UNIVERSITY OF SOUTHAMPTON

Faculty of Engineering, Science and Mathematics
School of Chemistry

Novel Nucleotide Analogues for Forming Stable DNA Triple Helices

by

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A thesis submitted for the degree of Doctor of Philosophy.

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DNA triple helices are an important tool in a variety of medicinal and biotechnological applications, such as gene therapy and chemotherapeutics. DNA triple helices are formed by binding of a triplex-forming oligonucleotide (TFO) to a DNA duplex, via specific recognition of the individual base pairs in the target sequence.

Mixed-sequence recognition of duplex DNA by TFOs is therefore an essential requirement for successful targeting. However, achieving strong, yet specific binding to the pyrimidine.purine (Py.Pu) base pairs CG and TA, by TFOs is a greater challenge than to the purine.pyrimidine (Pu.Py) base pairs (GC, AT), as fewer hydrogen bonds are presented for binding in the major groove of the double helix.

Selective recognition of CG, could be achieved by utilising additional interactions across the CG base pair, via amino-modified nucleosides, to form more stable, selective triplets than those which can be formed by the natural base T. Four modified phosphoramidite monomers, meta-aminophenyl-modified analogues of the bicyclic nucleosides, (2,3$^H$)-furano[2,3- $d$]pyrimidin-2(7$^H$)-one and N-methyl-(2,3$H$)-pyrrolo[2,3- $d$]pyrimidin-2(7$^H$)-one, were synthesised to address this potential hydrogen-bonding motif.

Biophysical studies demonstrate selective recognition of the CG base pair. Results indicate selectivity for CG and binding affinity are much improved on previous modifications. Their fluorescence properties and general oligonucleotide deprotection conditions were also studied.

In addition, the synthesis of a bis-amine modified 6-oxocytidine phosphoramidite monomer for GC recognition was re-investigated.

This research shows significant advances in the field of triplexes for therapeutic use.
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Declaration

I, Simon Richard Gerrard declare that this thesis entitled “Novel Nucleotide Analogues for Forming Stable DNA Triple Helices” and the work presented within are both my own and have been generated by myself as the result of my own original research.

I confirm that:

• this work was done wholly or mainly in candidature for a research degree at this University;

• where I have consulted/quoted the published work of others, this is always clearly attributed/referenced;

• I have acknowledged all main sources of help;

• I have made clear, where the thesis is based on work done in conjunction with another party, what my contribution to that work is;

• work described in Chapters 3 and 4 has been published in the paper below:

  − Gerrard, Simon R.; Srinivasan, Natarajan; Fox, Keith R.; Brown, Tom; “CG Base Pair Recognition Within DNA Triple Helices Using N-Methyl-3H-pyrrolo[2,3-d]pyrimidin-2(7H)-one Nucleoside Analogues”, Nucleosides, Nucleotides and Nucleic Acids, 26(10), 1363-1367. DOI: 10.1080/15257770701533958

Signed:………………………………..

Date: 21st June 2009
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<td>1-deazaC</td>
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<td>2-AE</td>
<td>2-aminopyridine</td>
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<td>3-EA</td>
<td>3-ethynylaniline, 3-aminophenyl acetylene</td>
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<td>3-HPN</td>
<td>3-hydroxypropionitrile, 2-cyanoethanol</td>
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<td>4H</td>
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dR  
2'-deoxyribofuranose

DSC  
disuccinimidyl carbonate

dT  
2'-deoxythymidine

dU  
2'-deoxyuridine

EDC  
ethyl (3-dimethylaminopropyl) carbodiimide

EDTA  
ethylenediamine tetra acetate/acetic acid

eq  
equivalents

ES  
ethylene sulfide

FAM  
carboxyfluorescein

FRET  
Förster Resonance Energy Transfer

FT  
Fourier Transform

G  
guanine / guanosine

GC  
gas chromatography

G^P, G^pdU  
5-(3-guanidinylprop-1-ynyl)-dU

G^PP  
* 6-(3-guanidinylphenyl)-N-methylpyrrolo-dC

GR  
guanidinylating reagent, N,N'-bis-[2-cyanoethoxy]carbonyl]-S-methyl-isothiourea

H, HEG  
hexaethylene glycol

HMBC  
heteromultinuclear bond correlation, long-range $^1$H–$^{13}$C COSY

HMDS  
hexamethyldisilazane

HMPA  
hexamethylphosphoramide

HMQC  
heteromultinuclear quantum correlation, $^1$H–$^{13}$C COSY

HPLC  
high-performance liquid chromatography

HRMS  
high resolution MS

IONEX  
ion-exchange

IR  
infra-red

J  
coupling constant (Hz)

$K_{assoc}$  
association constant

LC  
LightCycler®

LRMS  
low resolution MS

m  
medium (IR), multiplet (NMR)

m/z  
mass to charge ratio

MALDI-TOF  
Matrix Assisted Laser Desorption/Ionisation Time-of-Flight

MAP  
3-methyl-2-aminopyridine

MFP  
(2,3H)-furano[2,3-d]pyrimidin-2(7H)-one
MMT 4-monomethoxytrityl
\textsuperscript{MNH}_{6}\textsuperscript{P} 6-methyl-(2,3\textit{H})-pyrrolo[2,3-\textit{d}]pyrimidin-2(7\textit{H})-one, 6-methyl-pyrrolo-dC
\textsuperscript{MOE}_{5}\textsuperscript{pU} 5-(3-aminopropynyl)-2'-O-methoxyethyl-U
\textsuperscript{mox}C 5-methyl-\textsuperscript{6-\textit{exo}}C
Mp melting point
\textsuperscript{mpB} 5-methyl-2-pyridone
\textsuperscript{MP} * 6-methyl-\textsuperscript{N}-methylpyrrolo-dC
mRNA messenger RNA
MS mass spectrometry
n.d. not determined
N\textsuperscript{7}-G \textsuperscript{N}-guanine
N\textsuperscript{7}-I \textsuperscript{N}-inosine
NBS \textsuperscript{N}-bromosuccinimide
NCS \textsuperscript{N}-chlorosuccinimide
NHS \textsuperscript{N}-hydroxysuccinimide
NIS \textsuperscript{N}-iodosuccinimide
NMI \textsuperscript{N}-methylimidazole
NMR nuclear magnetic resonance
NPhth phthalamide
OD optical density (units)
OL oligonucleotide
P, pdU 5-(3-aminopropynyl)-2'-deoxyuridine
\textit{p}-TsOH \textit{para}-toluenesulfonic acid, \textit{p}-tosic acid
P\textsubscript{1} 8-aza-9-deaza-9-methyl-G
P\textsuperscript{B} 2-pyridone
PNA peptide nucleic acid
ppm parts per million
Pu purine base
Py pyrimidine base
pyDDA 6-aminopyrazin-2(1\textit{H})-one
pyr pyridine
q quartet (NMR)
Q\textsuperscript{B} 1-isoquinolone
R\textsubscript{f} retention factor
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rt</td>
<td>room temperature</td>
</tr>
<tr>
<td>S</td>
<td>(N)-[3-(4-acetamidothiazol-2-yl)phenyl]-acetamide, S base</td>
</tr>
<tr>
<td>s</td>
<td>strong (IR), singlet (NMR)</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
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<td>solid phase extraction</td>
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<td>thymine / thymidine</td>
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<tr>
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<td>TAEA</td>
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<td>TBAF</td>
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<tr>
<td>tC</td>
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</tr>
<tr>
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</tr>
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</tr>
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<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TFO</td>
<td>triplex-forming oligonucleotide</td>
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<tr>
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<td>TIPDS</td>
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<tr>
<td>(TUPP)</td>
<td>* 6-(3-thioureidophenyl)-N-methylpyrrolo-dC</td>
</tr>
<tr>
<td>U</td>
<td>uracil</td>
</tr>
<tr>
<td>UN(^I)</td>
<td>butylureido-naphthimidazole</td>
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<td>(UPP)</td>
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<tr>
<td>(\chiPP_f) (\chiPP_p)</td>
<td>(2,3(H))-furano[2,3-(d)]pyrimidin-2(7H)-one (furano-dT) phosphoramidite monomer</td>
</tr>
<tr>
<td>(\chiPP_p)</td>
<td>(2,3(H))-N-methylpyrrolo[2,3-(d)]pyrimidin-2(7H)-one (N-methylpyrrolo-dC) phosphoramidite monomer</td>
</tr>
<tr>
<td>(\psi^{iso}C)</td>
<td>pseudoisocytosine</td>
</tr>
</tbody>
</table>

* Monomers for CG recognition.
List of Monomers Used

Modified/non-natural monomers, labels (FAM, DABCYL) and spacer (HEG):
Chapter 1

Base Pair Recognition within DNA

Triple Helices – Introduction
1. **Base Pair Recognition within DNA Triple Helices: Introduction**

1.1 **Structure of DNA**

1.1.1 **Primary Structure**

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are macromolecular structures comprised of nucleotides (monomeric subunits). Each nucleotide is composed of a heterocyclic nucleobase, a furanose sugar and a phosphate moiety. Human genomic DNA is approximately $3.9 \times 10^9$ nucleotides, or 1.33 m in length, and each cell carries two copies.

In DNA, there are two purine bases, adenine 1 (A) and guanine 2 (G); and two pyrimidine bases, cytosine 3 (C) and thymine 4 (T). Nucleobases combine with a 2'-deoxy-ribofuranose sugar (dR) to give the nucleosides, 2'-deoxyadenosine 5 (dA), 2'-deoxyguanosine 6 (dG), 2'-deoxycytidine 7 (dC) and 2'-deoxythymidine 8 (dT) respectively. The bases are covalently bonded to C' of the sugar moiety by a glycosidic bond; for pyrimidine bases via the N1-atom, and for purine bases, via the N9-atom (Figure 1.1).

![DNA nucleobases and corresponding 2'-deoxyribonucleosides.](image)

**Figure 1.1.** DNA nucleobases and corresponding 2'-deoxyribonucleosides.

RNA differs from DNA in two ways. The sugar moiety is ribofuranose, namely it has a hydroxyl group in the 2’-position, and thymine is replaced by uracil 9, which lacks a methyl group at the 5-position (Figure 1.2).
Phosphodiesters link the nucleosides together to form the nucleic acid polymer. The sugar-phosphate backbone of this polymer is directional and chiral, by nature of the furanose ring structure. Nucleic acid sequences are therefore by definition, quoted as 5’ to 3’; the tetramer ACGT therefore is not the same as TGCA (Figure 1.3).

Geometry of nucleotides within the macromolecule or polymer, and of the nucleosides, is governed by sugar and sugar-base conformations. The furanose sugar ring can adopt two energy-minimised conformations or puckers, the $C^{2'}$-endo ($S$-type) and $C^{3'}$-endo ($N$-type) (Figure 1.4).

Figure 1.2. Uracil 9 and corresponding ribonucleoside, uridine 10 (U or rU).

Figure 1.3. Nucleic acid primary structure and examples of simple nucleotides 11,12.
The nucleobase lies almost perpendicular to the plane of the sugar allowing for different conformations. The nucleoside may adopt one of two principal orientations, either syn- or anti-conformations, by rotation around the C\(^1\)-N glycosidic bond (Figure 1.5).

Pyrimidine nucleobases always occupy the anti-conformation due to repulsion between the base carbonyl oxygen and furanose ether oxygen. Purine nucleobases, however, are also able to occupy the syn-conformation. Guanine occupies this orientation preferentially in mono-nucleotides, Z-DNA and some other oligomers, where favourable interactions are possible between the NH\(_2\) and 5’-phosphate.

**1.1.2 Secondary Structure**

The secondary structure of B-DNA was published in 1953 by Watson and Crick,\(^4\) following extensive X-ray diffraction studies by their associates, Franklin and Gosling,\(^5,6\) and Wilkins *et al*.\(^7\)

Two DNA strands are bound in an anti-parallel, double-helical structure by intramolecular hydrogen-bonding between bases. The four bases form Watson-Crick base pairs;\(^8\) G pairs with C *via* three intramolecular hydrogen bonds, and A pairs with T *via* two hydrogen-bonds.
Due to the antiparallel orientation of the two strands within the DNA duplex and non-symmetrical structure of deoxyribofuranose, GC is non-equivalent to CG, and AT is non-equivalent to TA. Therefore four orientation-specific Watson-Crick base pairs GC, CG, AT and TA can be described.

In the DNA double-helix or duplex, the sugar-phosphate backbones form the highly charged (poly-anionic), hydrophilic exterior of the structure, while the base pairs stack on top of each other within the hydrophobic core of the duplex, via π-π interactions. There are also two grooves running the entire length of the duplex, a major and a minor groove (Figure 1.6).

RNA exists in both single-stranded (within cell nucleii) and double-stranded forms (viral genome). It can form DNA.RNA hybrids, for example during transcription of genetic code from an unwound, single-strand of DNA to mRNA.

There are two principle conformations for the DNA double helix, A and B, both of which have been characterised by X-ray crystallography. B-DNA, which occurs in conditions of high humidity/low salt (e.g. in solution, intracellular), is a right-handed duplex. It has a periodicity of 10 base pairs; a pitch of 34 Å; a major groove 12 Å wide and 9 Å deep; and a minor groove 6 Å wide and 8 Å deep. The sugar pucker is C2'-endo and nucleosides adopt the anti-conformation.
A-DNA occurs under conditions of low humidity/high salt. This form is also adopted by RNA duplexes and DNA-RNA hybrid duplexes. It is a right-handed duplex like B-DNA but is wider; it has a periodicity of 11 base pairs; and the base pairs are tilted 20° from the helical axis. The major groove is now much narrower (3 Å) but very deep (14 Å), and the reverse is true for the minor groove. The sugars adopt the $C^{3\prime}$-endo pucker and the glycosidic bond has the anti conformation.

There are also a number of variants on A- and B-forms, some with synthetically-modified bases (methylated, brominated); all are right-handed, with different periodicities, dimensions and sugar conformations.

Z-DNA, a left-handed duplex, was first discovered by Rich et al. in 1979 during studies on the DNA hexamer d(CGCGCG). Z-DNA has a periodicity of 12 base pairs, a very narrow, deep minor groove, lacks a formal major groove, and is stable in conditions of high salt. Interestingly, for the left-handed duplex d(CG)$_n$, although all nucleosides should now adopt the syn-conformation, the pyrimidine nucleoside dC is unable to, hence adopts the anti. The sugar pucker is $C^{2\prime}$-endo for dC and $C^{3\prime}$-endo for dG. Due to these conformations, the backbone zigzags around the helical axis.

1.2 DNA Triplexes$^{1,2,10-13}$

The major and minor grooves of the DNA duplex are lined with potential hydrogen-bonding acceptors and donors. The major groove of B-DNA is accessible to DNA binding regions of various proteins and large drug molecules. The minor groove, although smaller, may also host a number of small molecules such as the pyrrole-amidine antibiotics distamycin and netropsin, which have a preference for AT-rich regions.$^{1,14}$ Importantly, it is possible for a third strand of DNA (or RNA) to bind inside the major groove to form a local triple-helix or triplex (Figure 1.7).

DNA triple helices were discovered by Felsenfeld and Rich in 1957.$^{15}$ In experiments using polyuridylic acid (polyU) and polyadenylic acid (polyA), they found a relatively weak complex (compared with the duplex A$_n$U$_n$) was formed in a 2:1 ratio when these were mixed in aqueous solution. Stabilised by Na$^+$/Mg$^{2+}$ cations, binding was specific
to polyU. No complexation was observed with polyC or other oligonucleotides. A decade later, the same observation was made for d(CT)<sub>n</sub> binding to d(GA)<sub>n</sub>d(CT)<sub>n</sub> and dG<sub>n</sub> binding to dG<sub>n</sub>dC<sub>n</sub>.<sup>1</sup>

![Figure 1.7. Schematic model (TFO in blue) and space-filling molecular model (TFO in red) of a parallel triple helix. Molecular model derived from NMR data on a triplex-duplex junction.<sup>16</sup>](image)

It is only in recent years, however, that the potential applications have been recognised. Triplexes could have important uses in gene therapy (antigene strategy),<sup>17-22</sup> site-directed cleavage<sup>17,23-25</sup> and repair,<sup>17,26-28</sup> or as a tool in molecular biology and biotechnology applications.<sup>17,29,30</sup> Binding of the third strand, or triplex-forming oligonucleotide (TFO), to a specific region of the genome blocks unwinding of the duplex by helicases. Importantly, this prevents transcription thereby inhibiting gene expression (gene knockout), and prevents DNA replication hence interrupts cell division (chemotherapeutics). TFOs for human therapeutic use should <i>statistically</i> be ca. 16-17 nucleotides long for the recognised duplex sequence to be unique, thus preventing undesired inhibition. In genomic DNA however, the base pairs are not statistically-distributed, hence TFOs may vary in length from 18 to 30 nucleotides or more, to account for local variation and repeating motifs.<sup>31</sup>

In a DNA triplex, the TFO binds in a helical manor around the duplex <i>via “Hoogsteen”</i> hydrogen-bonds<sup>32</sup> to exposed hydrogen-bond donors and acceptors in the major groove (Figures 1.7, 1.8).
Figure 1.8. Four Watson-Crick base pairs and hydrogen-bond acceptors (a) and donors (d) exposed in major groove.

