, NC**H**$_2$O), 1.48 (s, 18H, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT (100MHz, $CDCl_3$): δ 165.1, 158.6, 152.9 (**C**O), 139.9, 138.2, 130.3, 126.9 (**Ar**), 135.9, 135.0, 126.2, 121.6 114.7, 113.8 (**Ar**H), 84.3 (**C**(CH$_3$)$_2$), 62.0 (N**C**H$_2$O), 26.7 (C(**C**H$_3$)$_3$) ppm.

IR (neat) υ_{max}: 3352 (w), 1726 (m, C=O), 1706 (m, C=O), 1608 (w), 1538 (m), 1448 (w), 1355 (m, SO_2-N), 1309 (w), 1152 (s, SO_2-N), 1115 (w), 1061 (w), 997 (w), 865 (w), 751 (m), 583 (m) cm^{-1}

HRMS (ES$^+$): Calcd. for $C_{25}H_{29}N_3O_9S$ 570.1517 $(M+Na)^+$, found 570.1513.

4.7.33 Preparation of 4-*tert*-Butoxycarbonylamino-3-hydroxy-benzoic acid (113)



M.W. = 153.1 g Mol^{-1} M.W. = 218.3 g Mol^{-1} M.W. = 253.3 g Mol^{-1}

$C_7H_7NO_3$ $C_{10}H_{18}O_5$ $C_{12}H_{15}NO_5$

112 **113**

4-Amino-3-hydroxybenzoic acid (**112**) (2.41 g, 15.7 mmol) and Et_3N (1.67 g, 16.4 mmol, 2.1 mL) were stirred in THF:H_2O (2:1, 30 mL). Di-*tert*-butoxy dicarbonate (4.11 g, 18.8 mmol) was added. The reaction mixture was stirred overnight then concentrated *in vacuo*. 2M HCl was added and the resulting solid was extracted with

EtOAc (100 mL), washed with H_2O (2 x 100 mL), dried (anhydrous Na_2SO_4) and concentrated *in vacuo*. The resulting solid was crystallised (EtOAc/petroleum ether 40-60 °C) to give **113** as a red solid (0.62 g, 2.4 mmol, 16%), (m.p. 172 °C (decomposition)), (R_f = 0.44 (20% MeOH/80% EtOAc)).

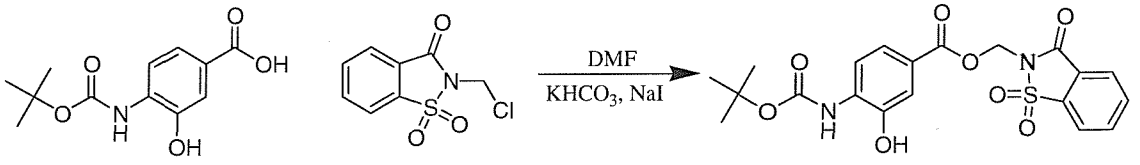
^{1}H NMR (400 MHz, d_6-DMSO): δ 7.87 (s, 1H, Ar**H**), 7.83 (d, 1H, *J* = 8.1 Hz, Ar**H**), 7.38 (d, 1H, *J* = 1.7 Hz, Ar**H**), 1.47 (s, 9H, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, d_6-DMSO): δ 165.7, 150.9 (**C**O), 144.5, 129.6, 117.0 (**Ar**), 121.5, 118.9, 115.7 (**Ar**H), 78.5 (**C**(CH$_3$)$_3$), 28.4 (C(**C**H$_3$)$_3$) ppm.

IR (neat) ν_{max}: 3500-2200 (Br, COOH), 3401 (m), 1704 (s, CO), 1666 (s, CO), 1604 (s), 1536 (s), 1504 (s), 1438 (s), 1408 (m), 1387 (m), 1370 (m), 1324 (m), 1283 (m), 1196 (m), 1148 (s), 1101 (s), 1047 (s), 1025 (s), 938 (s), 899 (s), 888 (s), 822 (m), 766 (s), 743 (m), 615 (m) cm^{-1}.

HRMS (ES$^+$): Calcd. for $C_{12}H_{15}NO_5$ 276.0842 $(M+Na)^+$, found 276.0847.

4.7.34 Preparation of 4-*tert*-butoxycarbonylamino-3-hydroxy-benzoic acid 1, 1, 3-trioxo-1,3-dihydro-1 λ^6-benzo[*d*]isothiazol-2-ylmethyl ester (114)



M.W. = 253.3 g Mol^{-1} M.W. = 231.6 g Mol^{-1} M.W. = 448.2 g Mol^{-1}

$C_{12}H_{15}NO_5$ $C_8H_6NO_3SCl$ $C_{20}H_{20}N_2O_8S$

113 **74** **114**

113 (0.30 g, 1.2 mmol), **74** (0.24 g, 1.1 mmol), $KHCO_3$ (0.12 g, 1.2 mmol) and NaI (0.10 g, 0.7 mmol) were stirred in DMF (10 mL) overnight then diluted with EtOAc (50 mL). The organic layer was washed with sat. $NaHCO_3$ solution (2 x 50 mL) then

H_2O (50 mL), dried (anhydrous Na_2SO_4) and the solution concentrated *in vacuo*. Purification by column chromatography (10% EtOAc/90% petroleum ether 40-60 °C, silica 60) gave **114** as a white solid (0.22 g, 0.49 mmol, 44%), (m.p. 69 °C), (R_f = 0.53 (50% EtOAc/50% petroleum ether 40-60 °C)).

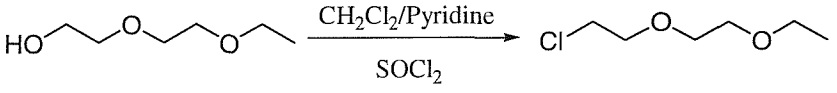
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.00 (d, 1H, *J* = 7.6 Hz, Ar**H**), 7.91-7.78 (m, 4H, *J* = 7.5 Hz, Ar**H**), 7.63 (d, 1H, *J* = 9.0 Hz, Ar**H**), 7.56 (dd, 1H, *J* = 6.2, 1.8 Hz, Ar**H**), 6.03 (NC**H**$_2$O), 1.51 (C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): 165.5, 158.9, 153.7 (**C**O), 145.3, 138.0, 132.4. 126.8, 123.6 (**Ar**), 136.2, 136.0, 135.2, 135.0, 128.9, 126.2, 123.9, 121.7, 119.1, 117.9 (Ar**H**), 82.2 (**C**(CH$_3$)$_3$), 61.5 (COO**C**H$_2$N), 28.6 (C(**C**H$_3$)$_3$) ppm.

IR (neat) ν_{max}: 1725 (m, C=O), 1604 (w), 1530 (m), 1502 (m), 1457 (w), 1429 (m), 1339 (m, SO_2-N), 1284 (m), 1256 (m), 1191 (s, SO_2-N), 1149 (s), 1073 (s), 1023 (m), 981 (s), 879 (w), 749 (s), 677 (m), 583 (s) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{20}H_{20}N_2O_8S$ 471.0833 $(M+Na)^+$, found 471.0835.