If the H-bond donors and acceptors on a nucleotide are correctly placed, it can bind to a base pair forming a triplet. Such triplets, written in the form X.YZ, can be formed from the natural bases.

There are two possible triplex configurations, which differ in the orientation of the third strand (Figure 1.9). In the parallel triplex, the third strand (TFO) is orientated in parallel with the strand to which it binds. In antiparallel triplexes, the reverse is true. Antiparallel triplexes are inherently less stable than parallel triplexes, hence most work in this area has involved parallel triple helices. DNA triplexes are structurally-characterised predominantly by NMR studies and circular dichroism (CD). Although the structure of DNA duplexes can be studied by X-ray crystallography (Section 1.1.2), DNA triplexes remain very difficult to crystallise.

Figure 1.9. TFO orientation in a Parallel and Antiparallel Triplexes.

The most stable, natural parallel triplets are C+GC, T.AT, T.CG and G.TA. With all natural triplets, binding can only occur to the nearest base in the pair, hence GC and AT, the purine-pyrimidine base pairs with two available purine H-bond acceptors/donors form the strongest triplets (Figure 1.10).
Antiparallel triplets include G.GC, T.CG, A.AT and T.AT (Figure 1.11). No natural base is capable of specifically recognising a TA base pair in an antiparallel triplex, although C.TA is probably the least unfavourable.34

The strongest and most selective natural base triplets, C+.GC and T.AT (parallel), have been the subject of much research. However, there are intrinsic problems with both of these when used in triplex base pair recognition.

Triplexes are inherently less stable than DNA duplexes due to electrostatic repulsion associated with the close proximity of the three polyanionic strands; Hoogsteen hydrogen-bonds are therefore longer and weaker. Protonation of cytosine at the N3-position is required for the C+.GC triplet to form. However, due to the low pKₐ of this protonated N3 atom (pKₐH4.5, as free nucleobase, cytosine), a low pH (<6.0) is required for partial protonation. At low pH, the triplet is very stable as the positive charge aids stabilisation of the negative charges on the phosphate groups, and there may be extra stabilisation from favourable π-stacking of the positively-charged cytosine ring in the complex. At physiological pH, however, few cytosines are protonated. Triplex stability
is then greatly reduced, such that TFOs containing many C bases or contiguous C bases will not form triplexes above pH 6.0. C.GC triplets are much less stable, lacking a second hydrogen-bond and favourable positive charge. The charge is the dominating factor governing stability. The triplet T.AT is less stable than C+.GC as it lacks a stabilising positive charge, but the stability is not dependent on pH as protonation is not required for its formation.

1.2.1 Purine Recognition

Recognition of purine-pyrimidine base pairs, AT and GC, is potentially easiest because two hydrogen-bonds are possible between the third base and the purine, giving greater selectivity over other base pairs. Pyrimidine bases are more difficult to recognise selectively as only one formal H-bond is possible. Indeed, both purine and pyrimidine recognition have been subject to much research. DNA sequence recognition is still, however, mainly restricted to tracts of contiguous purine-pyrimidine (homopurine.homopyrimidine, Pu.Py) base pairs (AT, GC), as these form the most stable and selective triplexes. Achieving four-base mixed-sequence recognition, however, is essential to be able to target any gene or genetic code of choice. The number of target Pu.Py genomic sequences is limited, and many contain one or several pyrimidine interruptions, or Py.Pu inversions, which must be accommodated.

1.2.1.1 AT Base Pair Recognition

Recognition of the AT base pair was achieved by Sollogoub and Osborne et al., with a bis-amino-modified analogue of T, 2'-O-aminoethyl-5-(3-aminoprop-1-ynyl)-uridine or bis-amino-U (BAU) (Figure 1.12).

Figure 1.12. Analogues of T and nucleosides for AT recognition; 2'-O-aminoethyl-dT (AT) 13, 5-(3-aminoprop-1-ynyl)-dU (pdU) 14 and bis-amino-U (BAU) 15.
Cuenoud et al.$^{38}$ demonstrated that addition of a 2′-aminoethoxy group to dT enhances triplex stability. The amine group is protonated at physiological pH (pH 7.0) hence imparts partial charge-stabilisation of the anionic sugar-phosphate backbone. The replacement of the methyl group of dT, with an aminopropynyl group at the 5-position, significantly enhances stability of the triplex via partial charge-stabilisation$^{39}$ as described above, and through increased π-π stacking interactions.$^{40}$ Combining these modifications afforded $^{8A}U$, which whilst retaining the hydrogen-bonding pattern of T, binds to AT more strongly and with much better selectivity.

Guanidinium-based groups have also been assessed as moieties with potential for charge-stabilisation. The guanidinium moiety remains protonated over a large pH range ($pK_a \sim 12.5$) and can form multiple hydrogen-bonds. Prakash et al.$^{41}$ reported that a 2′-O-guanidinylethyl group had significant benefits over a 2′-O-aminoethyl group. TFOs containing this modification showed high affinity for duplex DNA and RNA, and when in isolated positions, increased the triplex/third strand melting temperature ($T_m$) by $\sim 3.2 \, ^\circ C$ per modification. A novel protecting group, N-(2-cyanoethoxycarbonyl)-(CEOC) was also used, which is compatible with oligonucleotide synthesis conditions.$^{42}$ Sensitivity to base, however, even aqueous sodium bicarbonate or triethylamine, is an issue during synthesis. The guanidinyl group is often added post-synthetically, for example by heating the oligonucleotide with O-methylisourea chloride in aqueous ammonia.$^{43}$ This is not selective, however, as all exposed amines will be guanidinylated.

Roig et al.$^{44}$ also reported triplex formation using 2′-deoxyuridine with modification at the 5-position by a range of alkynyl linkers terminating in one or two guanidinium groups. These linkers gave an increase in triplex stability through charge-stabilisation. Incorporation of two non-contiguous monomers gave a small increase in $T_m$ over just one (1.5-2.0 $^\circ C$). The bis-guanidinyl version of $^{8A}U$ (synthesised in-house) was discovered to be as effective as the mono-guanidinylated version. This was thought to be due to steric hindrance factors. Both guanidinyl monomers were an improvement on $^{8A}U$ and these studies are continuing.
Many modifications could be made to cytosine to give potential nucleotides for GC recognition, and there have also been a variety of other strategies. The first logical analogue was 5-methylcytosine\(^{45-47}\) (\(5\text{-MeC}\)) (Figure 1.13). Combining properties of T and C, \(N^3\) has a slightly increased \(pK_a\) and triplexes containing a \(5\text{-MeC}^+\).GC triplet were stable at a higher pH than C, although still not at physiological pH. Thermodynamic studies indicated the enhanced stability was entropic in origin (methyl groups displacing water molecules from hydrophobic core), however, the methyl groups may also improve base-stacking.\(^{46,48}\)

Stable triplex formation has also been achieved using \(5\text{-MeC}dC\) modified at the 4\(N\)-position with spermine\(^{49-51}\) or tetraethyleneoxyamine.\(^{51}\) 4-Guanidino analogues of C\(^{52}\) were also synthesised and assessed against GC.\(^{53,54}\) However, although the predicted alignment was good, triplexes could not be formed (Figure 1.13).

![Cytosine analogues for GC recognition. R = furanose sugar](image)

Pseudoisocytosine (\(\psi^{\text{iso}}\text{C}\))\(^{55,56}\) was successfully incorporated into TFOs for recognition of GC. The 2\(^\prime\)-O-methyl-derivative of \(\psi^{\text{iso}}\text{C}\) formed pH-independent triplets, via two permanent N–H bonds. It could also be protonated at the \(N^1\)-position thereby increasing stability of \(\psi^{\text{iso}}\text{C}.GC\) triplets by charge-stabilisation. These triplets were also more stable than C\(^+\).GC, at sites containing several contiguous GC pairs. This was presumably due to reduced charge repulsion, as is present between contiguous C\(^+\).GC triplets.

Recently, \(\psi^{\text{iso}}\text{C}\) was incorporated into a peptidic analogue of DNA, aminoethylglycyl peptide nucleic acid (\(\text{aegPNA}\)) for use in PNA/DNA triplex systems.\(^{57}\) In PNA\(^{58,59}\), the
DNA sugar-phosphate backbone is replaced by a flexible, pseudo-polypeptide, to which the nucleobases (B) are attached (Figure 1.14).

![Figure 1.14. Example PNA structures, aegPNA and aepPNA.](image)

1-Deazacytosine\(^{60,61}\) (1-deazaC), similar in structure to \(\psi^{\text{iso}}\)C, was investigated by Sollogoub, Powers et al. as a non-protonated, C-nucleoside analogue of C. Unfortunately, due to the challenging synthesis of the monomer although finally successful, and problems in deprotection of subsequent 1-deazaC containing oligonucleotides, biophysical studies were abandoned. There were also potential problems with GC selectivity due to tautomeric ambiguity of the endocyclic amide (Figure 1.15).\(^{61}\)

![Figure 1.15. Tautomerism in 1-deazacytosine.\(^{61}\)](image)

2-Aminopyridine\(^{62-66}\) (2-AP) was successfully employed for recognition of GC. Triplexes containing this C analogue, as the 2'-deoxy-derivative, were found to be far more stable than C or 5-MeC at high pH. This is due to the much higher basicity of N\(^1\) (pK\(_{\text{aH}}\) 5.93), although it was still pH-dependent. This stability was demonstrated when a triplex containing six contiguous 2-AP.GC triplets formed at pH 7.0. In addition, it had greater stability at lower pH as well. Surprisingly, Bates et al.\(^{62}\) found the \(\alpha\)-anomer more effective in stabilisation of the parallel triplex than the \(\beta\)-anomer, although Cassidy et al.\(^{63}\) report otherwise. Cassidy does report, however, that the \(\alpha\)-anomer can bind in an otherwise \(\beta\)-anomeric triplex with minimal structural perturbation.

3-Methyl-2-aminopyridine (MAP)\(^{61,65}\) also exhibits excellent triplex stability to higher pH than 5-MeC. The methyl group, however, does not appear to exact a noticeable difference in properties, hence the hydrophobic/entropic effects associated with 5-MeC cannot be a factor in this circumstance. MAP also demonstrated excellent
selectivity for GC in a mixed four-base sequence, and forms very stable triplexes, together with BA, for alternating GC/AT base pairs.

A lesser known pyrazine-based C analogue (pyDDA), demonstrated pH-independent binding between pH 6.3 and 8.0, to GC base pairs. PyDDA was also analysed recently in epimerisation studies as a new base for incorporation into duplex DNA.

1.2.1.3 6-Oxocytosine in GC recognition

6-Oxocytosine (6-oxo-C) and derivatives were used successfully as non-protonated C-analogues, for pH-independent GC recognition (Figure 1.16).

Berressem and Engels examined 6-oxo-C and 5-methyl-6-oxo-C (mox-C), as the 2'-O-methyl derivatives (16,17), for efficacy in triplex formation. Both bases formed stable triplets with a GC inversion in a tract of contiguous AT base pairs. This binding was nearly pH independent with a small loss of stability in basic conditions around pH 8.0; the 5'-methyl group proving slightly destabilising. They also reported that at conditions of low pH, C.+GC formed the more stable triplet. This was proposed to be due to enhanced base-stacking by the positively-charged cytosine ring, although 6-oxo-C forms stronger hydrogen bonds to guanine, due to the electron-withdrawing C-6-carbonyl group. Cytosine therefore has a greater binding affinity at low pH due to the positive charge, but forms a much less stable triplet than 6-oxo-C and mox-C from pH 6.0-8.0. Parsch and Engels later reported results of a number of 6-oxo-C and 5-allyl-6-oxo-C derivatives designed for guanine recognition. UV melting studies showed that 5-allyl-6-oxo-C derivatives (inc. 19) destabilised the triplex. They gave a lower triplex melting temperature (Tm), than 6-oxo-C; hence, favourable hydrophobic/entropic properties that the 5-methyl group gave to C, also could not play a role in this case (c.f. MA). An
RNA TFO containing RNA monomers of $6^\text{oxo}C$ (inc. 20), however, did not form triplexes with duplex DNA.

Xiang et al. reported that 2’-deoxy-$m^\text{ox}C$ ($m^\text{ox}dC$) had the highest $T_m$ of all analogues tried, and demonstrated pH-independent binding from pH 6.4 to 8.5. However, triplex-formation was weaker over five contiguous GC base pairs. If this base was used alternatively with $5^\text{Me}C$ or in presence of the DNA binding agent, spermine, several contiguous GC pairs could be recognised.

Following this work, Xiang and McLaughlin reported use of a simple acyclic carbohydrate linker in place of 2’-deoxyribofuranose (dR) on $m^\text{ox}C$. The $T_m$ was increased for this compound at an isolated GC base pair, the linker flexibility aiding orientation for hydrogen-bonding, which may be compromised due to steric repulsions. Over several contiguous GC pairs, however, the $T_m$ was reduced. The triplex may be entropically destabilised by several contiguous flexible linkers.

6-Oxocytosine was even reported to be effective as a sequence-specific HIV-1 integrase competitive inhibitor in vitro, in duplex DNA. Integrase is responsible for integrating viral DNA into DNA of the host, thus enabling virus replication.

Studies conducted by Wang, Powers and Osborne on 2’-$O$-aminoethyl derivatives of $6^\text{oxo}C$ and $m^\text{ox}C$ indicated this base proved ineffective at GC recognition, because triplexes were weak or did not form; the 2’-$O$-aminoethyl group proving non-effective in triplex stabilisation. It was rationalised that a steric clash between the furanose oxygen and C6-carbonyl oxygen caused the base to twist around the glycosidic bond (Figure 1.17). This makes the triplet non-planar, thereby misaligning the Hoogsteen hydrogen-bonding motif. This repulsion may also have induced a change in sugar pucker.

Figure 1.17. Space-filling representation of steric clash and repulsion in 2’-$O$-aminoethyl-6-oxocytidine.
1.2.1.4 GC Base Pair Recognition using Purine Bases

The natural purine base G forms triplets with GC, in both parallel (anti-conformer) and antiparallel (syn-conformer) triplexes. Another logical approach therefore to analogue development, involved the use of purines as bases for guanine recognition (Figure 1.18).

![Figure 1.18. Purines as C analogues for GC recognition.](image)

The G-related compounds, N7-G77-80 and P1,81-83 form triplets with GC, independent of pH. Their stability as isolated X.GC triplets is similar to 5-MeC+GC, but have much greater stability over 5-MeC, in triplexes with contiguous GC base pairs. They also demonstrate selectivity for GC over other base pairs, as was detected by DNase I footprinting studies. D’Costa et al.84 have also incorporated N7-G into two types of PNA (aegPNA, aepPNA) as a C+ analogue for PNA/DNA triplex formation.

Marfurt et al.85 researched N7-inosine (N7-I) as a base for guanine recognition. This structure would form only one hydrogen bond, most likely between N1H of N7-inosine and N7 of guanine, but obtain selectivity over other base pairs by disfavoured steric interactions. The α-anomer was shown to significantly reduce binding affinity relative to a triplex containing a 5-MeC base at this position. The 2’-O-methyl- β-anomer slightly destabilised the triplex but the 2’-deoxy- β-anomer gave a ~10-fold increase in binding affinity on 5-MeC as determined by quantitative DNAse I footprinting analysis. Very high selectivity was also observed over the other base pairs. Further molecular modelling studies also suggested that H-bonding between N1H and C2H, with O6 of guanine cannot be ruled out, as these favourable electrostatic interactions may help stabilise the triplet.
8-Oxoadenine (8-oxoA) and N\(^6\)-methyl-8-oxoadenine\(^{86-90}\) were synthesised for GC recognition and again formed pH-independent triplets (syn-conformer). These triplets were almost as stable at a range of pHs, as C\(^+\).GC is at low pH, and could form triplets in DNA tracts containing several contiguous GC pairs. Selectivity for GC was achieved by a combination of steric and electronic factors.

1.2.1.5 Other Novel Base designs

Other heterocyclic bases have been designed, which aim to mimic cytosine in binding to the GC base pair. Christensen et al.\(^{57}\) used a novel 1,8-naphthyridine-2,7(1,8\(H\))-dione moiety (as a replacement for C\(^+\)) in Hoogsteen-like triplex formation, in a PNA.DNA/PNA system for recognition of a single-strand of DNA (Figure 1.19).

Figure 1.19. Bi- and tricyclic analogues of C; 1,8-naphthyridine-2,7(1,8\(H\))-dione, 1,3-diaza-2-oxophenothiazine (tC) and 1,3-diaza-2-oxophenoxazine (tC\(^0\)), and the tCG base pair.

Capable of binding with two permanent NH bonds (major tautomer), the larger aromatic system was proposed to improve base-stacking in the triplex, hence impart extra stability. In isolated positions on the PNA it performed comparably with C\(^+\) at pH 5.0, and better by 6.7 °C/modification with C\(^+\) and by 1.5 °C with \(\psi^{iso}\)C, at pH 7.0. In adjacent positions, although both C and \(\psi^{iso}\)C displayed slightly reduced stability over isolated positions, the naphthyridindione performed significantly better than in isolated positions at pH 7 and 9. This was attributed to the increase in base-stacking over C and \(\psi^{iso}\)C. Adjacent C\(^+\) and protonated \(\psi^{iso}\)C suffer some electrostatic repulsion due to positive charges. This modified base has been used in triplex recognition studies.\(^{91,92}\)

Related structures, 1,3-diaza-2-oxo-phenothiazine (tC) and –phenoxazine (tC\(^0\)), have also been used in various DNA systems (DNA and DNA.PNA duplexes), as their non-protonated form, as C-substitutes in base pairing.\(^{93-95}\) Tricyclic base tC has also been studied for its fluorescence properties.\(^{96,97}\)
1.2.2 Pyrimidine Recognition

Pyrimidine.purine base pairs TA and CG are more difficult to target than purine.pyrimidine base pairs GC and AT, as only one formal hydrogen-bonding residue is offered for binding in the major groove. It may be possible to achieve either selectivity or strong binding affinity, but achieving both in combination is a challenge.

1.2.2.1 TA Base Pair Recognition

The natural base G is capable of recognising TA with only limited affinity and selectivity, hence alternative monomers have been designed to utilise the full hydrogen-bonding pattern of the TA base pair. The most selective nucleoside for recognition of TA base pairs is the S nucleoside 21 (Figure 1.20).98,99 This non-natural, novel monomer was partly derived from the TA/CG recognition monomer D3 22,100 which was actually demonstrated to intercalate selectively between a TA-AT step.101,102

![Figure 1.20. TA recognition monomers; D3 22,100 S 2198,99 and 2’-aminoethoxy-S 23 (2-AE-S),76 and the S.TA triplet.](image)

2’-O-Aminoethyl-S (2-AE-S) 23, evaluated by Wang et al.,76 showed increased binding affinity to a TA interruption over S. The increase in triplex stability by introduction of a 2’-O-aminoethyl group is well-studied,37,38,103 and clustering of several 2’-O-aminoethyl-modified nucleosides affords significant triplex stabilisation and faster rates of triplex formation in vivo.104 The drawback with D3, S and 2-AE-S is a significant affinity for CG, although selectivity for TA is increased for S and 2-AE-S at higher pH.76 Despite selectivity issues, a TFO containing the recognition monomers, BAU, MAP, AP-P (Section 1.2.2.2) and S, was used successfully to target mixed-sequence duplex DNA at pH 7.67
TA recognition has also been achieved using 2′-O-aminoethyl-derivatives of G and 2-aminopurine, although the 2′-O-aminoethyl group in this case proved detrimental to selectivity and binding affinity. This may be due to the C3′-endo sugar pucker of these RNA-derived nucleosides.

1.2.2.2 CG Base Pair Recognition using modified Pyrimidine bases

The natural base T can recognise CG with a moderate affinity, but also forms triplets with AT. Thymine therefore cannot be used for CG recognition, but was a useful starting point for a series of modified pyrimidine-derived nucleosides, which aimed to utilise the full hydrogen-bonding pattern of the CG base pair.

The C3′-carbonyl group is important in recognition of CG within a parallel triplex. This is demonstrated by the moderate affinity of C, at lower pH, for CG.

Figure 1.21. 2′,4′-BNA-pyridinone nucleosides for CG recognition; 2-pyridone 24 (PB), 5-methyl-2-pyridone 25 (mpB) and 1-isoquinolone 26 (QB).

The simple, non-natural bases 2-pyridone 24 (PB) and 5-methyl-2-pyridone 25 (mpB) efficiently recognise CG, as the conformationally-locked 2′-O,4′-C-methylene bridged nucleic acid (2′,4′-BNA) nucleosides (Figure 1.21). The sugar is locked in the C3′-endo conformation, which also affords a generalised increase in triplex stability, presumable through favourable alignment of the third strand base.

Hari et al. investigated a benzo-derivative of PB, the 1-isoquinolone-2′,4′-BNA nucleoside, QB 26. PB was shown to have a slight affinity for AT, thereby reducing selectivity for CG. The analogue QB demonstrated a marked reduction in affinity for AT at pH 7.0, appearing no more selective for AT as an abasic site (a sugar with no
base at the 1’-position). They proposed that a steric clash between the isoquinolone C’H and 5-methyl group of T, when placed against an AT base pair, inhibited binding.

Prévot-Halter and Leumann\textsuperscript{111} realised the importance of the N\textsuperscript{3}-atom in addition to the C\textsuperscript{2}-carbonyl, in recognition of cytosine. Comparing 5-MeC, 2-A\textsubscript{P}, and 4H\textsuperscript{T} 27 (4-oxo-deletion mutant of T), the 4-amino group was shown to play no critical role in CG recognition. In UV melting experiments, 4H\textsuperscript{T} in fact showed much greater selectivity for CG over GC and AT, although binding affinity was reduced.

Later studies, which tested recognition of a homopurine strand with a single C interruption, suggested a weak C–H O interaction between H\textsuperscript{5} of C and O=C\textsuperscript{2} of 4H\textsuperscript{T} was key to the recognition properties.\textsuperscript{112} This was partly based on observation that the \textsuperscript{7}H.GC triplet is as stable as the C\textsuperscript{+}.GC triplet at pH 7.0, despite forming only one formal H-bond; the two weak C–H O interactions providing additional stability (Figure 1.22).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure122.png}
\caption{Pyrimidin-2-one nucleoside for CG recognition, 5-methylpyrimidin-2(1\textit{H})-one 27 (4H\textsuperscript{T})\textsuperscript{105,111-114} and putative 4H\textsuperscript{T}.CG and \textsuperscript{7}H.GC triplets.\textsuperscript{112}}
\end{figure}

Although the proposed interaction (pictured above) has not been confirmed by NMR studies, replacement of C (of the CG base pair) for 5-fluorocytosine destabilised the triplex by 8.3 °C, suggesting this interaction is highly significant.

Buchini and Leumann\textsuperscript{105,113,114} subsequently attempted to improve the binding affinity of 4H\textsuperscript{T} for CG and triplex stability, by replacing the deoxyribofuranose (dR) sugar with that of 2’-O-aminoethyl-ribofuranose. Targeting a homopurine.homopyrimidine duplex containing a single CG inversion, the 2’-O-aminoethyl (2’-AE) group increased the triplex stability by 1.3 °C at pH 7.0, whilst retaining selectivity against the other base pairs. Triplexes were formed using a 15mer TFO, (using AE\textsuperscript{T} 13 and 2’-O-aminoethyl-5-MeC), with up to five CG interruptions in the duplex; selectivity dramatically increasing
with increasing number of CG interruptions, as the $T_m$ of the other triplexes fell sharply. When using dT and $5\text{-Me}dC$ in the TFO under the same conditions, however, general triplex stability was poor, and with five interruptions, the $T_m$ could not be determined.

Recognition of CG inversions using a fully 2'-AE-modified TFO produced interesting results. Three and five consecutive CG inversions were tolerated using 2'-AE-4H-T (x), and position did not affect triplex stability. Neighbouring nucleosides, however, played an important role; 2'-$O$-aminoethyl-$5\text{-Me}C$ (ɛ) proving especially destabilising compared to 4H-T (t). Changing part of the TFO, from ‘tttttttttt’ to ‘cccccccccc’, (and the corresponding duplex sequence accordingly), reduced the triplex $T_m$ from 67 °C to 20 °C. This is likely to be due to electrostatic repulsion between neighbouring protonated cytosine nucleobases. Once the binding motif of 4H-T was established, alternative nucleosides were sought, which would increase triplex stability, and binding affinity and selectivity for CG.

The cyclisation reaction, (occurring via a 5-endo-dig mechanism),[115] which leads to the bicyclic nucleoside 3H-furano[2,3-d]pyrimidin-2(7H)-one (furano-dT) was first published by Robins,[116] then Cruickshank et al.[117] as a minor fluorescent side-product of the Sonogashira reaction on 5-iodo-2'-deoxyuridine nucleosides. The 6-unsubstituted furano-dT nucleoside, dF* 28 found use by Woo et al.[118] as a fluorescent C analogue, when converted to the 3H-pyrrolo[2,3-d]pyrimidin-2(7H)-one (pyrrolo-dC) nucleoside dP 29, during oligonucleotide deprotection/resin cleavage, using conc. aqueous ammonia. Although dP could not be directly incorporated into oligonucleotides due to instability during the oxidation step, the conversion of dF* to dP was quantitative. Nucleoside dP retains the hydrogen-bonding pattern of C and forms a stable base pair with G, also acting as a fluorescent marker (Figure 1.23).

![Figure 1.23. Bicyclic furano-dT/pyrrolo-dC nucleosides; dF* 28 and dP 29. 6-methyl-furano-dT 30 (MFp) and 6-methyl-pyrrolo-dC 31 (MNHP).][119]
The 6-methyl- \((\text{MNH}\text{P}, 31)\) and 6-phenyl analogues also found use as fluorescent markers; the fluorescence being sensitive to base-pairing with G.\(^{119,120}\) These could be directly incorporated into oligonucleotides as the pyrrolo-\(\text{dC}\) derivatives, or via post-synthetic conversion of the furano-\(\text{dT}\) nucleoside with conc. aqueous ammonia, as previously described. \(^{\text{MNH}\text{P}}\) and other 6-alkyl derivatives were also tested by McGuigan \textit{et al.} for anti-viral activity.\(^{121}\)

This core bicyclic structure was modified by Ranasinghe \textit{et al.},\(^{122}\) for use as a CG recognition monomer (Figure 1.24). Post-synthetic conversion of the furano-\(\text{dT}\) nucleotide to the \(N\)-methyl-pyrrolo-\(\text{dC}\) derivative using aqueous methylamine, instead of ammonia, alters the hydrogen-bonding pattern. The \(N\)-methyl group prevents the nucleoside acting as a C-analogue, blocking any recognition of the GC base pair whilst retaining the \(C^2\) carbonyl and \(N^3\), essential for selective binding to CG.\(^{111}\)

\textbf{Figure 1.24.} \(N\)-Methylpyrrolo-\(\text{dC}\) nucleosides for CG recognition; 6-methyl-\(N\)-methyl-pyrrolo-\(\text{dC} 32\) \((\text{MP})\), 6-(aminoethyl)-\(N\)-methylpyrrolo-\(\text{dC} 33\) \((\text{AE}\text{P})\) and 6-(aminopropyl)-\(N\)-methylpyrrolo-\(\text{dC} 34\) \((\text{AP}\text{P})\).\(^{122}\)

Utilising the Sonogashira cross-coupling\(^{123}\) conditions of Hobbs Jr.\(^{124}\) and the documented cyclisation reaction under reaction conditions published by McGuigan \textit{et al.},\(^{121}\) two 6-aminoalkyl-furano-\(\text{dT}\) nucleosides were synthesised. After incorporation into oligonucleotides, these, and the commercially-available 6-methyl-furano-\(\text{dT} 30\) \((\text{MF}\text{P})\), were post-synthetically converted to the corresponding \(N\)-methylpyrrolo-\(\text{dC}\) analogues 32-34 during deprotection with aqueous methylamine.
Figure 1.25. Proposed triplet motifs; \( \text{MP.CG} \) and \( \text{AP.P.CG} \).

The purpose of using this structure was three-fold; firstly, the bicyclic-nucleobase should enhance triplex stability by additional base-stacking; secondly, the amino group would be protonated at pH 7 thus providing stability through charge-stabilisation, and thirdly, it could form extra hydrogen-bonds to guanine. Using the extended hydrogen-bonding motif of the CG base pair could enhance selectivity and binding affinity (Figure 1.25).

Melting studies (fluorescence and UV) compared binding affinity and selectivity of the core structure \( \text{MP} \) and nucleosides \( \text{AE.P} \) and \( \text{AP.P} \) with the natural base T, against a single CG inversion. At pH 5.5, an increase in \( T_m \) for \( \text{MP} \), \( \text{AE.P} \) and \( \text{AP.P} \), of 1.9 °C, 1.1 °C and 2.6 °C respectively, confirmed the aminopropyl linker performed best, although the results are close in value. Quantitative DNase I footprinting studies confirmed selectivity for the duplex target for all three monomers.

In order to compare with \( \text{4H.T} \), UV melting was conducted using the same sequence and conditions as used by Buchini and Leumann. At pH 7.0, \( \text{MP} \) and \( \text{AP.P} \) were shown to be comparable with \( \text{4H.T} \), and \( \text{AE.P} \) comparable with T. Therefore, although an improvement on T, and whilst selective for CG, the aminoalkyl moieties did not significantly increase triplex stability. The pendant amino groups most likely did not form hydrogen-bonds with G as originally hoped but contributed some charge-stabilisation. However, this did set precedent for future analogues, which will be discussed in Section 3, and \( \text{AP.P} \) was used successfully as a CG recognition monomer in a modified TFO against a mixed-sequence DNA target.
Modification of cytosine and use of non-natural heterocyclic compounds have been assessed in CG recognition, in parallel and antiparallel triplexes, and in NMR studies in organic solution. This significantly widens the scope for achieving CG recognition, despite very variable binding affinities. Reduction in predicted stability was observed when moving from NMR studies of isolated triplets to melting studies on triplex systems.

**Figure 1.26.** Modified C analogues 35-43 for CG recognition in parallel triplex, and proposed 4N-6-aminopyrid-2-yl-dC.CG (apyC.CG) triplet.

Modification with pendant groups at the 4N-position of dC (Figure 1.26) was used to take advantage of the extended hydrogen-bonding motif of CG, and to afford extra stability through increased base-stacking. The 4N-carbamoyl-35 and 4N-ureidocarbamoyl-dC36 nucleosides of Guzzo-Pernell et al.128 were designed to form extra hydrogen-bonds with CG, and could be fitted into reasonable triplets with CG. However, no triplex could be observed, perhaps due to hydrophilicity of the pendant groups preventing binding within the hydrophobic groove. An anthraniloyl-derivative 37 was also evaluated, introducing a hydrophobic benzene ring, but with no triplex formation. A 4N-phenyl-carbamoyl derivative did form triplexes at pH 6.0, albeit very weakly, with CG and GC base pairs, but the triplex was too unstable to be of practical use.132

4N-Aminopropyl-38 and 4N-acetamidopropyl-dC39 nucleosides of Huang et al.,130 demonstrated selectivity for CG, but relatively weak binding affinity in UV melting studies against a single CG inversion. It was a surprise to the author that the flexible chain could support triplex formation, and more rigid modifications were considered.
Huang et al. later investigated a 4N-(6-aminopyrid-2-yl)-dC analogue \( \text{apyC} \), which forms a triplex with a target containing a single CG inversion. NMR and DMS alkylation studies indicated this base binds as the imino-tautomer, which is also capable of recognising AT. UV melting experiments indicated \( \text{apyC} \) had a much stronger binding affinity to CG than their previous modifications. Selectivity against GC and TA was maintained, although it also recognised AT to a lesser extent. A biphasic melting profile against CG was also observed, which indicated other triplex structures, such as intercalation rather than Hoogsteen hydrogen-bonding. Notably, removing the 6-amino group \( \text{apyC} \) essentially eliminated triplex formation, and replacing the pyridyl for a phenyl ring \( \text{apyC} \) had only a minor reduction on triplex stability.

Chin et al. also evaluated this monomer’s CG recognition properties, in an intermolecular (‘linear’) triplex and an intramolecular (‘paper-clip’) triplex, against a single CG inversion. The \( \text{apyC}.{\text{CG}} \) triplet was shown to be as stable as a C\(^{+}\).GC triplet, at pH 6.0, and gave pH-independent triplex binding when located internally. The more flexible ‘linear’ triplex could also tolerate the alternating sequence \( \text{apyC}.{\text{TPy}}.\text{apyC} \).

A novel 5-MedC derivative, tethered at the 4N-position with an N,N-dimethylaminoethyl-modified naphthalenediimide \( \text{apyC} \), achieves CG recognition through one hydrogen-bond (from N\(^{3}\) to N\(^{4}\)H of C), and intercalation. UV melting studies demonstrated good selectivity from pH 6.4-7.5, predominantly due to hydrogen-bonding; intercalation was responsible for a general increase in stability compared with C. Melting studies, monitoring at 383 nm (\( \lambda_{\text{max}} \) – naphthalenediimide), suggested a ‘cooperative’ un-stacking of the intercalator on third strand dissociation.

1.2.2.4 Novel Non-natural Heterocyclic Monomers for CG Base Pair Recognition

Several non-natural heterocyclic bases for CG recognition have also been studied in organic solution, by NMR of the isolated triplet. Although only an approximation of their behaviour in the aqueous environment, these studies presented some interesting potential candidates for future triplex studies. Some have also been progressed to triplex melting studies.
Figure 1.27. Novel heterocyclic CG recognition monomers; ureido-naphthimidazole 44 ([UN]1),
aminoo- 45, amido- 46,47, and ureido-phthalimides 49-50, ureido-isoindolin-1-one
51,52, urocanamide 53,54 and imidazole derivatives 55,56.

A butylureido-naphthimidazole 44 ([UN]1) was assessed in NMR binding studies in
forming a triplet with CG, with hydrogen bonds to 4-NH of C and C=O and N7 of G. Although incorporated into DNA, biophysical studies were not published.