4.7.35 Preparation of 1-chloro-2-(2-methoxy-ethoxy)-ethane (115)



M.W. = 120.2 g Mol^{-1}
$C_5H_{12}O_3$

M.W. = 138.6 g Mol^{-1}
$C_5H_{11}O_2Cl$
115

Di-ethylene glycol monoethyl ether (9.99 g, 82.4 mmol, 10.0 mL) and pyridine (10.0 mL) were stirred in CH_2Cl_2 (80 mL). $SOCl_2$ (14.80 g, 9.1 mL, 0.13 mol) was added drop-wise and the reaction mixture was refluxed for 5 hours. The reaction mixture was concentrated *in vacuo*, then diluted with CH_2Cl_2 (30 mL). The organic phase

was washed with 2M HCl (2 x 30 mL) then H_2O (30 mL), dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. The resulting residue was purified by distillation to give **115** as a yellow oil (6.52 g, 47.0 mmol, 57%), (b.p. 184 °C at 760 mmHg, Lit.[229] 180-181 °C at 760 mmHg), (R_f = 0.76 (Et_2O)).

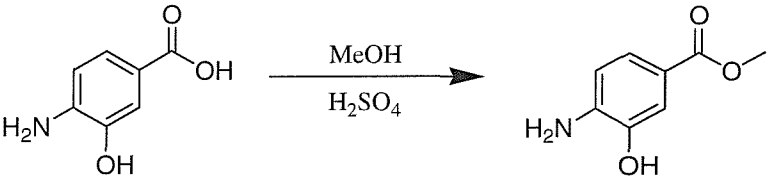
^{1}H NMR (300 MHz, $CDCl_3$): δ 3.76 (t, 2H, *J* = 7.0 Hz, *n*-C**H**$_2$), 3.69-3.57 (m, 6H, *n*-C**H**$_2$), 3.52 (t, 2H, *J* = 7.0 Hz, *n*-C**H**$_2$), 1.21 (t, 3H, *J* = 7.0 Hz, *n*-C**H**$_3$) ppm.

^{13}C NMR + DEPT (75 MHz, $CDCl_3$): δ 71.4, 70.7, 69.8, 66.7 (*n*-**C**H_2), 42.6 (**C**H_2Cl), 15.1 (CH_2**C**H_3) ppm.

IR (neat) υ_{max}: 2974 (w), 2867 (w), 1445 (w), 1351 (w), 1299 (w), 1109 (s), 1054 (w), 746 (w, C-Cl), 666 (m) cm^{-1}.

Unable to obtain LRMS.

4.7.36 Preparation of 4-amino-3-hydroxy-benzoic acid methyl ester (116)



M.W. = 153.1 g Mol^{-1}
$C_7H_7NO_3$
112

M.W. = 167.2 g Mol^{-1}
$C_8H_9NO_3$
116

4-Amino-3-hydroxy benzoic acid (5.60 g, 36.6 mmol) was refluxed in MeOH (50 mL) with conc. H_2SO_4 (10 mL) for 6 hours. $NaHCO_3$ was added until CO_2 evolution ceased. The resulting solid was extracted with Et_2O (100 mL). The organic phase was washed with sat. $NaHCO_3$ solution (100 mL) and H_2O (2 x 100 mL), dried (anhydrous Na_2SO_4) and concentrated *in vacuo* to give **116** as a solid (5.92 g, 35.4 mmol, 96%), (m.p. 114 °C, Lit.[230] 114-116 °C), (R_f= 0.66 (EtOAc)).

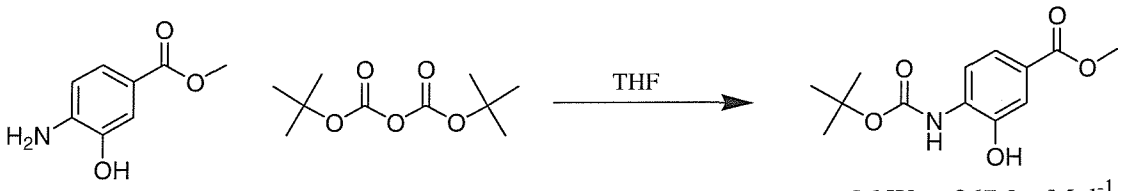
^{1}H NMR (400 MHz, d_6-DMSO): δ 7.27-7.25 (m, 2H, Ar**H**), 6.60 (d, 1H, *J* = 8.7 Hz, Ar**H**), 3.72 (s, 3H, OC**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, d_6-DMSO): δ 166.4 (**C**O), 142.8, 142.3, 116.3 (**Ar**), 122.5, 114.5, 112.5 (**Ar**H), 51.4 (O**C**H$_3$) ppm.

IR (neat) υ_{max}: 3396 (m), 3315 (m), 2946 (w), 2576 (w), 1704 (s, C=O), 1602 (m), 1524 (m), 1429 (s), 1289 (s), 1206 (s), 1100 (s), 999 (s), 905 (s), 862 (s), 764 (s), 713 (s), 632 (m) cm^{-1}.

LRMS (ES$^-$): *m/z* 166.2 ([M-H]$^-$, 100%, 167.2, 10%).

4.7.37 Preparation of 4-*tert*-butoxycarbonylamino-3-hydroxy-benzoic acid methyl ester (117)



M.W. = 167.2 g Mol^{-1} M.W. = 218.3 g Mol^{-1} M.W. = 267.3 g Mol^{-1}

$C_8H_9NO_3$ $C_{10}H_{18}O_5$ $C_{13}H_{17}NO_5$

116 **117**

116 (4.49 g, 26.8 mmol) was stirred overnight in THF (50 mL) with di-*tert*-butoxy dicarbonate (6.44 g, 29.5 mmol). The reaction mixture was concentrated *in vacuo* and the residue was dissolved in CH_2Cl_2 (50 mL). The organic phase was then washed with 2M HCl (50 mL), H_2O (2 x 50 mL), dried (anhydrous Na_2SO_4) and concentrated *in vacuo*. The residue was purified by column chromatography (Et_2O, silica 60) to give an oil from which a solid crystallised overnight. The solid was filtered hot from petroleum ether 40-60 °C and washed further with hot petroleum

ether 40-60 °C to give **117** as a red solid (4.64 g, 11.7 mmol, 65%), (m.p. 106 °C), (R_f = 0.78 (EtOAc)).

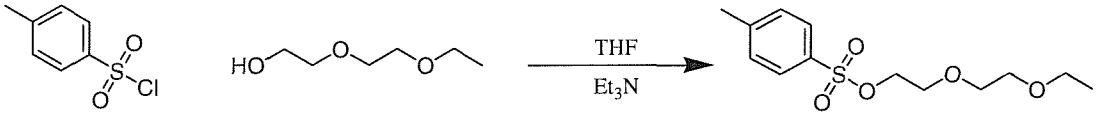
^{1}H NMR (300 MHz, d_6-DMSO): δ 7.57 (dd, 1H, *J* = 8.8, 2.1 Hz, Ar**H**), 7.76 (d, 1H, *J* = 2.1 Hz, Ar**H**), 6.76 (d, 1H, *J* = 8.8 Hz, Ar**H**), 3.75 (s, 3H, OC**H**$_3$), 1.50 (s, 9H, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT 135 (75 MHz, d_6-DMSO): δ 165.7, 151.1 (**C**O), 145.5, 135.8, 116.0 (**Ar**), 128.2, 123.6, 114.6 (**Ar**H), 83.1 (**C**(CH$_3$)$_3$), 51.4 (O**C**H$_3$), 27.2 (C(**C**H$_3$)$_3$) ppm.

IR (neat) υ_{max}: 3480 (w), 3380 (w), 1762 (m), 1702 (C=O), 1637 (m), 1605 (m), 1525 (w), 1432 (w), 1296 (s), 1235 (s), 1132 (s), 1096 (s), 1049 (m), 862 (m), 763 (s), 748 (m) cm^{-1}.

HRMS (ES$^+$): Calcd. for $C_{13}H_{17}N_1O_5$ 290.0999 $(M+Na)^+$, found 290.1006.

4.7.38 Preparation of toluene-4-sulfonic acid 2-(2-ethoxy-ethoxy)-ethyl ester (118)



M.W. = 190.7 g Mol^{-1}
$C_7H_7ClO_2S$

M.W. = 120.2 g Mol^{-1}
$C_5H_{12}O_3$

M.W. = 288.4 g Mol^{-1}
$C_{13}H_{20}O_5S$
118

p-Toluene sulphonyl chloride (25.92 g, 0.14 mol) and di-ethylene glycol monoethyl ether (16.35 g, 0.14 mol) were stirred with Et_3N (13.75 g, 0.14 mol, 17.4 mL) in THF (100 mL) overnight. The reaction mixture was then concentrated *in vacuo* and dissolved in EtOAC (50 mL), washed with 2M HCl (50 mL), H_2O (50 mL), sat. $NaHCO_3$ solution (50 mL) and then H_2O (50 mL). The organic phase was then dried

(anhydrous Na_2SO_4) and concentrated *in vacuo* to give **118** as an oil (32.06 g, 0.11 mol, 82%), (R_f = 0.40 (80% Et_2O/20% petroleum ether 40-60 °C)).

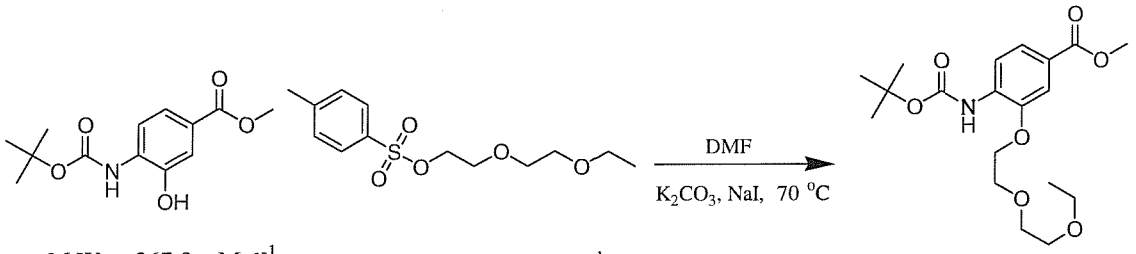
^{1}H NMR[231] (400 MHz, $CDCl_3$): δ 7.76 (d, 2H, *J* = 8.0 Hz, Ar**H**), 7.31 (d, 2H, *J* = 8.0 Hz, Ar**H**), 4.14 (t, 2H, *J* = 6.8 Hz, *n*-C**H**$_2$), 3.66 (t, 2H, *J* = 6.8 Hz, *n*-C**H**$_2$), 3.56 (t, 2H, *J* = 6.8 Hz, *n*-C**H**$_2$), 3.56-3.44 (m, 4H, *n*-C**H**$_2$), 1.16 (t, 3H, *J* = 7.0 Hz, OCH_2CH_3) ppm.

^{13}C NMR + DEPT (100 MHz, $CDCl_3$): δ 145.2, 133.5 (**Ar**), 130.2, 128.4 (**Ar**H), 71.2, 70.1, 69.7, 69.1, 67.0 (*n*-**C**H$_2$), 22.0 (Ar**C**H$_3$), 15.5 (*n*-**C**H$_3$) ppm.

IR (neat) υ_{max}: 2869 (w), 1598 (w), 1451 (w), 1352 (m), 1189 (m), 1174 (s), 1110 (m), 1096 (m), 1013 (m), 916 (m), 815 (m), 773 (m), 661 (s), 552 (s) cm^{-1}.

LRMS (ES^+): *m/z* 289.2 ($[M+H]^+$, 100%, 290.2, 20%, 311.2, 90% $[M+Na]^+$, 312.2, 10%).

4.7.39 Preparation of 4-*tert*-butoxycarbonylamino-3-[2-(2-ethoxy-ethoxy)-ethoxy]-benzoic acid methyl ester (119)



M.W. = 267.3 g Mol^{-1}
$C_{13}H_{17}NO_5$
117

M.W. = 288.4 g Mol^{-1}
$C_{13}H_{20}O_5S$
118

M.W. = 383.4 g Mol^{-1}
$C_{19}H_{29}NO_7$
119

117 (2.00 g, 7.5 mmol) and **118** (3.20 g, 11.1 mmol) were heated in DMF (30 mL) at 70 °C with K_2CO_3 (5.50 g, 34.8 mmol) and NaI (1.20 g, 0.30 mol) overnight. The reaction mixture was then diluted with H_2O (50 mL) and the product was extracted with EtOAc (50 mL). The organic phase was washed with 2M HCl (50 mL) and

H_2O (50 mL), dried (anhydrous Na_2SO_4) and concentrated *in vacuo*. The residue was purified by column chromatography (20% Et_2O/80% petroleum ether 40-60°C, silica 60) to give **119** as an oil (1.98 g, 5.2 mmol, 69 %), (R_f = 0.52 (70% Et_2O/30% petroleum ether 40-60 °C)).

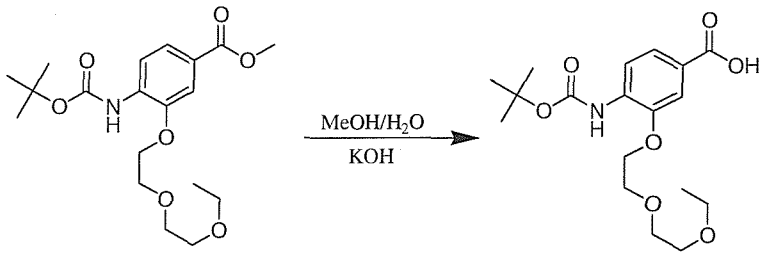
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.16 (d, 1H, *J* = 8.8 Hz, Ar**H**), 7.66 (dd, 1H, *J* = 8.8, 2.1 Hz, Ar**H**), 7.53 (d, 1H, *J* = 2.1 Hz, Ar**H**), 4.24 (t, 2H, ArOC**H**$_2$), 3.88 (t, 2H, OC**H**$_2$), 3.87 (s, 3H, OC**H**$_3$), 3.71 (t, 2H, OC**H**$_2$), 3.62 (t, 2H, OC**H**$_2$), 3.53 (q, 2H, *J* = 6.8 Hz, OC**H**$_2$$CH_3$), 1.53 (s, 9H, C(C**H**$_3$)$_3$), 1.20 (t, 3H, *J* = 6.8 Hz, OCH_2C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 167.2, 152.8 (**C**O), 146.5, 134.0, 117.5 (**Ar**), 124.5, 124.0, 113.7 (**Ar**H), 81.3 (**C**$(CH_3)_3$), 71.2, 70.3, 69.8, 69.4 (O**C**H_2), 67.1 (O**C**H_2CH_3), 52.3 (COO**C**H_3), 28.7 (C(**C**$H_3)_3$), 15.5 (OCH_2C**H**$_3$) ppm.