Several modified phthalimide structures were also evaluated in NMR binding studies
with CG. Whereas amino- 45, acetamido- 46 and benzamido- 47 modifications
gave relatively weak binding, ureido- 48 and N-alkylureido- groups 49,50 demonstrated
strong binding via three hydrogen-bonds from the phthalimide C=O to 4-NH of C, and
from the urea to C=O and N7 of G.

A hexylureido-isoindolin-1-one structure 51,135 analogous to the above monomer, also
effectively bound to CG with an association constant (\(K_{assoc}\)) ten-times that of
Zimmerman’s naphthimidazole 44.133 This was also progressed into triplex melting
studies as the ethylureido-isoindolin-1-one-methylene monomer 52,137 however, it
proved no more effective at CG recognition than an abasic site, with a stability of
approximately 1.8-5.0 kcal/mol lower than the natural T.AT and C’.GC triplets.

Derivatives of urocanamide 53,54 and related imidazole structures 55,56 were evaluated
by Purwanto et al.138-140 in NMR studies and UV melting experiments. NOE experiments indicated binding to CG occurred through two hydrogen-bonds, one to C7
and one to C=O of G. NMR binding studies of the primary amide 53, N-propylamide
54, alkyl- 55 and phenyl- 56 derivatives indicated strongest binding from the primary
amide (urocanamide). Reduced affinity for the other three were attributed to rotation
around the amide bond hindering binding for the N-ethylamide, too much flexibility for
alkyl derivatives, and too little flexibility for phenyl derivatives.
The *N*-propyl urocanamide 54 was progressed to triplex melting studies, targeting a single CG inversion in an AT rich duplex. Despite showing a sharp third strand melting transition, a broad melt transition against TA of a similar *T* _m_ was also detected, possibly due to binding to A through protonation of the imidazole ring. An increase in selectivity for CG over TA was observed on increasing pH to 6.5, confirming this pH dependence of TA binding. Triplex stability, however, was approximately 10 °C lower than for the C+GC triplet, at pH 6.0, due in part to lack of π-π stacking area and hydrophilicity of the amide moiety. These observations once again highlighted the importance of base-stacking, hydrophobic interactions and conformation within the triplex, which cannot be well approximated in NMR studies of isolated triplets.

![Figure 1.28. Novel carbohydrate and antiparallel CG recognition monomers/triplets.](image)

Recognition of the CG base pair within antiparallel triplexes has also been employed despite issues of stability. The azole monomers 57-60 of Durland *et al.*141 generally bound to two or more base pairs with moderate affinity, and the purine nucleoside 2'-deoxynebularine 61 (dN) demonstrated equal binding affinity to CG and AT at pH 7.4 via one hydrogen bond. The conformationally-locked WNA-βC nucleoside of Sasaki *et al.*142 effectively recognised CG, whilst the G and T β-anomers, WNA-βG and WNA-βT, bound selectively to TA within an antiparallel triplex, as determined by gel-shift assays. A novel abasic carbohydrate was also assessed in NMR studies as a CG binding agent.143 Recognition occurs via two hydrogen-bonds, although it is doubtful this could be in useful in a triplex context.

Although some success has been afforded within the antiparallel triplex, CG recognition is still most likely to be achieved through the parallel motif. Despite some degree of pH-dependence parallel triplexes are more stable and easier to work with, but antiparallel recognition is still of interest.
1.3 Conclusion

Genomic DNA is a promising target for many small-molecule and oligonucleotide-based therapeutics, aimed at treating a wide variety of genetic diseases and cancers.

The sequence-specificity of DNA allows for oligo therapeutics to be synthesised that target only specific sequences, to block a particular gene function, target cancer cells, and for a range of site-specific DNA strand modifications. It is for these reasons that extensive research has been carried out over a number of years, to achieve selective recognition of each of the four base pairs, whilst understanding the effect of interaction of neighbouring modified nucleosides on the efficacy of triplex formation, and enhancing resistance to in vivo degradation.

The therapeutic use of DNA triplexes is an expanding, exciting field, which continues to produce promising results in biophysical studies and against real targets in some biological systems.
Chapter 2

Modified 6-Oxocytidine Nucleosides for GC Recognition
2. Modified 6-Oxocytidine Nucleosides for GC Recognition

2.1. Introduction

Recognition of GC base pairs using the natural base C is both selective and strong below pH 6.0. Due to the low pK\textsubscript{aH} of N\textsuperscript{3} of cytosine, protonation of which is required for Hoogsteen hydrogen-bonding, the C\textsuperscript{+}.GC or C.GC triplet is not stable, however, at physiological pH (7.0). This severely limits the number of biological applications where this can be used, especially against targets containing contiguous GC base pairs, where repulsion between positive charges destabilises the triplex further.

A variety of pyrimidine- and purine-based monomers for GC recognition have been evaluated as described in Section 1.2.1.2. These include the pyrimidine nucleosides, 5-methylcytosine (5-MeC\textsuperscript{45-47}) and the C-nucleoside 3-methyl-2-aminopyridine (MAP\textsuperscript{61,65}), providing stability through a higher pK\textsubscript{aH} and hydrophobic methyl group; and N\textsuperscript{6}-methyl-8-oxoA\textsuperscript{87} and pyDDA\textsuperscript{68}, which form pH-independent triplets with permanent hydrogen-bonds.

The non-natural base 6-oxocytosine (6-oxoC, 62\textsuperscript{70-74}) also found use as a GC recognition monomer, which forms triplets independent of pH. Unlike some other GC recognition monomers, 6-oxoC could potentially be modified at the 5-position on the nucleobase (and 2’-position of the sugar), with pendant amino groups, such as for bis-amino-U (BA\textsubscript{U}).

![Figure 2.1. Structures of 6-oxocytosine 62, 2’-O-(2-aminoethyl)-5-(3-aminoprop-1-ynyl)-6-oxocytidine 63 (BA\textsubscript{ox}C), and proposed hydrogen-bonding and electrostatic interactions within the putative BA\textsubscript{ox}C.GC triplet.](image-url)
In the above diagram (Figure 2.1), charge-stabilising interactions are shown between the protonated 2'-amine moiety and guanidine 5'-phosphate, and also between the protonated 5'-amine moiety and $\text{BAoxC}$ phosphates. Hoogsteen hydrogen-bonds are also shown between $\text{BAoxC}$ and G.

The initial project aim therefore, was synthesis of a direct analogue of $\text{BAU}$, a $\text{bis}$-amino-modified derivative of 6-oxocytidine, 2'-O-(2-aminoethyl)-5-(3-aminoprop-1-ynyl)-6-oxocytidine 63 ($\text{BAoxC}$) (Figure 2.1). This would combine the triplex-stabilising effect of the amine groups as exemplified by $\text{BAU}$ and potentially enhanced $\pi$-stacking interactions from the alkynyl moiety. GC recognition would be achieved, as previously described, via two permanent hydrogen bonds between N$^3$H and N$^4$H of the base and N$^7$ and C$^6$=O of guanine, giving pH-independent triplex binding. Although poor binding affinity was observed when using 2'-O-aminoethyl-modified 6-oxoC and moxC compared to 2'-O-methyl- or 2'-deoxy- derivatives (Section 1.2.1.3), combining both amine modifications was proposed to enhance alignment with the GC base pair in addition to the properties described above.

2.2. $\text{Bis}$-amino-6-oxocytidine: Synthesis

The key steps in synthesis of a functionalised 6-oxocytidine monomer are coupling of the persilylated 6-oxocytosine (6-aminouracil) base to the modified sugar; sugar deprotection and reprotection of the 5'-position; halogenation at the 5-position; protection of the 4-NH$_2$ group; and subsequent base modification. The order in which these are to be carried is open to variation. The final compound is then converted into a 3'-O-phosphoramidite$^{144}$ for incorporation into triplex-forming oligonucleotides (TFOs).

Dr. Osborne conducted much work in house, into synthesis of a $\text{BAoxC}$ monomer 64, for incorporation into TFOs. After the sugar-base coupling using the 2'-O-phthalimidoethyl-modified sugar 65, and three protection/deprotection steps, problems were encountered in two key steps in the synthesis (Scheme 2.1).

Iodination under the above conditions required excess NIS and several days to effect the transformation. The subsequent Sonogashira reaction under Hobbs’ conditions$^{124}$ was
unsuccessful, thought at the time to be affected by neighbouring functionality rather than electronic factors, and the route was abandoned for re-evaluation.

Scheme 2.1: Final three steps in original synthetic route to $^{18}$O-at C monomer 64.

2.2.1 Synthesis of 5-Halo-6-oxocytidine nucleosides

It had been observed by Prof. T. Brown that during automated oligonucleotide synthesis, approximately 60% of unmodified $^{6\text{oxy}}$C nucleosides were iodinated at the 5-position in the emerging oligonucleotide, during the oxidation of P$_{\text{III}}$ to P$_{\text{V}}$. Following this observation, Osborne successfully used the iodine/THF/pyridine solution, employed in this oxidation step, to iodinate an acetylated 2'-O-phthalimido-$^{6\text{oxy}}$C derivative. Once reaction feasibility was established, Osborne reacted a 5'-O-(4,4'-dimethoxytrityl)-protected $^{6\text{oxy}}$C derivative 66 with N-iodosuccinimide (NIS) (Scheme 2.1). It was later established that the aqueous workup of 5-iodo-$^{6\text{oxy}}$C derivatives with sat. aq sodium thiosulfate to remove excess iodine, as used by Dr. Osborne, caused significant deiodination of the product, perhaps a reason for the moderate 56% yield.

Iodine, NIS$^{145-147}$ and iodine monochloride, $^{148,149}$ are well-known as iodinating agents for nucleosides, although ICl has potential for chlorination$^{150}$ and is more difficult to handle. For optimisation of yield/reaction time, an alternative iodinating reagent was sought. Bromination at this point was considered unnecessary as iodinated substrates are more reactive to Pd-catalysed cross-coupling conditions.
Benzyltriethylammonium dichloroiodate (BTEA-ICl\textsubscript{2}) 71 (Figure 2.2), was used by Harrowven \textit{et al.}\textsuperscript{151} to iodinate a substituted anisole intermediate in synthesis of a target macrocycle, in 99% yield over 5 hours. A co-catalyst ZnCl\textsubscript{2} was used, which also helped to solubilise BTEA-ICl\textsubscript{2} in the solvent acetic acid.

![Figure 2.2. Benzyltriethylammonium dichloroiodate (BTEA-ICl\textsubscript{2}) 71.](image)

Most ammonium polyhalide-derived reactions such as this, in the literature, have utilised the benzyltrimethylammonium (BTMA) salt, which is commercially available, for halogenation reactions; for example, the dichloroiodate, tetrachloroiodate and tribromide or chlorobromate for iodination, chlorination and bromination respectively. The preferred solvents for halogenation, using BTMA dichloroiodate (BTMA-ICl\textsubscript{2}), were methanol or CH\textsubscript{2}Cl\textsubscript{2}/methanol and several inorganic co-catalysts, CaCO\textsubscript{3},\textsuperscript{152,153} NaHCO\textsubscript{3},\textsuperscript{152,154} and ZnCl\textsubscript{2}\textsuperscript{151,155} have been used. Although the BTEA ion has been exploited as a range of salts, it has rarely been used as the dichloroiodate salt. Mitra \textit{et al.}\textsuperscript{156-159} however, published several papers discussing halogenation using solid-supported BTEA-polyhalide salts. The BTEA-based reagent was used in preference to BTMA-ICl\textsubscript{2} by the Harrowven group, as it was believed to afford improved yield and selectivity.

2.2.1.1 \textit{Synthesis and Reaction of Benzyltriethylammonium dichloroiodate}

Literature methods for synthesising BTMA-ICl\textsubscript{2}, include reacting BTMA-chloride with iodine monochloride in 86% yield,\textsuperscript{152} or with iodine and silver trifluoroacetate.\textsuperscript{160} The first of these methods was attempted using BTEA-Cl\textsubscript{72} instead, however, only a gum containing a mixture of salts was isolated. Attention was subsequently turned to a cleaner route, published by Kosynkin and Tour,\textsuperscript{154} which used standard-grade reagents (bleach, NaI, HCl) in aqueous medium. This was tested on a 10 g scale, affording BTEA-ICl\textsubscript{2} 71 in 96% yield.

The solution phase iodination conditions of Kosynkin \textit{et al.}\textsuperscript{154} and the solvent-free conditions of Hajipour \textit{et al.}\textsuperscript{161} (in which the reagent Me\textsubscript{4}N\textsuperscript{+} ICl\textsubscript{2} was ground to a fine
powder with the substrate), were both tested in TLC-scale reactions using BTEA-ICl; 71, although the latter method proved inefficient for the BTEA salt. When scaled up on a TIPDS-protected 6-oxo-C derivative 73, synthesised by Dr. Osbourne, despite a clean conversion, only the chlorinated nucleoside 74 was afforded in 46% yield.

This could be rationalised by dissociation of the dichloroiodate anion forming ICl (brown colouration observed) in situ, which then acted as a chlorinating agent. Boden et al.\textsuperscript{150} published the ability of ICl to chlorinate very electron-rich or sterically hindered substrates, via a single electron transfer from substrate to ICl.

2.2.1.2 Other Methods of Halogenation of 6-Oxocytidine derivatives

Pan et al.\textsuperscript{162} published the halogenation of TIPDS-protected 6-amino-dU derivatives, (structural isomers of 6-oxocytidine) using bromine/iodine in moderate to good yields. Reporting higher yields for bromination, this method was tested on acetylated 6-oxo-C derivatives. The bromine solution was added dropwise via a steel syringe needle, and became darker with a black precipitate of FeBr\(_3\), which was thought to catalyse the reaction. Concurrently, Dr. Y. Wang attempted to brominate 6-oxocytosine 62 with no success or very long reaction time by adding the bromine solution via glass pipette. Addition of a small quantity of FeBr\(_3\) significantly accelerated the reaction, thus supporting this hypothesis. An anomeric mixture of furanose/pyranose 6-oxo-C nucleosides 75-78 was reacted with bromine, catalysed by 1.0 mol\% FeBr\(_3\), to afford the brominated products 79-82 in 51% total yield.

After obtaining the anomerically pure 6-oxocytosinyl-β-ribofuranoside 75 (Section 2.2.3), a cleaner and higher yielding halogenation reaction was sought, to produce a large quantity of brominated/iodinated 6-oxo-C. Despite the moderate yield and long
reaction time necessary for iodination of 6-oxoC using NIS, attention was turned back to N-halosuccinimides using the alternative conditions of Hirota et al.\textsuperscript{163}

Hirota et al. brominated several 4N-alkyl-1,3-dimethyl-6-oxocytosine derivatives (Figure 2.3) using N-bromosuccinimide (NBS), with the radical initiator AIBN, suspended in THF, in 80-95% yield. 6-Oxocytidine derivative 75 was transformed cleanly under these conditions, using only 2.0 mol% AIBN in 30 minutes, to afford the product 79 in 95% yield.

Iodination using N-iodosuccinimide (NIS) under the same conditions resulted in a brown colouration. Presumed to be due to decomposition of NIS in storage, a large quantity was recrystallised (dioxane/chloroform)\textsuperscript{164} and the reaction was repeated. Despite the same brown colouration whether conducted at rt or 0 °C, on a 5 g scale the reaction was complete within 15 minutes. Partial or almost complete deiodination of product occurred after aqueous workup, using dilute aq Na$_2$S$_2$O$_3$ or Na$_2$S$_2$O$_5$ respectively, as previously observed (Section 2.2.1). The sodium thiosulfate (Na$_2$S$_2$O$_3$) workup, however, was used successfully on a 0.5 g quantity of the reaction mixture, separating the layers as soon as decolourisation occurred. Suspension of the remainder in ethyl acetate and soaking over 2 days, however, removed all excess iodine and succinimide by-product, affording the product 83 in 82% combined yield.

2.2.2 Synthesis of 2’-O-Phthalimidoethyl-modified Ribofuranose Sugar

The 2’-O-phthalimidoethyl-modified ribofuranose sugar 65, synthesised and used by Osborne et al.\textsuperscript{37} in preparation of the AT-recognition monomer BA\textsubscript{u}, was prepared for coupling to 6-oxocytosine 62 (Scheme 2.2), as part of the synthetic route to the BAoxC monomer 64.
Sonogashira reactions were optimised. This would then be coupled with 6-oxocytosine sugar 89 via Phthalimide substitution bromoacetate impurity which co-eluted with the product. Subsequent reduction of the methylethanoyl sugar removed during the following steps (Scheme 2.3).

The sugar synthesis starts with formation of the 1’-O-methyl acetal of D-ribose 85, followed by protection of the 3’- and 5’-OH groups with the bidentate tetraisopropylsiloxane-1,3-diyl (TIPDS) group, using Markiewicz Reagent. The reaction proceeded well over two steps, with minor impurities and solvent traces, which were removed during the following steps (Scheme 2.3).

Alkylation of the 2’-OH with methyl bromoacetate gave the product 2’-O-methylethanoyl sugar 87 in a moderate yield, containing a significant methyl bromoacetate impurity which co-eluted with the product. Subsequent reduction of the ester moiety using LiBH₄ therefore, afforded a lower yield of 88 than in the literature. Phthalimide substitution via Mitsunobu conditions gave the TIPDS-protected sugar 89 (α-anomer only) and subsequent acetalysis gave the final 2’-modified ribofuranose sugar 65 in good yield.