IR (neat) υ_{max}: 3433 (w), 2976 (w), 2870 (w), 1714 (s), 1594 (m), 1524 (s), 1481 (s), 1436 (w), 1422 (w), 1289 (m), 1236 (s), 1150 (s), 1102 (s), 1047 (s), 1024 (s), 997 (m), 763 (s) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{19}H_{29}N_1O_7$ 406.1836 $(M+Na)^+$, found 406.1828.

4.7.40 Preparation of 4-*tert*-butoxycarbonylamino-3-[2-(2-ethoxy-ethoxy)-ethoxy]-benzoic acid (120)



M.W. = 383.4 g Mol^{-1}
$C_{19}H_{29}NO_7$
119

M.W. = 369.4 g Mol^{-1}
$C_{18}H_{27}NO_7$
120

119 (1.95 g, 5.0 mmol) was dissolved in MeOH (10 mL). A solution of KOH (0.84 g, 15.0 mmol) in H_2O (15 mL) was added and the reaction mixture was stirred for 30 minutes at 60 °C. The reaction mixture was then diluted with H_2O (30 mL) and the aqueous phase washed with EtOAc (50 mL). 2M HCl (50 mL) was added to the aqueous phase and the product extracted with EtOAc (50 mL). The organic phase was washed with H_2O (50 mL), dried (anhydrous Na_2SO_4) and concentrated *in vacuo* to give **120** as an oil which solidified upon standing (1.62 g, 4.4 mmol, 88%), (m.p. 88 °C), (R_f = 0.34 (70% Et_2O/30% petroleum ether 40-60 °C)).

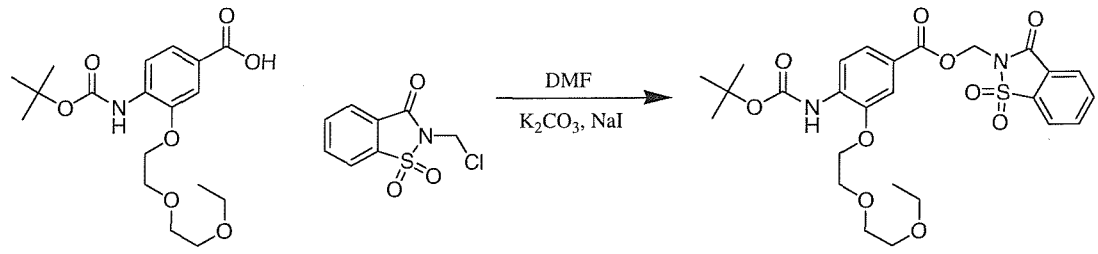
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.21 (d, 1H, *J* = 8.8 Hz, Ar**H**), 7.75 (dd, 1H, *J* = 8.8, 2.1 Hz, Ar**H**), 7.58 (d, 1H, *J* = 2.1 Hz, Ar**H**), 4.25 (t, 2H, ArOC**H**$_2$), 3.89 (t, 2H, OC**H**$_2$), 3.72 (s, 3H, OC**H**$_3$), 3.64 (t, 2H, OC**H**$_2$), 3.56 (t, 2H, OC**H**$_2$), 3.53 (q, 2H, *J* = 6.8 Hz, OC**H**$_2$CH$_3$), 1.53 (s, 9H, C(C**H**$_3$)$_3$), 1.21 (t, 3H, *J* = 6.8 Hz, OCH$_2$C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 181.5, 171.8 (**C**O), 152.8, 146.5, 134.8 (**Ar**), 125.3, 117.5, 114.0 (**Ar**H), 81.5 (**C**(CH$_3$)$_3$), 71.2, 70.3, 69.8, 69.4, 67.1 (O**C**H$_2$), 67.1 (O**C**H$_2$CH$_3$), 28.7 (C(**C**H$_3$)$_3$), 15.5 (OCH$_2$C**H**$_3$) ppm.

IR (neat) υ_{max}: 3427 (w), 2980 (w), 2868 (w), 1722 (m), 1674 (s), 1598 (m), 1527 (m), 1484 (s), 1434 (m), 1456 (m), 1286 (m), 1238 (s), 1128 (s), 1108 (s), 1048 (s), 1022 (m), 969 (m), 877 (m), 768 (m), 569 (m) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{18}H_{27}N_1O_7$ 392.1680 $(M+Na)^+$, found 392.1672.

4.7.41 Preparation of 4-*tert*-butoxycarbonylamino-3-[2-(2-ethoxy-ethoxy)-ethoxy]-benzoic acid 1,1,3-trioxo-1,3-dihydro-1λ^6-benzo[*d*]isothiazol-2-ylmethyl ester (110)



M.W. = 369.4 g Mol^{-1}
$C_{18}H_{27}NO_7$
120

M.W. = 231.6 g Mol^{-1}
$C_8H_6NO_3SCl$
74

M.W. = 564.6 g Mol^{-1}
$C_{26}H_{32}N_2O_{10}S$
110

120 (0.89 g, 2.4 mmol) and **74** (0.50 g, 2.3 mmol) were stirred in DMF (10 mL) overnight with K_2CO_3 (1.88 g, 12.0 mmol) and NaI (0.10 g, 0.7 mmol). The reaction mixture was diluted with H_2O (50 mL) and the product was extracted with EtOAc (50 mL). The organic phase was washed with sat. $NaHCO_3$ solution (50 mL), 2M HCl (50 mL), H_2O (50 mL), dried (anhydrous Na_2SO_4) and concentrated *in vacuo* to give **110** as a viscous oil (0.62 g, 1.1 mmol, 48%), (R_f = 0.49 (Et_2O)).

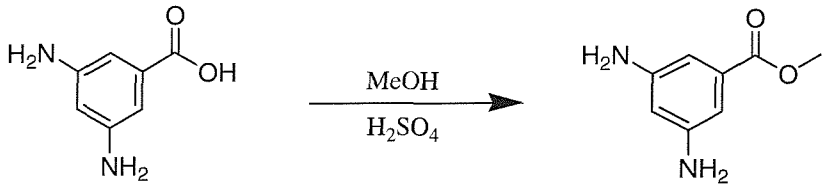
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.15-8.12 (m, 2H, Ar**H**), 7.98-7.86 (m, 4H, Ar**H**), 7.75 (dd, 1H, J = 8.8, 2.1 Hz, Ar**H**), 7.58 (d, 1H, J = 2.1 Hz, Ar**H**), 6.07 (s, 2H, NC**H**$_2$OOC), 4.23 (t, 2H, J = 6.0 Hz, ArOC**H**$_2$), 3.89 (t, 2H, J = 6.0 Hz, OC**H**$_2$), 3.72-3.68 (m, 2H, OC**H**$_2$), 3.64-61 (m, 2H, OC**H**$_2$), 3.53 (q, 2H, J = 6.8 Hz, OC**H**$_2$CH$_3$), 1.53 (s, 9H, C(C**H**$_3$)$_3$), 1.21 (t, 3H, J = 6.8 Hz, OCH$_2$C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 165.3, 158.6, 152.7 (**C**O), 146.4, 138.2, 134.9, 126.9, 122.5 (**Ar**), 135.9, 134.7, 126.2, 125.2, 121.6, 117.5, 114.0 (**Ar**H), 81.4 (**C**$(CH_3)_3$), 71.2, 70.3, 69.7, 69.4 (O**C**H_2), 67.1 (O**C**H_2CH_3), 61.8 (N**C**H_2OOC), 28.7 (C(**C**$H_3)_3$), 15.6 (OCH_2C**H**$_3$) ppm.

IR (neat) υ_{max}: 1723 (s), 1594 (m), 1526 (s), 1481 (m), 1343 (s), 1278 (m), 1237 (s), 1192 (s), 1150 (s), 1129 (s), 1074 (s), 984 (s), 748 (s), 677 (m), 583 (s) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{26}H_{32}N_2O_{10}S$ 587.1670 $(M+Na)^+$, found 587.1677.

4.7.42 Preparation of 3,5-diamino-benzoic acid methyl ester (121)



M.W. = 152.2 g Mol^{-1}
$C_7H_8N_2O_2$
103

M.W. = 166.2 g Mol^{-1}
$C_8H_{10}N_2O_2$
121

3,5-Diaminobenzoic acid (**103**) (10.0 g, 65.7 mmol) and conc. H_2SO_4 (20 mL) were refluxed in MeOH (50 mL) for 4 hours. $NaHCO_3$ was added until gas evolution ceased. The product was extracted with EtOAc (50 mL) and the organic phase was washed with sat. $NaHCO_3$ solution (50 mL), H_2O (2 x 50 mL), dried (anhydrous Na_2SO_4) and then concentrated *in vacuo* to give **121** as an off-white solid (7.92 g, 72%), (m.p. 126 °C, Lit.[232] 123-126 °C), (R_f = 0.54 (EtOAc)).

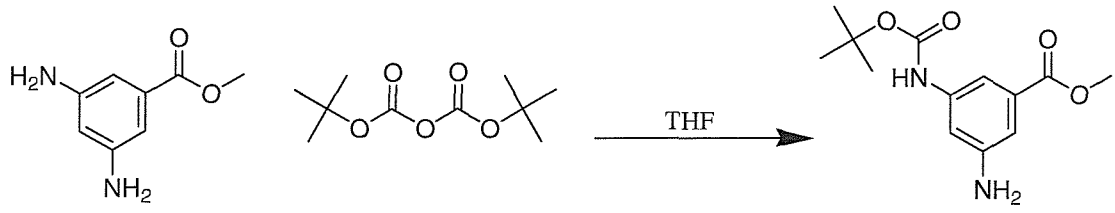
^{1}H NMR (400 MHz, d_6-DMSO): δ 6.44 (d, 2H, *J* = 2.0 Hz, Ar**H**), 6.03 (t, 1H, *J* = 2.0 Hz, Ar**H**), 3.74 (s, 3H, COOC**H**$_3$) ppm.

^{13}C NMR + DEPT (100 MHz, d_6-DMSO): δ 166.5 (CO) 148.6, 129.8 (<u>Ar</u>), 102.9 (<u>Ar</u>H), 50.7 (O<u>C</u>H$_3$) ppm.

IR (neat) υ_{max}: 1704 (s, C=O), 1601 (s), 1255 (s), 881 (m), 886 (m), 769 (s), 680 (m), 669 (m) cm^{-1}.

LRMS (ES$^+$): *m/z* 167.2 ([M+H]$^+$, 100%, 168.2 10%).

4.7.43 Preparation of 3-amino-5-*tert*-butoxycarbonylamino-benzoic acid methyl ester (122)



M.W. = 166.2 g Mol^{-1} M.W. = 218.3 g Mol^{-1} M.W. = 266.3 g Mol^{-1}

$C_8H_{10}N_2O_2$ $C_{10}H_{18}O_5$ $C_{13}H_{18}N_2O_4$

121 **122**

121 (5.92 g, 35.6 mmol) and di-*tert*-butoxy dicarbonate (7.77 g, 35.6 mmol) were stirred together in THF (50 mL) overnight. The reaction mixture was concentrated *in vacuo* and the residue then dissolved in $CHCl_3$ and washed with 2M HCl (2 x 50 mL). The aqueous phase was then washed with $CHCl_3$ (50 mL). $NaHCO_3$ was added to the aqueous phase until the evolution of gas ceased. 10% Citric acid solution (200 mL) was added and the product extracted with $CHCl_3$ (100 mL). The organic phase was washed with H_2O (2 x 100 mL), dried (anhydrous Na_2SO_4) and concentrated *in vacuo* to give **122** in the form of an off-white solid (5.84 g, 21.9 mol, 60%), (m.p. 128 °C), (R_f = 0.77 (EtOAc)).

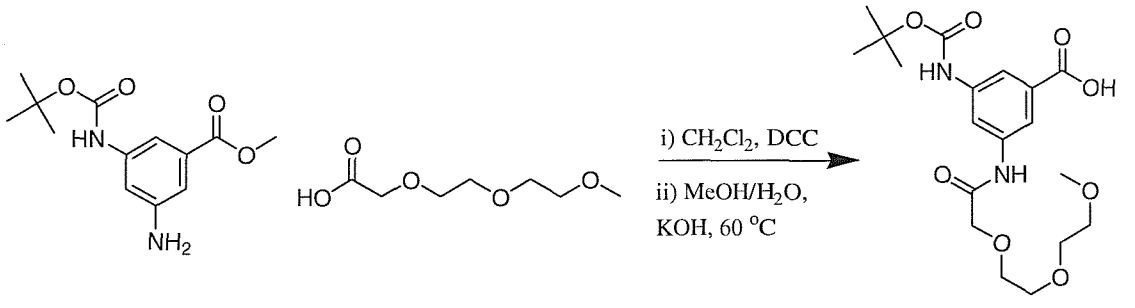
^{1}H NMR (400 MHz, d_6-DMSO): δ 9.22 (s, 1H, CON<u>H</u>), 7.28 (s, 1H, Ar<u>H</u>), 6.98 (s, 1H, Ar<u>H</u>), 6.83 (s, 1H, Ar<u>H</u>), 5.31 (s, 2H, N<u>H</u>$_2$), 3.78 (s, 3H, OC<u>H</u>$_3$), 1.46 (s, 9H, C(C<u>H</u>$_3$)$_3$) ppm.