This would then be coupled with 6-oxocytosine 62 (Scheme 2.3), once halogenation and Sonogashira reactions were optimised.

Scheme 2.2. Synthesis of 2’-modified ribofuranose sugar 65 (lit. yields in parentheses).

Scheme 2.3. Sugar-base coupling under Vorbrüggen’s conditions (in house).
Due to the expensive nature of Markiewicz reagent (£100/g, now ~£25/g), an alternative sugar moiety was needed for synthesis of a 6-oxocytidine nucleoside, on which halogenation and Sonogashira reactions could be evaluated. 1',2',3',5'-Tetra-O-acetyl-D-ribose 91 was chosen, as the synthesis is straightforward and the reagents are cheap.

The first stage was acetylation of D-ribose 84, which was first effected using acetic anhydride in pyridine, with a catalytic amount of DMAP (from in house method). Following purification, this afforded a 97% yield, of a 1:1.6 ratio of ribofuranose (α:β ca. 1:7) to ribopyranose (α:β ca. 1:6.5). Persilylation of 6-oxocytosine 62 by refluxing in HMDS, followed by coupling to the sugar mixture 91-94 with trimethylsilyl triflate in DCE at rt, afforded an inseparable mixture of β-ribofuranoside 75 and β-ribopyranoside 77 in a 49% yield. Only trace amounts of the α-anomers 76,78 were isolated due to neighbouring group participation from the 2'-acetato group (Scheme 2.4).

Scheme 2.4. Synthesis of 2',3',5'-tri-O-acetyl-6-oxocytosinyl-ribosides 75-78.

This β-riboside mixture 75,77 was subsequently brominated, as described in Section 2.2.1.2, for Sonogashira cross-coupling.

The desired β-ribofuranoside 75 was synthesised selectively by converting D-ribose 84 into the 1'-O-methylribofuranoside 85, used in synthesis of the 2'-O-phthalimidoethyl-β-ribofuranose sugar 65 in Section 2.2.2, followed by acetylation to give the β-anomer of the product sugar 91, in 39% yield. After crystallisation, the remaining syrup, containing ca. 65:35 ratio of α,β-anomers, was not repurified, and further crystallisation of the β-anomer could not be induced by ice-cold ethanol or by seeding.
The sugar-base condensation was conducted under Vorbrüggen’s conditions as described in Scheme 2.2. No chromatography was required, as the product nucleoside 75 crystallised out of solution during workup, in 70% yield (16.9 g). The nucleoside was brominated/iodinated using NBS/NIS, as described Section 2.2.1.2.

2.2.4 Sonogashira Cross-Coupling

There has been much research on palladium-catalysed alkynylation reactions. Modifying Sonogashira’s original conditions (CuI, (Ph3P)2PdCl2, Et2NH) for nucleosides, Robins and Barr’s use of warm triethylamine and Hobbs’ conditions for alkynylation of nucleosides are two popular methods employed in synthesis of modified nucleosides.

Prior to carrying out Sonogashira reactions, the stability of the iodinated 83 and brominated nucleosides 79 to heat, light and base (Et3N, piperidine) in THF, DMF and methanol was tested. TLC and MS analysis indicated no decomposition of any kind (deacetylation, dehalogenation), hence suggesting they should be stable under Sonogashira conditions.

A range of conditions and reagents for the Sonogashira cross-coupling of a protected aminoalkyne, were tested on the brominated derivatives of the β-riboside mixture 79,81, brominated nucleoside 79 and iodinated nucleoside 83.

The brominated β-riboside mixture 79,81 was treated with N-trifluoroacetyl propargylamine 68 (2 eq) under Hobbs’ conditions, Pd(PPh3)4 (10 mol%), CuI (20 mol%) and Et3N (2 eq) in DMF (Scheme 2.5).

Scheme 2.5. Sonogashira cross-coupling reaction between N-trifluoroacetyl propargylamine 68 and brominated 6-oxocytosinyl β-ribosides 79,81.
TLC analysis indicated a complex mixture and only starting material and debrominated starting material were isolated after purification (analysed by MS). It is unusual, yet possible that debromination occurred via hydride-insertion after Pd-insertion into the C-Br bond.

Triethylamine was the base of choice, because it is non-nucleophilic and there is no possibility of substitution of bromine. It was shown by Wang et al.,\textsuperscript{169} that triethylamine also inhibits alkyne dimerisation/homo-coupling and promotes the desired cross-coupling reaction. Diethylamine, as used by Kenkichi Sonogashira, was shown to promote homo-coupling under the right conditions.

Using a large excess of Et\textsubscript{3}N (20 eq) or as a solvent, on 50 mg and 500 mg scale, resulted only in partial debromination, deacetylation, and triphenylphosphine oxide. A similar observation was made for the iodinated nucleoside \textsuperscript{83}.

The last reactions on the brominated β-riboside mixture \textsuperscript{79,81} were carried out under microwave heating. The cross-coupling reactions were conducted in DMF or THF at 60 °C for 10 mins, and in DMF at 100 °C for 10 mins. Once again, only complex mixtures were formed.

A fresh quantity of \textit{N}-trifluoroacetyl propargylamine\textsuperscript{117,170} \textsuperscript{68} (Scheme 2.6) was synthesised for subsequent Sonogashira reactions; the quality of which was confirmed by standard characterisation methods and in a test Sonogashira reaction using 5'-O-(4,4'-dimethoxytrityl)-5-iodo-2'-deoxyuridine (DMT-IdU) \textsuperscript{97} under Hobbs’ conditions.\textsuperscript{124} However, subsequent test reactions using the brominated \textsuperscript{79} and iodinated \textsuperscript{83} \textit{6-oxo-C} derivatives, were unsuccessful. Only partial de-iodination of starting material \textsuperscript{83}, and formation of triphenylphosphine oxide (in both cases) were observed.

\textbf{Scheme 2.6.} Synthesis of \textit{N}-trifluoroacetyl propargylamine \textsuperscript{68}, (lit.\textsuperscript{117} yield in parentheses).

It was proposed that the above problems might be exacerbated by the presence of acetyl groups hence a suitable method for deacetylation was sought. The brominated \textsuperscript{79} and iodinated \textsuperscript{83} \textit{6-oxo-C} nucleosides were deacetylated by heating in 0.4 M K\textsubscript{2}CO\textsubscript{3} in
methanol/water (4:1, v/v) (mild reagent occasionally used in oligonucleotide deprotection) until fully dissolved. Treatment with DOWEX 50WX8 ion exchange resin (200/400 mesh, pyridinium form) monitoring the pH until <7, soaking the resulting solid (after filtration and concentration) in cold ethanol or isopropanol and drying, afforded the ‘free’ bromo-99 and iodo-nucleosides 100 in good to high yield. If too little DOWEX resin was used, this resulted in a hygroscopic potassium salt of the nucleoside, which required further treatment.

A 0.5 g scale Sonogashira reaction was executed using the deacetylated iodinated nucleoside 100 and alkyne 68, under the same conditions. However, complete deiodination was observed over 2 hours, to give the deiodinated starting material, 6-oxocytidine 101 in 44% yield (Figure 2.4).

![Figure 2.4. Starting material 100, desired Sonogashira product 102 and 6-oxocytidine 101.](image)

As little success had been achieved using modifications of Hobbs’ conditions, other catalysts and conditions were assessed. Recently, Alami et al.\textsuperscript{171} published cross-coupling reactions effected by the strongly active (bis-benzonitrile)palladium (II) dichloride – copper (I) iodide catalyst system. Using this system they were able to conduct cross-couplings of vinyl chlorides with a variety of alkynes, in dry piperidine. The weakly-coordinated PhCN ligands, allow for much faster oxidative insertion of Pd into the C–X bond (even less reactive C–Cl bonds, at room temperature), and with excellent yields (62-97%) and reaction times (~30 minutes).

The above reaction conditions \(((\text{PhCN})_2\text{PdCl}_2 \ 5 \ \text{mol\%}, \ \text{CuI} \ 10 \ \text{mol\%}, \ \text{alkyne} \ 68 \ - \ 2 \ \text{eq}, \ \text{dry piperidine}, \ \text{rt}, \ \text{argon atmosphere})\), however proved ineffective on 6-oxocytidine substrates. TLC analysis of the reaction with iodinated nucleoside 100, indicated only deiodination. TLC and MS analysis of the reaction with 5-bromo-6-oxocytidine 99, indicated nothing had occurred, with or without DMF as a co-solvent (to aid dissolution). The reactions could not be left longer than 90 minutes or heated due to catalyst sensitivity.
The bis-(triphenylphosphine)palladium (II) dichloride (10 mol%) – copper (I) iodide (30 mol%) catalyst system, as used by Robins and Barr\cite{116,168} for cross-coupling reactions with 5-iodo-2’-deoxyuridines in warm triethylamine (50 °C) was also assessed. Even when using 4 equivalents of alkyne, only deiodination and alkyne dimerisation were detected for substrate 100 and nothing was observed for the brominated substrate 99.

Alternatively, instead of conducting cross-couplings in absence of water, the reaction was conducted in hot water, using 0.5 mol% (Ph$_3$P)$_4$Pd, as published by Bhattacharya and Sengupta.\cite{172} The authors’ principal idea was to utilise the hydrophobic effect, and although the 6-oxocytidine substrates are (partially) water-soluble, it was hoped these conditions might be beneficial. However, again only deiodination of 100 was observed.

These reactions, using various catalysts, solvents, temperatures and ratios of reagents were all unsuccessful. Brominated 6-oxoC nucleosides (β-anomers) did not react, and iodinated 6-oxoC nucleosides were deiodinated. This illustrates the surprising strength of the C–Br bond and susceptibility of the R–Pd(PPh$_3$)$_2$–I intermediate to decomposition. There may also be other steric and electronic factors preventing this transformation, such as the neighbouring C$^6$-carbonyl and C$^4$-amino groups. Although other alkynes were not tested, N-trifluoroacetyl propargylamine 68 couples to protected and unprotected 5-iodo-2’-deoxyuridine nucleosides in excellent yield.\cite{117,170}

Sonogashira-like palladium-catalysed cross-coupling reactions were subsequently abandoned. It is still possible, however, that results could be obtained using a triflato-nucleoside,\cite{59} alkynylzinc reagents\cite{173,174} or even Stille coupling,\cite{175} which have been successfully used to alkynylate other nucleosides.

2.3 Alternative Modifications at 5-position

Attempts to enhance base-stacking by attachment of an alkynyl group failed as described above. It was also found that the 5-allyl-group of 6-oxo$d$C caused destabilisation of the triplex, relative to 6-oxo$d$C alone. An alternative method was sought therefore for attachment of a pendant amine group at the 5-position of 6-oxoC, with or without additional base-stacking enhancement, which would increase triplex-stability by charge-stabilisation.
2.3.1 Halide-Amine Exchange

Halide-amine exchange was proposed as an alternative method for introduction of amine functionality to the base. There is much literature precedent for this transformation on aromatic compounds, uridine nucleosides, uracils and aminouracils, using primary and secondary amines and even ammonia. In 1951, Arthur Phillips published a series of substitution reactions on 5-bromouracil, using amines such as methylamine, butylamine, cyclohexylamine, piperidine and benzylmethylamine in typically good to quantitative yields. Benzylmethylamine was used for introduction of a methylamino group (following hydrogenolysis of the benzyl group), as reaction of similar heterocycles with methylamine and ammonia often requires elevated temperatures or high pressure, with long reaction times.

5-Bromo-6-oxocytidine 99 was heated at 85 °C in a sealed tube with four different amines (piperidine, benzylamine, propargylamine, TAEA 103) for 3.5 hours (Figure 2.5). TLC analysis indicated loss of starting material, most likely through degradation, and no positive identification of desired products was made by MS analysis. A tan-coloured solid was isolated from the last reaction with the polyamine tris(2-aminoethyl)amine, TAEA 103, which was confirmed by NMR, however, to be a salt of TAEA.

Azam et al. attached the polyamine TAEA 103 to an anthraquinone intercalator-modified chimeric TFO, by a simple chloride-amine exchange reaction. The polyamine moiety demonstrated increased triplex stability without altering selectivity, and has since been used, in house, by Dr. Z. Zhao, for synthesising simple polyamine-anthraquinone intercalators for non-specific triplex stabilisation.
Initially, test reactions were performed on $2',3',5'$-O-triacetyl-5-bromo-6-oxocytidine 79, heating the nucleoside at 80 °C in a sealed tube, for 2.5 hours in piperidine or benzylamine. Following treatment with DOWEX 50WX8-400 (pyridinium form) and diethyl ether, a white gum containing some deacetylated piperidinyl compound 104, and deacetylated starting material 101 were obtained respectively. Following this, to avoid complex mixtures due to partial deacetylation, the free nucleoside 5-bromo-6-oxocytidine 99 was used. This was treated with propargylamine, benzylamine and TAEA 103.

Varying the reaction conditions for bromide-TAEA exchange was unsuccessful. Heating 5-bromo-6-oxocytidine 98 with TAEA 103 at 180 °C for 20 minutes, heating at 75 °C in a 30% aq solution of the amine overnight, and refluxing in distilled pyridine with 5 eq of TAEA 103 produced similar results as obtained previously and isolation of amine salts.

In order to confirm that exchange had occurred successfully originally, when using piperidine, nucleoside 99 was heated in the amine alone, or with pyridine, water or DMF as co-solvent at 70 °C for 17 hours. This proved partially successful, with an estimated 20-30% conversion (TLC analysis) for all reactions, except in water. These results were also confirmed by MS analysis.

The amine-halide exchange was proposed to occur via an S$_\text{N}$Ar mechanism, which if true would suggest a faster, more efficient reaction with chloro- and fluoro-, rather than bromo- or iodo-groups. To take advantage of this, and to avoid problems due to cleavage of acetyl protecting groups, a large quantity of unprotected 5-chloro-6-oxocytidine 108 was synthesised for comparison with the other 5-halo-6-oxocytidine derivatives. $2',3',5'$-Tri-O-acetyl-6-oxocytidine 75 was treated with N-chloro-succinimide (NCS) based on the method published by Hirota et al.\textsuperscript{163} as described in Section 2.2.1.2 (Scheme 2.7). The product was purified by column chromatography, not soaked in ethyl acetate to remove the succinimide by-product, as it is partially soluble in this solvent.
Scheme 2.7. Synthesis of 5-chloro-6-oxocytidine 108.

The product 109 was deacetylated as previously described to afford the desired free nucleoside 108 in 81% yield over two steps, containing 1.8 mol % succinimide from the previous step and 35.6 mol % KOAc, not observed for previous deacetylations by this method.

In the next series of test exchange reactions, chlorinated 74,108 and iodinated 100 6-oxoC nucleosides were treated with piperidine, and brominated nucleoside 99 with five 1,n-diaminoalkanes (n = 2, 6, 8, 10, 12), at 70 °C for 18-20 hours (Figure 2.6).

Figure 2.6. Substrates for amine-halide substitution reactions 74,99,100,108.

5-Iodo-6-oxocytidine 100 reacted partially with piperidine but less cleanly than 5-bromo-6-oxocytidine 99, suggesting an S_N_Ar mechanism might apply. The protected chlorinated 6-oxoC derivative 74, however, underwent deprotection of the 4N-dimethylformamidine protecting group, with no desired reaction, and none of the 1,n-diaminoalkanes reacted with 5-bromo-6-oxocytidine 99.

Chloro-6-oxoC derivative 74 was treated with ethylene diamine (42 eq) and TAEA 103 (18 eq) at 75 °C for 90 minutes and also for 18-20 hours. Only formamidine deprotection and amine degradation were observed, and trifluoroacetylation of the latter reaction mixture yielded only trifluoroacetylated amine.

So far, successful amine-halide exchange had been limited to the secondary amine piperidine, which is probably not beneficial to triplex formation. Attempts to couple
with primary amines, and in particular, polyamines, although unsuccessful thus far, were continued using 5-chloro-6-oxocytidine 108. It was thought that protection of two of the primary amine moieties of tris(2-aminoethyl)amine 103, could potentially aid the reaction by presenting only one centre for reaction, although the bis-protected product was finally not used. Asseline et al.182 published the synthesis of the bis-trifluoroacetylated polyamine 110 via alkylation of the trifluoroacetic acid salt of bis-trifluoroacetylated diethylene triamine 111 with bromoethanol, followed by Mitsunobu reaction with LiN3 and hydrogenation over Pd/C, in 23% overall yield.

Scheme 2.8. First route to N-(2-aminoethyl)-bis-N,N-[2-(trifluoroacetamido)ethyl]amine 110.

The first synthetic procedure attempted (Scheme 2.8) began with mono-protection with a 4,4′-dimethoxytrityl (DMT) group, by addition of a solution of DMTCl 112 in distilled pyridine, dropwise over 20 minutes, to a stirred, ice-cold solution of TAEA 103 (6 eq) in distilled CH2Cl2. After aqueous workup, an off-white foam was afforded, which unfortunately contained only bis- and tris-tritylated products.

Scheme 2.9. Other routes to N-(2-aminoethyl)-bis-N,N-[2-(trifluoroacetamido)ethyl]amine 110.

The reaction was repeated using MMTCl 115 due to issues of stability with DMT, using 7 eq of polyamine 103 and dropwise addition of the MMTCl/pyridine solution over a 5½ hour period. This afforded, after workup, a green syrup/resin. Trifluoroacetylation using ethyl trifluoroacetate (10 eq), with excess Et3N to prevent detritylation, afforded the fully-protected compound 116 in a low 20% overall yield. The detritylation step was carried out on a test scale, using acetic acid (30 eq) in methanol with heating to afford a white oil, which could not be identified by MS analysis (Scheme 2.9).
Free amine 103 was also trifluoroacetylated directly using only 2 equivalents of ethyl trifluoroacetate, which selectively protected only two amines in 37% yield. Indeed, the selectivity obtained using ethyl trifluoroacetate is known, as published by Yang et al.,\textsuperscript{183} who described the selective protection of two and three chemically-equivalent amines in 1,4,7-triazacyclonane and cyclen respectively, despite using excess reagent.

Finally, after testing the substrate’s stability to DMF and pyridine, at 100 °C overnight, a number of test amine-substitution reactions on 5-chloro-6-oxocytidine 108 were carried out. The substrate was heated, under argon as described previously, at 100 °C in DMF, with TAEA 103 (10 eq), diethylene triamine (10 eq) and piperidine (60 eq) for 21 hours; and with piperidine (30 eq), piperidine (30 eq) with KF (2.5 eq), TAEA 103 (30 eq) and 6-aminocaproic acid (30 eq) for up to 2½ days. Once again, only piperidine proved suitably nucleophilic to react. It was difficult to determine if KF had any effect on this reaction.

These studies indicated only the strongly nucleophilic amine piperidine was able to displace a halogen at the 5-position of 6-oxocytidine.

There is literature precedent for reaction of primary amines with 5-bromo-6-oxocytosine (5-bromo-6-aminouracil) derivatives (e.g. 5-bromo-1,3-dimethyl-6-aminouracil, 117). Brederick et al.\textsuperscript{184} achieved efficient reaction with benzylamine, n-butylamine and ethanolamine and Searle & Co.\textsuperscript{185} with 2-(N,N-dimethylamino)ethylamine, by heating in ethanolic solution. Rybar et al.\textsuperscript{186} report yields of 50-80%, using simple amines such as propylamine, allylamine and benzylamine, but encountered further reaction with the product to form bis-6-oxocytosinylamine compounds, even when using vast excess of the amine. Zhang and Zhang\textsuperscript{187} also reported efficient reaction at 120 °C, under microwave irradiation, to afford yields of 70-95% for several arylethylamines (Scheme 2.10).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{Scheme2.10.png}
\caption{Bromide-amine exchange reaction on 5-bromo-1,3-dimethyl-6-oxocytosine 117.\textsuperscript{184-187}}
\end{figure}
Based on the literature evidence, it was thought possible to attach a polyamine or similar amine moiety to the base then couple to the sugar to form the modified nucleoside. However, reaction with the desired amine TAEA \(103\) may give multiple additions, hence a protecting group strategy or methylated-analogue of TAEA \(118\) would be needed, synthesis of which could be envisaged to proceed via a tetramethyl diethylene triamine \(119\).\(^{188}\)

![Scheme 2.11. Proposed synthetic route to \(N,N,N',N'\)-tetramethyl-tris-(2-aminoethyl)amine \(118\).](image)

It is also unknown whether such a group would affect the sugar-base coupling and this route would require much optimisation. It may also be possible therefore to exchange Br with \(NH_3\), for further base-modification (4\(\pi\),5\(\pi\)-cyclisation reactions to increase base-stacking), however, the products (5,6-diaminouracils or 5-amino-6-oxocytosines) are unstable due to oxidative self-condensation.\(^{189}\)

2.3.2 Halide-Thiol Exchange

Amine-modified alkanethiols were chosen as the next candidate for nucleophilic substitution of chloride. Being more nucleophilic than amines and with evidence of chloride-thiol exchange in the literature,\(^{190}\) this was considered the logical next route. Attachment of a mercaptocarboxylic acid was considered for coupling of various amines, including TAEA \(103\) or its bis-trifluoroacetyl derivative \(110\). The amine could be coupled to the acid, before or after halide-thiol exchange (Scheme 2.12).

![Scheme 2.12. Proposed synthesis of thiol-modified amine \(123\) and coupling with 5-chloro-6-oxocytidine \(108\).](image)
5-Chloro-6-oxocytidine 108 was treated with mercaptoacetic acid (10 eq) in DMF at 100 °C for 18 hours, resulting in formation of a new product and near complete consumption of starting material. A hygroscopic powder was obtained on precipitation with methanol and diethyl ether, and a second powder was obtained on ether-induced precipitation from the filtrate, the coupled product could not be detected by MS.

The second proposal was synthesis of mercapto-analogues of TAEA 125,126, the attempted synthesis of which is described below (Scheme 2.13). The seven-step synthesis of the non-acetylated analogue 126 was published by Cheng et al.193 in 13% overall yield. Either thiol could then be reacted with the desired 5-chloro-6-oxoC derivative.

Selective trifluoroacetylation of the primary amine groups of diethylene triamine, DETA 127 was first achieved by trifluoroacetylation in presence of water to give the trifluoroacetate salt182,192 111. The large quantity of salt 111 could not be converted to the free amine using Et₃N/heat or polyamine-modified Amberlite IRA-96 resin, hence the free amine 128 was synthesised.194 This was achieved by reaction of DETA 127 with a slight excess of ethyl trifluoroacetate, under anhydrous conditions. Neither the salt 111 nor free amine 128, however, reacted with ethylene sulphide (ES) to introduce the 2-mercaptoethyl side-chain, despite strong literature precedent for alkylation of secondary amines with ES,195,196 and of the salt 111 with alkyl bromides such as bromoethanol.182. The alternative route would proceed via alkylation using S-acetyl-2-bromoethane thiol 129, synthesised from ES and AcBr (lit. 67% yield),191 followed by selective cleavage of the S-acetyl group, using borohydride-functionalised Amberlite IRA-400 resin and Pd(OAc)₂.197 The resin is prepared by stirring the wet chloride-form with an aqueous solution of NaBH₄. The deprotection of octyl thioacetate was reported

**Scheme 2.13.** Proposed synthetic routes to polyaminethiol derivatives 125,126, (lit.182,191,192 yields in parentheses).
to proceed cleanly in 98% yield. Alternatively, full deprotection using 3M aq NaOH in acetone\textsuperscript{198} should afford the free mercapto-polyamine \textbf{126}.

5-Chloro-6-oxocytidine \textbf{108} was also treated with cysteamine (2-aminoethane thiol) (10 eq) to assess whether a free amine moiety would affect this transformation. The starting material was consumed over 16 hours and low yields of a white powder and a very hygroscopic tan-coloured powder were obtained by recrystallisation from methanol and precipitation of the filtrate with methanol/diethyl ether respectively. No components could be identified by MS, but this does not disprove possible reaction, which, however, would need substantial optimisation.

Further work was halted at this stage, as attention was transferred to the CG recognition project (Chapters 3-7), which was running alongside this research, and obtaining positive results synthetically and in biophysical studies.

\subsection*{2.4 Alternative Sugar Modification at the 2’-Position}

As an alternative sugar moiety, synthesis of a 2’-O-methyl-6-oxo-C derivative was also investigated, for comparison with published biophysical studies, by a different route to that already published.\textsuperscript{70,71} Whilst lacking the charge-stabilising properties of the 2’-O-aminoethyl group, the 2’-O-methyl moiety still increases stability to enzymic degradation \textit{in vivo}.\textsuperscript{199}
The 2′-O-phthalimidoethyl moiety of the monomer BAU was introduced onto the sugar via a six-step synthesis, before coupling to the base.\textsuperscript{37} There is scope, however, for introducing 2′-O-aminoalkyl modifications on the nucleoside itself, via a tricyclic nucleoside intermediate.

There has been much published into cyclisation reactions of uridine derivatives to form their tricyclic anhydouridine intermediates. In most cases, HMPA is the chosen solvent, however, due to issues of carcinogenicity, DMA and DMF have been successfully employed, although in varying yields.

In work reported by Reese \textit{et al.},\textsuperscript{200,201} when uridine 131 is heated with diphenyl carbonate (1.1 eq) and a catalytic amount of NaHCO\textsubscript{3} (5 mol\%) in dry DMF or DMA at 100 °C, for 4-5 hours, the tricyclic compound 2,2′-anhydrouridine 132 can be formed in quantitative yield (Scheme 2.14). The reaction was executed successfully using 5-iodouridine by Dr. L. Brennan, in house, and a methyl group at the 5-position can also be tolerated.\textsuperscript{202,203}

\begin{center}
\textbf{Scheme 2.14.} Cyclisation of uridine 131 to form 2,2′-anhydouridine 132.\textsuperscript{200,201}
\end{center}

Manoharan \textit{et al.}\textsuperscript{42} and Ross \textit{et al.}\textsuperscript{203} describe introduction of a 2′-O-phthalimidoethyl group by the following method. 2-(Phthalimido)ethanol is converted into the trialkoxyborane, by slowly dissolving in a solution of diborane in THF, then reacted with 2,2′-anhydro-5-methyluridine 133 or 2,2′-anhydouridine 132 in presence of NaHCO\textsubscript{3} (cat.) in a steel bomb, at 150 °C for 24 hours or 175 °C for 72 hours. Following purification by column chromatography, only ~20% yield is achieved (Scheme 2.15).
Scheme 2.15. 2’-Modification via anhydouridine derivatives 132,133.42,203

Blommers et al.103 also reported the same coupling using Ti(OiPr)₄ instead of diborane, to form the titanium alkoxide in situ, forming an isotopically-labelled version of 134 in 32% yield.

Reese et al.200,201 have also reported the reaction of 2-methoxyethanol with 2,2’-anhydouridine 132, via the aluminium alkoxide. Refluxing coarsely divided aluminium foil in dry 2-methoxyethanol until dissolution, then reaction with the 2,2’-anhydronucleoside 132, afforded the 2’-O-modified nucleoside after 48 hours in 91% yield. This simpler, yet more effective reaction was executed in house, by Dr. L Brennan, to insert an N,N-dimethylaminoethoxy group in ∼95% crude yield.

After testing the Ti(OiPr)₄ coupling (mentioned above), 2-(phthalimido)ethanol was successfully coupled to DMT-protected anhydro-5-methyl-uridine in ∼45% yield, in house, via formation of the aluminium alkoxide, by heating aluminium foil in molten 2-(phthalimido)ethanol. This, however, has yet to be repeated.

Cyclisation of cytidine 136 can be achieved via the inexpensive reagent, 2-acetoxybenzoyl chloride 137, and 2,2’-anhydrocytidine (cyclocytidine) 138 is also commercially available. Reichman et al.204 report a 65% yield of 3’-O-acetyl-2,2’-anhydrocytidine 139 after refluxing for 25 minutes in acetonitrile. Deacetylation then afforded the desired product 138. This method can also be applied to uridine. Heating at 50 °C for 3 minutes affords the cyclised product 132. Refluxing for 30 minutes affords the 2’-deoxy-2’-chloro- derivative 140 (Scheme 2.16).

Scheme 2.16. Cyclisation of cytidine 136 and 2’-deoxy-2’-chlorouridine 140.204
It was possible that both methods of cyclisation could be employed for 6-oxocytidine 101 and derivatives, as two carbonyl groups are available for reaction. It was also feasible that two products 141,142 are possible through either reaction, although either product could be used for subsequent 2’-modification.

Scheme 2.17. Cyclisation reaction of 6-oxocytidine 101.

The cyclisation reaction was carried out twice (Scheme 2.17), using diphenyl carbonate, to afford each time a 1:1 mixture of 2,2’- 141 and 6,2’-anhydro-6-oxocytidine 142 in quantitative yield. The 1H-NMR spectrum was complicated, but the sugar protons could be assigned and the spectrum contained two 1’-H signals corresponding to the two isomers.

2’-O-Methyl modification could then have been achieved by a similar method to Reese,200,201 reacting 4 equivalents of Mg(OMe)2 in methanol,205 with DMT-protected derivatives of 2,2’- 141 and 6,2’-anhydro-6-oxocytidine 142. The anhydro-nucleosides 141,142 were, however, not modified any further at this point.

2.5 Conclusion

Several synthetic investigations have been carried out, in order to assess reaction feasibility and subsequently to synthesise a bis-amino-modified 6-oxocytidine monomer 64, for GC base pair recognition studies within DNA triple helices. Palladium-catalysed alkynylation was ruled out as a method of introduction of amine functionality at the 5-position due to substrate inactivity or degradation. Instead, modification by amine- or thiol-halide exchange was investigated with some success. Feasibility of 2’-modification was also investigated, although this requires further work.

Synthesis of a bis-amino-modified 6-oxocytidine monomer is reliant on several key factors. Attachment of an amine moiety to the 5-position must be achieved, either via a
thiol on the nucleoside or an amine on the base. This functionality must then also be
tolerated by sugar-base coupling, cyclisation and methoxide insertion reactions. Due to
the large number of steps involved, and extensive optimisation needed, unless large
quantities of the initial intermediates can be synthesised, poor-moderate yields cannot
be tolerated.

It is now unlikely that synthetic work on 6-oxoC will continue. However, should this not
be the case, the 2'-O-methyl group is the most feasible 2'-modification. Whichever
route would be chosen, large quantities of some intermediates can be synthesised in
good yield.
Chapter 3

Amino-Modified Furano-dT Nucleosides for CG Recognition
3. **Amino-Modified Furano-dT Nucleosides for CG Recognition**

### 3.1 Introduction

The pyrimidine-purine base pairs CG and TA remain most difficult to target, as only one formal hydrogen-bond is presented for binding in the major groove. Thymine is the only natural base that can effectively recognise CG, but it cannot be used for CG recognition due to lack of selectivity. This discovery lead to a variety of monomers, including a series of modified pyrimidine-based nucleosides, designed to target the CG base pair, utilising new and extended Hoogsteen hydrogen-bonding patterns.

![Figure 3.1. Pyrimidine-based CG recognition monomers.](image)

The first generation pyrimidine-based nucleosides $P_B$ 24 (and $\text{mpB} 25\text{107,108}$) and $4HT 27\text{,105,111-114}$ demonstrated the importance of the $C^2$ carbonyl group and $N^3$ for effective binding to $C$ of the CG base pair via a formal hydrogen-bond and a weaker $C$–$H$–$O$ interaction.

![Figure 3.2. Proposed binding motifs for $\text{mpB.CG, 4HT.CG, MP.CG and AP.P.CG triplets.}$](image)

The second generation nucleosides used the fluorescent bicyclic $N$-methylpyrrolo-dC core of $\text{MP 32}$, which retains the $C^2$ carbonyl and $N^3$ for binding. An aminooalkyl group was introduced at the 6-position to target $C^6$=O and $N^7$ of guanine.$\text{122}$ Combining the extended aromatic system and amine moiety was proposed to enhance binding affinity.
by increased base-stacking and charge-stabilisation due to protonation of the amine group, and also selectivity due to extra hydrogen-bonding to G. A small increase in $T_m$ was obtained for $^{AP}P_{34}$ over T and $^{MP}_{32}$, against a single CG inversion, indicating the pendant amino group may only contribute stability via charge-stabilisation, rather than through additional binding to G.

Modification of $^{AP}P$ with a 2′-O-aminoalkyl-group did not enhance CG binding affinity as expected (through charge-stabilisation), but demonstrated some affinity for AT, presumed to be due to binding of an imino-isomer of the base.61

A third generation of monomers was then developed; replacing the flexible alkyl linker with a meta-phenylene group, which could increase the potential base-stacking interaction.206 The new rigid linker may also have entropic benefits, and may place the amino group in the correct position for binding. The new monomer $^{APP}_{143}$ (aminophenyl-) (Figure 3.1) showed a small increase in $T_m$ over $^{AP}P_{34}$ (aminopropyl-) in UV melting studies at pH 5.8,206 to give the following order of stability:

$$^{APP} > ^{AP}P > ^{MP} > T$$

Other modifications were also considered ($^{XP}$P), and these and alternative methods of synthesis/incorporation of these monomers are discussed below.

3.1.1 Monomer Nomenclature

Furano-dT monomers have been subsequently described by the abbreviation $^{XP}_{PP}$. ‘X’ describes the aryl functional group (-C$_6$H$_4$NH$_2$ – A, -C$_6$H$_4$NHCOCH$_3$ – Ac, -C$_6$H$_4$NHCONH$_2$ – U) and the subscript ‘f’, that these monomers were incorporated into TFOs as the furano-dT analogue and post-synthetically modified. The 6-methyl-furano-dT derivative $^{144}$ also carries the notation, $^{MP}_{f}$ (Figure 3.4).
Figure 3.3. Commercially available 6-methyl-furano-dT (\(^6\text{P}_3\)) phosphoramidite monomer 144.

The subscript ‘p’ denotes \(N\)-methylpyrrolo-dC monomers (\(^3\text{P}_p\)), which require no further modification after incorporation into TFOs. In addition to the three aryl moieties described by \(X\) above, it also describes the guanidinylphenyl moiety (-C\(_6\)H\(_4\)NHC(N)NH\(_2\) – G). The synthesis and biophysical studies of these are discussed in Chapters 5 and 6.