^{13}C NMR + DEPT (100 MHz, d_6-DMSO): δ 167.2, 153.2 (<u>C</u>O), 148.7, 140.8 130.8 (<u>Ar</u>), 109.2, 108.3, 107.4 (<u>Ar</u>H), 79.6 (<u>C</u>$(CH_3)_3$), 52.2 (O<u>C</u>H_3), 28.6 (C(<u>C</u>$H_3)_3$) ppm.

IR (neat) υ_{max}: 3355 (w), 1693 (m, C=O), 1600 (m), 1542 (s), 1479 (w), 1434 (s), 1361 (w), 1286 (w), 1250 (s), 1150 (s), 1066 (w), 987 (m), 868 (m), 771 (s), 685 (m) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{13}H_{18}N_2O_4$ 289.1159 $(M+Na)^+$, found 289.1162.

4.7.44 Preparation of 3-*tert*-butoxycarbonylamino-5-{2-[2-(2-methoxy-ethoxy)-ethoxy]-acetylamino}-benzoic acid (124)



M.W. = 266.3 g Mol^{-1}	M.W. = 178.2 g Mol^{-1}	M.W. = 412.4 g Mol^{-1}
$C_{13}H_{18}N_2O_4$	$C_7H_{14}O_5$	$C_{19}H_{28}N_2O_8$
122	**123**	**124**

[2-(2-Methoxyethoxy)-ethoxy]-acetic acid (**123**) (0.74 g, 4.1 mmol, 0.64 mL) was stirred in CH_2Cl_2 (10 mL) with DCC (0.85 g, 4.1 mmol) for 30 minutes. **122** (1.00 g, 3.8 mmol) was added and the reaction mixture stirred for 1 hour. The resulting precipitate was filtered off and washed with CH_2Cl_2. The filtrate was washed with sat. $NaHCO_3$ solution (50 mL) followed by 2M HCl (50 mL) and then H_2O (50 mL). The organic phase was then dried (anhydrous Na_2SO_4) and concentrated *in vacuo*. The residue was dissolved in MeOH (15 mL) and KOH (0.42 g, 7.5 mmol) in H_2O (15 mL) was added. The reaction mixture was stirred at 60 °C for 1 hour, concentrated *in vacuo* then diluted with H_2O. The resulting precipitate was filtered off and washed with H_2O. The aqueous phase was then washed with CH_2Cl_2 (2 x 50

mL). 2M HCl (50 mL) was added to the aqueous and the product extracted with EtOAc (50 mL). The organic phase was washed with H_2O (50 mL), dried (Na_2SO_4) and concentrated *in vacuo* to give **124** as a white solid (1.21 g, 2.93 mmol, 78%), (m.p. 38 °C), (R_f = 0.29 (EtOAc)).

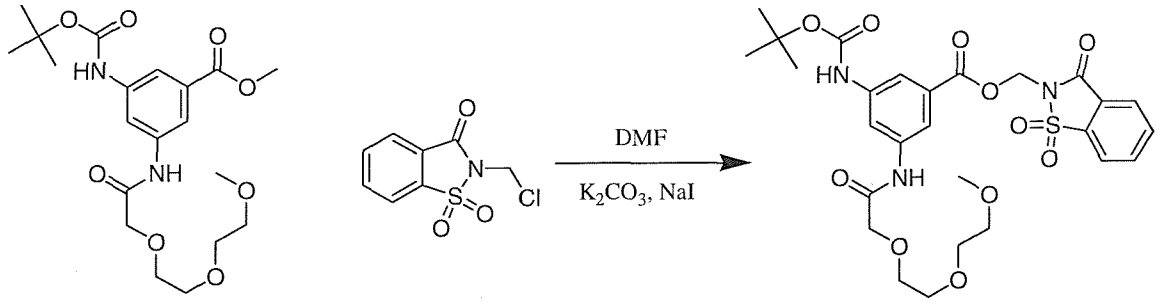
^{1}H NMR (300 MHz, $CDCl_3$): δ 8.07, 7.95, 7.88 (t, 1H, *J* = 2.0 Hz, Ar**H**), 4.13 (s, 2H, NHCOC**H**$_2$), 3.78-3.74 (m, 6H, OC**H**$_2$), 3.65-3.62 (m, 2H, OC**H**$_2$), 3.34 (s, 3H, OC**H**$_3$), 1.52 (s, 9H, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT (75 MHz, $CDCl_3$): δ 170.2, 168.8, 152.8 (**C**O), 139.6, 138.1, 130.8 (**Ar**), 116.0, 115.9, 114.9 (**Ar**H), 80.9 (**C**(CH$_3$)$_3$), 71.9 (NHCO**C**H$_2$), 71.3, 70.5, 70.4, 70.2 (O**C**H$_2$), 58.8 (O**C**H$_3$), 28.3 (C(**C**H$_3$)$_3$) ppm.

IR (neat) ν_{max}: 3300-2500 (br, COOH), 3304 (w), 2928 (w), 1694 (s), 1605 (s), 1541 (s), 1452 (m), 1426 (m), 1392 (w), 1367 (w), 1227 (s), 1153 (s), 1101 (s), 868 (m), 773 (m), 678 (m) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{19}H_{28}N_2O_8$ 435.1738 $(M+Na)^+$, found 435.1739.

4.7.45 Preparation of 3-*tert*-butoxycarbonylamino-5-{2-[2-(2-methoxy-ethoxy)-ethoxy]-acetylamino}-benzoic acid 1,1,3-trioxo-1,3-dihydro-1λ^6-benzo[*d*]isothiazol-2-ylmethyl ester (111)



M.W. = 426.5 g Mol^{-1} M.W. = 231.6 g Mol^{-1} M.W. = 607.6 g Mol^{-1}

$C_{20}H_{30}N_2O_8$ $C_8H_6NO_3SCl$ $C_{27}H_{33}N_3O_{11}S$

124 **74** **111**

74 (0.62 g, 1.5 mmol) and **124** (0.30 g, 1.4 mmol) were stirred in DMF (10 mL) overnight with NaI (0.10 g, 0.7 mmol) and K_2CO_3 (1.11 g, 7.5 mmol). The reaction mixture was then diluted with EtOAc (50 mL) and washed with H_2O (3 x 50 mL). The organic phase was dried (anhydrous Na_2SO_4) and concentrated *in vacuo*. The residue was purified by column chromatography (50% EtOAc/50% petroleum ether 40-60 °C, Silica 60) to give **111** in the form of a white solid (0.20 g, 0.3 mmol, 22%), (m.p. 65 °C), (R_f = 0.36 (EtOAc)).