3.2 Synthesis of Furano-dT Monomers for CG Recognition

The first synthetic targets were modified furano-dT nucleosides. In previous work by Ranasinghe et al.\(^{122}\), the modification was synthesised as the furano-dT phosphoramidite monomer.

Following incorporation into oligonucleotides, the nucleotide is post-synthetically modified during deprotection and cleavage from the solid support, as described in Section 3.3.
3.2.1 Synthesis of 6-(3-Trifluoroacetamidophenyl)-furano-dT monomer 145\textsuperscript{206,207}

The first aim was to repeat the synthesis of the \textit{meta}-trifluoroacetamido-furano-dT monomer 145, first synthesised, in house, by Sunil Vadhia,\textsuperscript{206} from 5'\textsuperscript{-}O-(4,4'\textsuperscript{-}dimethoxytrityl)-5-iodo-2'-deoxyuridine (DMT-IdU) 97 (DMT-on method). The key steps in the synthetic route to all the furano-dT monomers, are a Sonogashira cross-coupling of an arylalkyne with DMT-IdU 97 or the ‘free’ nucleoside, 5-iodo-2'-deoxyuridine 148, followed by a copper (I)-catalysed cyclisation reaction to form the furanopyrimidin-2-one nucleobase core.

![Scheme 3.1. Synthesis of 3-ethynylaniline/3-aminophenyl acetylene 152, (lit.\textsuperscript{206} yields in parentheses).](image)

The arylalkyne, 3-ethynylaniline (3-aminophenyl acetylene, 3-EA) 152 was synthesised (Scheme 3.1), by the same route as used by Vadhia,\textsuperscript{206} which follows a revised version of a method published by Melissaris \textit{et al.}\textsuperscript{208} 3-Iodoaniline 149 was reacted with 2-methyl-but-3-yn-2-ol 150 via a Sonogashira cross-coupling reaction, using the catalyst system, Pd(PPh\textsubscript{3})\textsubscript{2}Cl\textsubscript{2} – PPh\textsubscript{3} – CuI (3.0:3.0:1.0 mol %), in triethylamine at 50 °C/reflux. Triethylammonium iodide was removed during a lengthy workup with diethyl ether. Stirring in glacial acetic acid, concentration then addition of water, however, did not induce crystallisation or precipitation as described by Melissaris, and aqueous workup with 2M aq HCl did not remove residual starting material. Upon repeating this reaction triethylammonium iodide was removed by trituration/precipitation using diethyl ether, and the protected alkyne 151 was isolated, following purification, in a moderate 53% yield.

Alkyne 151 was deprotected by refluxing in a dry solution of KOH in isopropanol for 3.5 hours, to afford 3-EA 152 in 39% yield. Following this work, a much cheaper commercial supply of 3-ethynylaniline 152 became available, and this compound was subsequently purchased instead.
Sonogashira cross-coupling of 5'-O-(4,4'-dimethoxytrityl)-5-iodo-2'-deoxyuridine 96 (Section 3.2.2) with 3-ethynylaniline 152 was carried out under Hobbs' conditions for alkynylation of nucleosides (Scheme 3.2). Larger quantities of CuI (0.4 eq) and alkyne (3.0 eq) were necessary for successful reaction. Trituration with diethyl ether, to remove unreacted alkyne, and column chromatography afforded the Sonogashira product 153 in 76% yield. Vadhia conducted this cross-coupling using the trifluoroacetyl-protected alkyne followed by deacetylation to give the same product 153. The NH$_2$ group was subsequently discovered not to affect this Sonogashira reaction and therefore did not need prior protection, although a slightly higher yield was obtained when protected.

Cyclisation was effected by heating at 50 °C overnight with CuI (1.0 eq) in methanolic triethylamine in absence of light. The reaction could not be monitored by TLC, as the starting material 153 and product 154 had the same R$_f$, and on isolation, the product contained 3.5 mol % uncyclised material, as determined by $^1$H-NMR analysis.

The aniline moiety of 154 was protected with a trifluoroacetyl group, by refluxing with ethyl trifluoroacetate (4 × 2.0 eq) and DMAP (4 × 1.0 eq) in THF with Et$_3$N for greater than 2 days. Despite adding triethylamine to protect against detritylation, the necessary prolonged heating lead to general degradation and a 35% yield. This demonstrates the low reactivity of this aniline and instability to prolonged heating. Vadhia achieved a moderate yield of 62% using a larger excess of ethyl trifluoroacetate. The alkyne amine could have been trifluoroacetylated using trifluoroacetic anhydride, before Sonogashira
cross-coupling, thus avoiding selectivity/reactivity issues later on. However, it is doubtful that this protecting group would survive reflux in methanol/triethylamine, hence necessitating protection after cyclisation.

Finally, the protected, cyclised nucleoside 155 was phosphitylated at the 3’-position using 2-O-cyanoethyl-N,N-diisopropyl chlorophosphoramidite 70 (1.2 eq) with DIPEA in THF,\textsuperscript{144} strictly under an argon atmosphere and anhydrous conditions, and using argon-degassed or distilled solvents at every stage of preparation thereafter (standard procedure for phosphitylation reactions). The air- and acid-sensitive phosphoramidite monomer 155 was obtained in 64% yield after separation of a small amount of uncyclised phosphitylated material carried through from the cyclisation reaction.

3.2.2 Synthesis of 6-(3-Ureidophenyl)-furano-dT Monomer 146\textsuperscript{207}

The anilino-NH$_2$ of \textsuperscript{8}PP is only able to form one hydrogen-bond to G (\textit{via} C$^6$=O). Formation of two hydrogen-bonds with G would not only increase binding affinity, but also selectivity for the CG base pair \textit{via} this extended Hoogsteen binding. A primary urea moiety was proposed as a suitable candidate for this binding motif. Urea moieties have also been used to target G in NMR binding studies of isolated X.CG triplets, using various heterocyclic monomers (Section 1.2.2.4).\textsuperscript{133-137} Vadhia had previously attempted the synthesis of the 3-ureidophenyl monomer 146 (Figure 3.3), using the DMT-off synthetic route, where the nucleoside is tritylated after Sonogashira cross-coupling and cyclisation, which proved difficult.

The arylalkyne for synthesis of this monomer, 3-ureidophenyl acetylene 156 was originally synthesised by Vadhiya, by heating 3-ethynylaniline 152 with phenyl carbamate 157 (10.6 eq) in DMF.\textsuperscript{206} The large quantity of phenyl carbamate used made purification difficult, hence the alkyne/carbamate mixture was used directly in Sonogashira cross-coupling with the free nucleoside 5-ido-2’-deoxyuridine 148 followed by cyclisation. The phenyl carbamate was removed after cyclisation to give a 43% yield over two steps. Tritylation of the free nucleoside, however, proved problematic affording only a 12% yield.\textsuperscript{206} This poor yield could be rationalised by the very high polarity/low solubility of the free, cyclised ureidophenyl nucleoside in the reaction solvent (pyridine).
Conducting the carbamidation in a sealed tube, excluding moisture, without solvent, allowed for use of only a slight excess of phenyl carbamate, and the pure product 156 could be isolated by column chromatography (Scheme 3.3). The reaction was carried out several times at 80-100 °C, using no more than 1.1 eq of phenyl carbamate to ensure adequate mixing of components, however, the yield was limited to a maximum of ~70% due to oxidation of the starting material during the reaction.

Alternatively, reaction with benzoyl isocyanate and deprotection may have afforded the product 156 in higher yield (Section 5.2.5). Using the benzoyl-protected urea in the Sonogashira cross-coupling would be a further improvement. The benzoyl group would not only reduce the polarity of the subsequent products thus aiding purification, but would also block phosphitylation of the urea thereby increasing the potential yield for this reaction. Additionally, the benzoyl group can be removed during oligonucleotide deprotection using ammonia, as was demonstrated, in house, by Dr. Edrees-Abdou in synthesis of the 3-thioureidophenyl-N-methylpyrrolo-dC analogue 158 (Section 5.2.5).

Prior to Sonogashira cross-coupling, a large quantity of 5’-O-(4,4’-dimethoxytrityl)-5-iodo-2’-deoxyuridine 97 was synthesised by reaction of a slight excess of 4,4’-dimethoxytrityl chloride (DMTCl) 112 with 5-iodo-2’-deoxyuridine 148 (25 g) in 76% yield (Scheme 3.4). Yields varied from 95-75% on a 5-25 g scale, and some bis-tritylated compound was usually formed as the reaction is not fully regioselective. The alkyne 156 was coupled to 97 (DMT-on method) in 80% yield, an 89% yield being obtained on a smaller scale, when triethylammonium iodide could be removed by soaking in acetonitrile.

Initially, the product was cyclised by heating at 60 °C overnight. Only 50% conversion was observed, but extra CuI and raising the temperature to 80 °C pushed the reaction to completion, affording only a 39% yield due to significant degradation. A higher yield may also have been attained using column chromatography instead of recrystallisation. Heating at 80 °C under argon pressure for only 2-3½ hours afforded a 69% yield.
Phosphitylation was pushed to ~90% completion using $2 \times 1.2$ eq of the chloro-phosphitylating agent 70. Some of the phosphoramidite co-eluted with the starting material thus reducing the yield slightly further, and some material was lost during precipitation. The phosphoramidite was precipitated after chromatography from CH$_2$Cl$_2$ into hexane, to remove remaining hydrolysed reagent (difficult to visualise by TLC), and triethylamine, which would adversely affect subsequent DNA synthesis. A minor nucleoside-based impurity was also detected after reaction, which was removed during purification, presumed later to be due to further phosphitylation of the primary urea in presence of excess reagent. Product 146 was finally afforded in 69% yield, containing negligible impurities.

3.2.3 Synthesis of 6-(3-Acetamidophenyl)-furano-dT Monomer 147

The third target, a 3-acetamidophenyl-modified furano-dT phosphoramidite 147 was proposed as an alternative to the urea. Although the acetamide can form only one hydrogen-bond with G (c.f. 5’PP), the amide NH is more acidic than the aniline NH, and may potentially form a stronger H-bond thereby increasing binding affinity.

The arylalkyne for this synthesis, 3-acetamidophenyl acetylene 161, was synthesised in 94% yield from 3-ethynylaniline 152 following a similar method to that published by Kabalka et al.$^{210}$ for synthesis of $o$-iodoacetanilide. The product was purified by trituration with water as described by Arnold et al.$^{211}$ (who prepared 161 using acetic anhydride and acetic acid), followed by aqueous extraction. Using only 0.15 eq Et$_3$N on
a 1.8 mmol scale, reduced the yield by 11%, and when repeated on an 18 mmol scale, un-neutralised HCl caused degradation to afford a 43% yield, reduced to 38% after recrystallisation (chloroform/hexane) (Scheme 3.5).

Scheme 3.5. Synthesis of 3-acetamidophenyl acetylene 161.

Sonogashira cross-coupling proceeded to completion using alkyne 161, but the product 162 proved difficult to separate from a close-running unknown impurity, affording a final yield of 75%. Cyclisation and phosphitylation under the same conditions as previously described afforded the cyclised nucleoside 163 then phosphoramidite monomer 147 in 89% and 69% yields respectively. The phosphitylation was not pushed to completion with extra reagent beyond ~80%, as the hydrolysed reagent can prove difficult to fully remove after purification (Scheme 3.6).

Scheme 3.6. Synthetic route to 6-(3-acetamidophenyl)-furano-dT phosphoramidite monomer 147.

3.3 Post-Synthetic Conversion of Furano-dT to N-Methylpyrrolo-dC

The three furano-dT phosphoramidite monomers 145-147 were incorporated into triplex-forming oligonucleotides (TFOs) for triplex melting studies, by the automated DNA synthesis method on solid-support (CPG), as detailed in Section 9.2.
Following DNA synthesis, the oligonucleotides were deprotected and cleaved from the solid-support using 30 wt % aqueous methylamine at room temperature for 6-24 hours (Scheme 3.7).

Scheme 3.7. General deprotection/cleavage of oligonucleotides highlighting key groups.

Methylamine, in addition to deprotection and resin-cleavage, ring-opens the furano-dT nucleobase to form a 5-arylmethyl-N-methyl-dC derivative. The methylamine insertion reaction occurred quantitatively in 6 hours, although some TFOs were allowed to deprotect overnight or longer to ensure complete reaction (Scheme 3.8).

Scheme 3.8. Post-synthetic conversion of furano-dT to N-methylpyrrolo-dC in oligonucleotide.

After removal and washing of the resin, the remaining aqueous solution was concentrated in vacuo at 50-60 °C and the residue was re-dissolved in distilled water (1 mL). The oligonucleotide was then treated with DOWEX 50WX8 sulfonic acid resin (200/400 mesh, H⁺-form), at room temperature for 18-36 hours, to recycle the keto-N-methyl-dC nucleotide to form the desired N-methylpyrrolo-dC derivative (Scheme 3.8).

Initially, oligonucleotides were HPLC-purified before acid-catalysed (DOWEX 50) cyclisation and analysed by MALDI-TOF to check extent of reaction (acidic matrix for MALDI can cause limited additional cyclisation). Once the methylamine insertion reaction was determined to be quantitative, oligonucleotides were treated with DOWEX resin straightaway after deprotection/insertion as described above.
After treatment with DOWEX, the resin was removed by filtration, washed with water and the oligonucleotide solution was concentrated in vacuo and desalted (Sephadex NAP™ column). The post-synthetically modified oligonucleotides were analysed by analytical HPLC, capillary gel electrophoresis (CE) (Section 7.3.1), MALDI-TOF and electrospray MS (negative mode). Unfortunately, the acid-catalysed re-cyclisation was not quantitative, only proceeding to 50-80% completion.

The desired cyclised TFO could be isolated by HPLC for oligonucleotides to be used in UV melting studies (Section 4.1). However, HPLC could not resolve TFOs used for fluorescence melting studies. These TFOs are labelled with a large, lipophilic quencher molecule (DABCYL), which dominates in the HPLC chromatogram thus reducing resolution. The two components were resolved by CE, but cannot be purified by this method hence were used in fluorescence melting studies as a mixture. The uncyclised/ring-opened modification, as a C analogue, could potentially bind to GC not CG as desired.

The standard method for deprotection/cleavage of oligonucleotides is treatment with conc. aqueous ammonia at room temperature or 55 °C for several hours. If these TFOs were treated in this way, ammonia would ring-open the furano-dT modification. The ammonia-insertion product spontaneously recyclises to form the NH-pyrrolo-dC derivative, which as a C analogue, could bind preferentially with GC. The N-methyl group, however, prevents the methylamine ring-opened nucleotide from cyclising spontaneously, necessitating acid catalysis. This is presumably due in part to steric bulk of the methyl group.

The post-synthetic conversion of these modifications, although not ideal due to incomplete cyclisation, afforded TFOs that were successfully used in UV and fluorescence melting studies. The results from these are discussed in Chapter 4. Further discussion of oligonucleotide deprotection conditions are given in the study, detailed in Chapter 7.
3.4 Synthesis of 5-(3-aminoprop-1-ynyl)-dU (pdU) monomer 164

Prior to melting studies, approximately 5 g of 5-(3-aminoprop-1-ynyl)-dU (pdU) phosphoramidite monomer 164 was synthesised. 5’-O-(4,4’-Dimethoxytrityl)-5-iodo-2’-deoxyuridine 97 and N-trifluoroacetyl propargylamine 68 were coupled under Sonogashira Pd-catalysed cross-coupling conditions in two 10 g scale reactions, to afford, after combined purification, the alkynyl-nucleoside product 165 in 80% yield (16.6 g). Following phosphitylation of 5 g of 165 using the chloro-phosphitylating reagent 70, under standard conditions, the phosphoramidite monomer 164 was afforded in 84% yield (43 bottles for oligonucleotide synthesis).

Scheme 3.9. Synthesis of 5-(3-aminoprop-1-ynyl)-dU (pdU) phosphoramidite monomer 164.

This phosphoramidite monomer 164 was used for synthesis of TFOs for melting studies (Chapter 4).

3.5 Conclusion

Three furano-dT phosphoramidite monomers 145-147 were synthesised for assessment as monomers for recognition of the CG base pair within a DNA triple helix motif. The common synthetic route (Sonogashira cross-coupling, Cu(I)-catalysed cyclisation and phosphitylation) was successful for each monomer, affording moderate to very good yields for almost every step.

Two main issues were encountered during synthesis. The high polarity of the substrates, once cyclised, introduced problems in purification, even when tritylated. Removal of close-running impurities and starting material also presented minor difficulties. In addition, the cyclised nucleosides demonstrated significantly reduced
reactivity at the 3’-OH and aniline NH$_2$. This presented problems in trifluoroacetylation and necessitated forcing the phosphitylation to completion with extra reagent, which is not ideal. However, all monomers were synthesised in high purity and incorporated into TFOs with high coupling efficiency (as determined by trityl cation conductivity monitoring – see Section 9.2.1) for triplex melting studies (Chapter 4).
Chapter 4

Biophysical Studies of Furano-dT Modified TFOs for CG Recognition
4. Biophysical Studies of Furano-dT Modified TFOs for CG Recognition

The three modified furano-dT phosphoramidite monomers ($\text{AP}P_f$ 145, $\text{AP}P_f$ 146, $\text{UP}P_f$ 147) and commercially available 6-methyl-furano-dT $\text{MP}_f$ monomer 144 were incorporated into unlabelled/labelled TFOs for UV and fluorescence melting studies. These studies were designed to test binding affinity for CG and selectivity against the other base pairs (GC, AT, TA) at several pHs. These properties are assessed by determining triplex melting temperatures from graphs of, change in UV absorption or fluorescence emission of fluorophore label respectively, on triplex melting, for different sequences, in several buffer conditions. After incorporation, the modification was post-synthetically converted in the oligonucleotide from a furano-dT to an N-methylpyrrolo-dC nucleotide. The procedure for this transformation is described in Section 3.3.