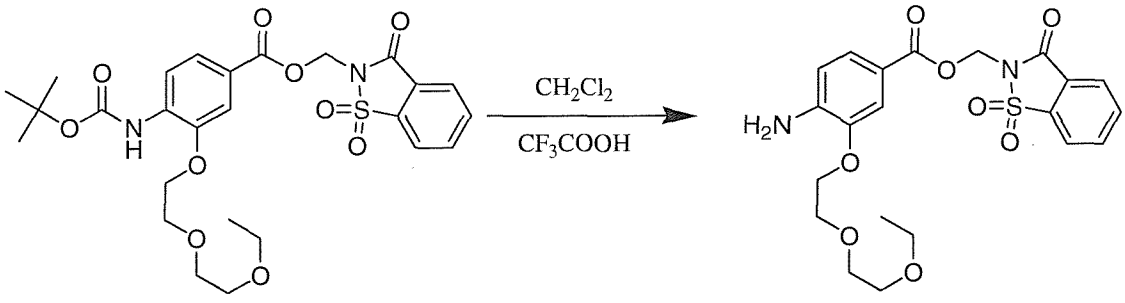
^{1}H NMR (300 MHz, $CDCl_3$): δ 8.12-8.10 (m, 2H, Ar**H**), 7.94-7.79 (m, 5H, Ar**H**), 6.06 (NC**H**$_2$O), 4.09 (s, 2H, NHCOC**H**$_2$), 3.76-3.71 (m, 6H, OC**H**$_2$), 3.60-3.57 (m, 2H, OC**H**$_2$), 3.32 (s, 3H, OC**H**$_3$), 1.49 (s, 9H, C(C**H**$_3$)$_3$) ppm.

^{13}C NMR + DEPT (75 MHz, $CDCl_3$): δ 168.5, 164.7, 158.1, 152.4 (**C**O), 139.5, 138.4, 137.8, 130.0, 126.6 (**Ar**), 135.5, 134.6, 125.8, 121.2, 115.7, 115.6, 114.9 (**Ar**H), 80.9 (**C**(CH$_3$)$_3$), 71.9 (NHCO**C**H$_2$), 71.3, 70.5, 70.4, 70.2 (O**C**H$_2$), 58.8 (O**C**H$_3$), 28.3 (C(**C**H$_3$)$_3$) ppm.

IR (neat) υ_{max}: 1724 (m, C=O), 1607 (m), 1541 (m), 1452 (m), 1341 (m, SO_2-N), 1194 (s), 1153 (s, SO_2-N), 1098 (s), 982 (s), 877 (w), 749 (m), 584 (m) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{27}H_{33}N_3O_{11}S_1$ 630.1728 $(M+Na)^+$, found 630.1726.

4.7.46 Preparation of 4-amino-3-[2-(2-ethoxy-ethoxy)-ethoxy]-benzoic acid 1,1,3-trioxo-1,3-dihydro-1λ^6-benzo[*d*]isothiazol-2-ylmethyl ester (125)



M.W. = 564.6 g Mol^{-1}
$C_{26}H_{32}N_2O_{10}S$
110

M.W. = 464.5 g Mol^{-1}
$C_{21}H_{24}N_2O_8S$
125

110 (0.47 g, 0.83 mmol) was dissolved in CH_2Cl_2 (10 mL). Trifluoroacetic acid (5 mL) was added drop-wise and the reaction mixture was stirred for 2 hours then diluted with CH_2Cl_2 (50 mL). Sat. $NaHCO_3$ solution was added until gas evolution ceased. The organic phase was separated and washed with H_2O (50 mL), dried (anhydrous Na_2SO_4) and concentrated in vacuo to give **125** as a viscous oil (0.34 g, 0.74 mmol, 89%), (R_f = 0.51 (EtOAc)).

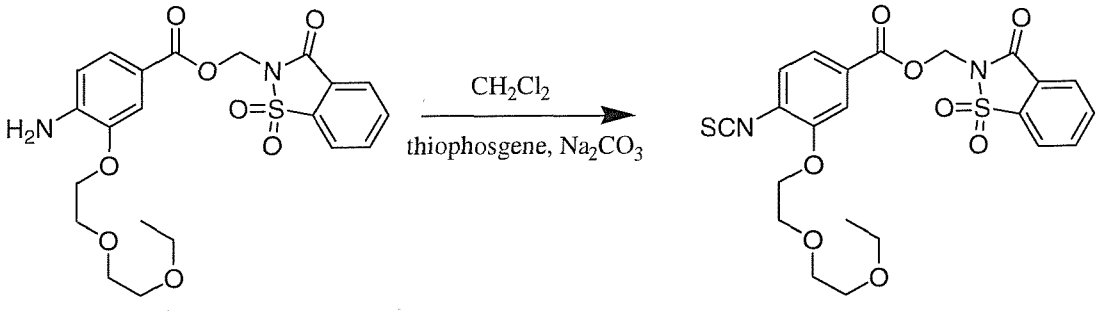
^{1}H NMR (300 MHz, $CDCl_3$): δ 8.06 (d, 1H, *J* = 7.2 Hz, Ar**H**), 7.98-7.80 (m, 4H, Ar**H**), 7.52 (dd, 1H, *J* = 8.8, 2.1 Hz, Ar**H**), 7.43 (d, 1H, *J* = 2.1 Hz, Ar**H**), 6.58 (d, 1H, *J* = 8.8 Hz, Ar**H**), 6.00 (s, 2H, NC**H**$_2$OOC), 4.15 (t, 2H, *J* = 4.5 Hz, ArOC**H**$_2$), 3.80 (t, 2H, *J* = 4.5 Hz, OC**H**$_2$), 3.67-3.63 (m, 2H, OC**H**$_2$), 3.58-3.55 (m, 2H, OC**H**$_2$), 3.48 (q, 2H, *J* = 6.8 Hz, OC**H**$_2$CH$_3$), 1.16 (t, 3H, *J* = 6.8 Hz, OCH$_2$C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (75 MHz, $CDCl_3$): δ 165.2, 158.2 (**C**O), 145.0, 143.0, 137.7, 126.5, 117.3 (**Ar**), 135.5, 134.5, 125.7, 125.5, 121.2, 114.0, 113.2 (**Ar**H), 70.7, 69.8, 69.5, 68.4, 66.6 (O**C**H_2), 61.2 (N**C**H_2OOC), 15.1 (OCH_2C**H**$_3$) ppm.

IR (neat) υ_{max}: 3480 (w), 3369 (w), 2872 (w), 1743 (s, C=O), 1709 (s, C=O), 1615 (s), 1593 (m), 1520 (m), 1440 (m), 1341 (s, SO_2-N), 1315 (s), 1285 (s), 1243 (s), 1190 (s), 1142 (s, SO_2-N), 1070 (s), 986 (s), 876 (m), 829 (m), 749 (s), 677 (s), 583 (s) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{21}H_{24}N_2O_8S$ 487.1146 $(M+Na)^+$, found 487.1133.

4.7.47 Preparation of 3-[2-(2-ethoxy-ethoxy)-ethoxy]-4-isothiocyanato-benzoic acid 1,1,3-trioxo-1,3-dihydro-1λ^6-benzo[*d*]isothiazol-2-ylmethyl ester (126)



M.W. = 464.5 g Mol^{-1}
$C_{21}H_{24}N_2O_8S$
125

M.W. = 506.5 g Mol^{-1}
$C_{22}H_{22}N_2O_8S_2$
126

Thiophosgene (0.22 g, 1.9 mmol, 0.15 mL), and Na_2CO_3 (0.68 g, 6.5 mmol) were stirred in CH_2Cl_2 (5 mL) at -10 °C. **125** (0.30 g, 0.65 mmol) in CH_2Cl_2 (20 mL) was added drop-wise over 1 hour. The reaction mixture was left to warm to room temperature and stirred for 2 hours. It was then washed with H_2O (3 x 20 mL), dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. Purification of the residue by

column chromatography (80% Et_2O/20% petroleum ether 40-60°C, silica 60) gave **126** as an off white solid (0.30 g, 0.60 mmol, 92%), (m.p. 72 °C), (R_f = 0.63 (Et_2O)).

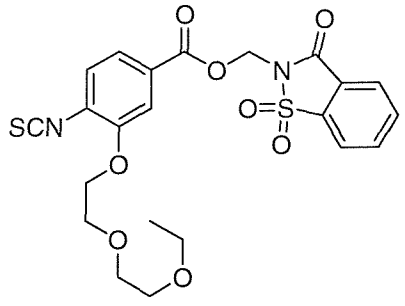
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.13 (d, 1H, *J* = 7.2 Hz, Ar**H**), 7.96-7.87 (m, 4H, Ar**H**), 7.61 (dd, 1H, *J* = 8.8, 2.1 Hz, Ar**H**), 7.59 (d, 1H, *J* = 2.1 Hz, Ar**H**), 7.07 (d, 1H, *J* = 8.8 Hz, Ar**H**), 6.07 (s, 2H, NC**H**$_2$OOC), 4.24 (t, 2H, *J* = 4.5 Hz, ArOC**H**$_2$), 3.93 (t, 2H, *J* = 4.5 Hz, OC**H**$_2$), 3.76-3.74 (m, 2H, OC**H**$_2$), 3.63-3.60 (m, 2H, OC**H**$_2$), 3.52 (q, 2H, *J* = 6.8 Hz, OC**H**$_2$CH$_3$), 1.19 (t, 3H, *J* = 6.8 Hz, OCH$_2$C**H**$_3$) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 165.2, 158.2 (**C**O), 154.4 (N**C**S), 142.2, 133.6, 125.4, 124.8, 120.3 (**Ar**), 136.0, 135.1, 126.3, 125.0, 123.4, 121.7, 113.7 (**Ar**H), 71.6, 70.3, 69.8, 69.5, 67.1 (O**C**H$_2$), 60.7 (N**C**H$_2$OOC), 14.2 (OCH$_2$C**H**$_3$) ppm.

IR (neat) υ_{max}: 2870 (w), 2014 (s, N=C=S), 1745 (s), 1598 (m), 1428 (m), 1345 (s, SO2-N), 1318 (m), 1283 (s), 1261 (m), 1234 (s), 1211 (s), 1195 (s, SO_2-N), 1081 (s), 983 (s), 878 (w), 750 (s), 677 (m), 584 (m) cm^{-1}.

HRMS (ES^+): Calcd. for $C_{22}H_{22}N_2O_8S_2$ 529.0710 $(M+Na)^+$, found 529.0710.

4.7.48 Preparation of 5.0 [EDA]-128-[3-[2-(2-ethoxy-ethoxy)-ethoxy]-4-thioureido-benzoic acid 1,1,3-trioxo-1,3-dihydro-1λ^6-benzo[*d*]isothiazol-2-ylmethyl ester] (127)



M.W. = 506.5 g Mol^{-1}

$C_{22}H_{22}N_2O_8S_2$

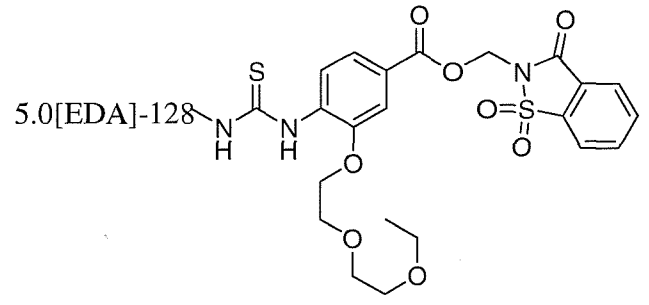
126

5.0[EDA]-128-amine

M.W. = 28225.1 g Mol^{-1}

$C_{1262}H_{2528}N_{506}O_{252}$

$CHCl_3$/DMSO, 40 °C



M.W. = 93662.8 g Mol^{-1}

$C_{4078}H_{5344}N_{762}O_{1276}S_{256}$

127

5.0 [EDA]-128-amine (93.4 mg, 3.3 x 10^{-6} mol) was dissolved in DMSO (5 mL) and added to a solution of **126** (0.25 g, 0.50 mmol) dissolved in $CHCl_3$ (5 mL). The reaction mixture was stirred at 40 °C overnight then diluted with Et_2O (100 mL). The resulting residue was dissolved in $CHCl_3$ (5 mL) and left to stand for 1 hour. Et_2O (100 mL) was added and the resulting precipitate was filtered and washed with Et_2O to give **127** in the form of an off-white solid (0.23 g, 2.4 x 10^{-6} mol, 73%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.35-7.37 (m, Ar**H**), 6.03 (s, NC**H**$_2$OOC), 4.20-1.91 (m), 1.18-0.86 (m) ppm.

^{13}C NMR + DEPT 135 (100 MHz, $CDCl_3$): δ 165.1, 158.6 (**C**O), 148.9, 138.0, 135.9, 126.8, 124.5, 123.3 (**Ar**), 136.0, 135.0, 126.2, 123.6, 121.7, 114.3, 113.6 (**Ar**H), 70.9, 70.0, 69.6, 68.7, 66.9 (*n*-**C**H$_2$), 61.9 (N**C**H$_2$OOC), 50.4, 49.4, 39.4, 34.3 (*n*-**C**H$_2$), 15.5 (OCH$_2$**C**H$_3$) ppm.

IR (neat) υ_{max}: 1747 (s, C=O), 1653 (m), 1537 (s, CS-NH), 1433 (m), 1341 (s, SO_2-N), 1277 (m), 1258 (m), 1194 (s, C=S), 1027 (s), 986 (s), 663 (m), 585 (m) cm^{-1}.

Anal. Calcd. for $C_{4078}H_{5344}N_{762}O_{1276}S_{256}$: C, 52.29; H, 5.75; N, 11.39; S, 8.76 Found: C, 51.30; H, 5.51; N, 10.97; S, 9.45.

5. References and Footnotes

5.1 Footnotes

¶ The terminology of Schecter and Berger [I. Schecter, A. Berger, *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157-162.] has been widely adopted to describe the interactions between enzyme and substrate binding. This nomenclature has been applied to identify all the relevant interactions between the amino acid side chains of a natural peptide substrate and the specificity pockets of the enzyme. The amino acid residues of the substrate (or inhibitor) are designated P_1, P_2 etc. numbering from the carbonyl end of the scissile amide bond in the left hand direction of the amino terminal. The corresponding subsites of the enzyme are termed S_1, S_2, etc. The residues in the right hand direction of the carboxy terminal from the scissile bond are designated P_1', P_2', etc. and the corresponding subsites S_1', S_2' etc.

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