4.1 UV Melting Studies

Methyl- (144, $\text{MP}_f$), trifluoroacetamidophenyl- (145, $\text{AP}P_f$) and ureidophenyl- (147, $\text{UP}P_f$) furano-dT phosphoramidite monomers were incorporated into TFOs (OL3-5) to assess binding affinity for a single CG inversion in a homopurine tract, in the parallel motif. A schematic representation of the UV melting triplex, used originally by Leumann et al.\textsuperscript{111} is shown in Figure 4.1.

![Figure 4.1](image_url)

**Figure 4.1.** UV triplex melting experiment. TFO shown in bold. $\text{M} = 5\text{-Me dC}$, $\text{X} = \text{MP}$ (OL3), $\text{AP}$ (OL4), $\text{UP}$ (OL5), purine duplex strand ($\text{C}$, OL1), pyrimidine duplex strand ($\text{G}$, OL2).

UV melting experiments were performed, (as described in detail in Section 9.2), on a Varian Cary 400 Scan UV-Vis spectrophotometer monitoring at 260 nm. A 5:1 ratio of TFO:duplex (5:1 µM) was used. Experiments were conducted at pH 6.07 and pH 6.40 (10 mM sodium phosphate, 200 mM NaCl, 1 mM Na$_2$EDTA). A standard melt programme was used (Programmes 1/2: 10-50-80 °C at 0.5 °C/min) with 3 or 4 heat/anneal cycles in total (Section 9.2). Condensation was not a major problem at low temperature, affecting only the first heat/anneal cycle, hence purging the sample
chamber with argon was not necessary. Disodium EDTA is used to scavenge divalent cations (e.g. \( \text{Mg}^{2+} \)), which cause general triplex stabilisation.

The results (obtained over six experiments) indicated \(^{\lambda}\text{PP}\) demonstrates enhanced binding affinity to CG compared to \(^{\text{M}}\text{P}\) (\(\Delta T_m +4.0 \, ^\circ\text{C}/+4.5 \, ^\circ\text{C}\) at pH 6.07/6.40) (Table 4.1). This represents a significant improvement over \(^{\text{M}}\text{P}\), which Ranasinghe et al.\(^{122}\) demonstrated to perform better than T, using the same triplex motif, at pH 7.0 in 10 mM sodium cacodylate buffer (with 200 mM NaCl, 0.25 mM spermine) (Table 4.1).

<table>
<thead>
<tr>
<th>pH</th>
<th>(T_m/, ^\circ\text{C},) melt/anneal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(^{\text{M}}\text{P}) (6 runs)</td>
</tr>
<tr>
<td>6.07</td>
<td>31.6/23.1, (62.7)</td>
</tr>
<tr>
<td>6.40</td>
<td>24.5/n.d., (63.0)</td>
</tr>
</tbody>
</table>

**Table 4.1.** UV triplex melting experiment: \(^{\lambda}\text{PP}\) against CG at pH 6.07 and 6.40 (10 mM sodium phosphate, 200 mM NaCl, 1 mM Na\(_2\)EDTA) using UV melting programme No. 1 (Section 9.2). Average \(T_m\) values given in \(^\circ\text{C}\) for TFO melt/anneal and for duplex in parentheses (average of melt and anneal \(T_m\)). n.d. = not determined (\(T_m < 17 \, ^\circ\text{C}\)).

The \(^{\uparrow}\text{PP}\) TFO (OL5), however, demonstrated reduced affinity compared to \(^{\lambda}\text{PP}\), yet performed better than the control \(^{\text{M}}\text{P}\), by +0.6 \(^\circ\text{C}\) and +1.1 \(^\circ\text{C}\), at pH 6.07 and pH 6.40 respectively. The MALDI-TOF analysis of OL5 showed the molecular ion to be 42 Da higher than expected, indicating the oligonucleotide may have acetylated during the capping step of oligonucleotide synthesis (found \(m/z\) 4697.2, expected 4656.2 \([\text{M} + \text{H}]^+\) ). It was presumed that acetylation had occurred on the primary urea, thereby reducing the \(T_m\) due to increased steric bulk directed into the side of the major groove. The urea was expected to perform better than the aniline, as it has the potential to form two hydrogen-bonds with G instead of one; the NHs would also be more acidic lending to formation of stronger hydrogen-bonds.

In further UV melting studies (Section 6.1), in which TFOs were synthesised without capping, the same order of binding affinity and similar \(\Delta T_m\)s were observed. It is unlikely therefore that the urea was acetylated, although when synthesised without capping, no peak for \([\text{M} + 43]^+\) was observed. Acetylation may have occurred on the 4-\(N\) position of one of the 5-methyl-dC nucleotides, but this cannot be confirmed.
Significant hysteresis was observed for the melting temperature using this melt programme, with $\Delta T_m$ values between 5.4 and 9.2 °C. This could be decreased by reducing the rate of heating/cooling. The duplex hysteresis was within experimental error, at less than 0.5 °C.

A graph displaying melting curves and their derivatives for $^\wedge$PP (OL4) at pH 6.07, is shown in Figure 4.2 below. The hysteresis between melting and annealing curves is clearly visible.

![Graph of melting curves and derivatives](image)

**Figure 4.2.** UV Melting curves/derivatives for $^\wedge$PP (OL4) against CG at pH 6.07 (10 mM sodium phosphate, 200 mM NaCl, 1 mM Na$_2$EDTA). Data is smoothed and un-normalised.

These initial studies indicated that the 3-aminophenyl moiety significantly enhanced binding affinity over a methyl group. The urea moiety, unexpectedly demonstrated poorer binding than the amine. This result necessitated further UV melting and other studies for confirmation and to test other analogues.

4.2. **Fluorescence Melting Studies**

Fluorescence melting studies were conducted to assess binding affinity and selectivity, and the sequence-dependency of these recognition properties. Fluorescence melting
measures change in fluorescence due separation of fluorophore and fluorescence quencher moieties on duplex and TFO respectively, on triplex melting.

4.2.1 Binding Affinity/Selectivity study

Methyl- (144, MP), trifluoroacetamidophenyl- (145, APP), acetamidophenyl (147, App) and ureidophenyl- (146, UPP) furano-dT phosphoramidite monomers were incorporated into labelled TFOs for initial fluorescence melting studies. The target was a single CG inversion in a homopurine tract of a hairpin duplex, in the parallel motif. The initial triplex motif is represented below in Figure 4.3.

\[
\begin{align*}
5' &-Q-PMM TPM TXP TPT PTM PT \\
5' &-F-GT GTT AGG AAG A \\
&Y AAA AAG AAC TGG T-H \\
&CA CAA TCC TTC T \\
&Z T TTT TTC TTG ACC A-H
\end{align*}
\]

Figure 4.3. Fluorescence triplex melting experiment. TFO shown in bold. (M = 5-Me dC, P = 5-(3-aminoprop-1-ynyl)-dU (pdU), X = MP (OL10), APP (OL11), App (OL12), UPP (OL13), Q = DABCYL (quencher), F = FAM (fluorophore), H = HEG (hexaethylene glycol), hairpin duplex YZ = CG (OL6), GC (OL7), AT (OL8), TA (OL9).

Fluorescence melting studies were conducted (as described in detail in Section 9.2), on a Roche LightCycler®. Initial experiments were performed at pH 6.0 (20 mM NaOAc, 200 mM NaCl), and a 10:1 ratio of TFO:duplex (5:0.5 µM) was used. Runs in these experiments were performed up to three times in duplicate, using LC Programme 1 (95-30-95 °C at 0.2 °C/min, see Section 9.2) (Table 4.2). These sequences were used for fluorescence melting only.

There is a stark contrast between some of these results and the previous UV melting study. Whereas APP previously demonstrated a significantly higher \(T_m\) than MP against CG (\(\Delta T_m +4.0-4.5 \, ^\circ C\)), these results place the \(T_m\) of APP, 1.2 °C lower than MP. This observation could not be easily explained, although the buffer and sequence had been changed. Despite these observations, the urea (UPP) showed the strongest binding affinity towards CG as originally expected and the order of stability (UPP>App≈APP), excluding MP, correlates with the predicted hydrogen-bonding motif.
Table 4.2. Fluorescence triplex melting experiment: \(^{3}\text{PP}_f\) against all four base pairs at pH 6.0 (20 mM NaOAc, 200 mM NaCl) using LC Programme 1 (Section 9.2). Average \(T_m\) values given in °C for TFO melt/anneal and for secondary melt transition in parentheses. n.d. = not determined (\(T_m < 36 \, ^\circ\text{C}\)), bp = base pair target.

<table>
<thead>
<tr>
<th>X (^{3}\text{PP}_f) bp</th>
<th>(T_m) / °C, melt/anneal</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG</td>
<td>M(^{3}\text{P})</td>
</tr>
<tr>
<td>53.1/50.6</td>
<td>51.9/47.9</td>
</tr>
<tr>
<td>GC 40.7/40.3, (67.0)</td>
<td>38.0/36.3, (51.3)</td>
</tr>
<tr>
<td>AT 40.4/38.2</td>
<td>40.3/38.0</td>
</tr>
<tr>
<td>TA 40.1/38.7</td>
<td>42.3/39.7</td>
</tr>
</tbody>
</table>

All monomers demonstrated good selectivity for CG over GC, AT and TA. \(^{3}\text{PP}\) was most selective against GC (\(\Delta T_m +14.2 \, ^\circ\text{C}\)) and \(^{3}\text{P}\) proved most selective against AT and TA (\(\Delta T_m +12.7 \, ^\circ\text{C}, +13.0 \, ^\circ\text{C}\)). Selectivity for CG over TA is most difficult to achieve as both present only one formal hydrogen-bond for binding. Therefore, achieving selectivity against TA is an important criterion in choosing a monomer for CG recognition. However, even the poorest selectivity (\(^{3}\text{PP}\) vs TA, \(\Delta T_m +7.3 \, ^\circ\text{C}\)) is still satisfactory.

Hysteresis of 0.42-4.97 °C was observed (average 2.66 °C), despite using a low heating/cooling rate of 0.2 °C/min (total run time - 12.5 hours). Hysteresis could once more have been reduced by reducing the rate further. However, this would necessitate a run time in excess of 20 hours, which might cause problems due to degradation/evaporation of sample. \(T_{m}s\) differed by no more than 1.2 °C.

The \(^{3}\text{PP}_f\) modification was evaluated previously by Dr. Powers in a different fluorescence melting triplex motif, at lower pH and a higher buffer concentration. It was shown to be selective for CG, although the order of selectivity over the other base pairs was different from that detected here.\(^{51}\)

A biphasic melting profile was also observed, against GC, but not the other base pairs or indeed in annealing against any base pair. The presence of the weaker, secondary transition can be rationalised by the proportion of monomer (20-40%), which was not re-cyclised during treatment of TFO with DOWEX-H\(^{+}\) resin. This C-analogue could, as
previously mentioned (Section 3.3) form a triplet with GC, affording a higher \( T_m \) than expected, for a triplex that should contain a disfavourable X.GC triplet (Figure 4.4).

![Figure 4.4. Putative X.GC and X\textsubscript{uncyclised}.GC triplets.](image)

The observed secondary \( T_m \) value against GC should be similar to that against CG (\( \sim 52 ^\circ C \)) as was observed for \( ^APP, ^AcPP \) and \( ^UPP \). However, the value is significantly higher for \( ^MP \) (67.0 \(^\circ C \)), which is difficult to rationalise.Replacing the aryl group for a methyl group should reduce the potential for beneficial interactions within the triple helix, unless the aryl group in fact confers disfavourable steric/hydrophobic interactions. Furthermore, comparing with the many modifications that have been made to the 5-position of C-derivatives for achieving GC recognition (Section 1.2.1.2), it is highly unlikely that the 5-(acetylmethyl)- group of uncyclised/ring-opened \( ^MP_f \) is stabilising enough to account for this \( T_m \). Other secondary structures and interactions may also be contributing factors.

4.2.2 Sequence study

The two possible explanations for reversal in the order of stability of \( ^MP \) and \( ^APPP \) between the initial UV and fluorescence melting studies were a change in buffer/pH, and changing the sequence. It was hypothesised, that altering the neighbouring nucleosides to the modification under examination within the TFO may affect binding affinities and selectivity.

New TFOs were therefore synthesised, containing the central trimers, TXT (OL14), PXT (OL15) and PXP (OL16) (\( X = ^APPP, P = pdU \)) to compare with the TXP-containing TFO (OL11) already investigated. It was thought that presence of pdU next to the \( ^XPp \)
modification may change binding affinity/selectivity or mask the effects of the modification, and may account for the differences between the two methods.

\[
\begin{align*}
5’-Q-P\text{PMM TPM VXW TPT PTM PT} \\
5’-F-GT GTT AGG AAG ACA AAA AAG AAC TGG T-H \\
CA CAA TCC TTC TG \text{T TT TTC TTG ACC A-H}
\end{align*}
\]

Figure 4.5. Fluorescence triplex melting experiment. TFO shown in bold. (\(M = ^{5}\text{Me}dC, \ P = 5-(3\text{-aminoprop-1-ynyl})\text{-dU (pdU), } X = ^{\text{AP}}P \ & \ VXW = \text{TXT (OL14), PXT (OL15), TXP (OL11), PXP (OL16); VXW = TXP} \ & \ X = ^{\text{MP}}P \ (OL10), ^{\text{AP}}PP \ (OL12), ^{\text{UP}}P \ (OL13), Q = \text{DABCYL (quencher); } F = \text{FAM (fluorophore), } H = \text{HEG (hexaethylene glycol), hairpin duplex (CG, OL6).}\)

The initial fluorescence melting experiments (Figure 4.5) were repeated using LC Programmes 1 and 2 (95-30-95 °C at 0.2 °C/min) at pH 6.2 (10 mM sodium phosphate, 200 mM NaCl). Experiments were run once or twice in triplicate and a 10:1 ratio of TFO:duplex (5:0.5 µM) was used as before. The aims of these experiments were not only to obtain confirmation of previous results, but to investigate how the change of sequence described above affects binding, and for comparison between analogous furano-dT (\(^{\text{XPP}}f\)) and \(N\)-methylpyrrolo-dC (\(^{\text{XPP}}p\)) modified TFOs (see Section 6.1 for results from \(^{\text{XPP}}p\)-modified TFOs).

<table>
<thead>
<tr>
<th>X Seq.</th>
<th>(T_m / ^\circ C, \text{melt/anneal})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(^{\text{MP}}P)</td>
</tr>
<tr>
<td>TXT</td>
<td>–</td>
</tr>
<tr>
<td>PXT</td>
<td>–</td>
</tr>
<tr>
<td>TXP</td>
<td>49.7/43.7</td>
</tr>
<tr>
<td>PXP</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 4.3. Fluorescence triplex melting experiment: \(^{\text{XPP}}f\text{ against CG in four TFOs (OL14, OL15, OL11, OL16) at pH 6.2 (10 mM sodium phosphate, 200 mM NaCl) using LC Programme 1 and 2 (Section 9.2). Average } T_m \text{ values given in } ^\circ C \text{ for TFO melt/anneal. * Data obtained for } ^{\text{XPP}}p\text{-modified TFOs is not shown (Section 6.2).}\)

Hysteresis was much greater than previously observed (4.08-6.91 °C, average 5.61 °C) and annealing data was of poorer quality than in previous experiments. However, the melting data was clear and \(T_m\) values differed by no more than 1.0 °C.
These experiments confirm that the urea ($^U$PP) has the highest binding affinity in the TXP-containing TFO. $^SP$ and $^AP$PP appear as effective as each other and slightly better than the acetamide ($^{Ac}$PP), although experimental error may account for variation as the difference in $T_m$ values is small.

When T is replaced by pdU, the $T_m$ increases because pdU forms additional charge-stabilising interactions with the sugar-phosphate backbone (Section 1.2.1.1). In the $^AP$PP-modified series of TFOs, as for any modification, for each substitution the $T_m$ would be expected to increase by the same amount, and $T_m$ should be equal for the TXP- and PXT-containing TFOs (OL11, OL15) as only the order is changed. However, the $T_m$ for TXP (OL11) is 0.6 °C higher than PXT (OL15). Also the increase in $T_m$ from TXT to TXP is greater than for PXT to PXP.

These observations indicate that placing pdU on the 5’-side of $^X$PP is slightly destabilising compared to the 3’-side. It is reasonable therefore, to suggest there may be interaction between the 3-aminoprop-1-ynyl group of pdU and the anilino group of $^AP$PP, slightly affecting recognition of CG. This difference was most significant for $^UP$PP ($^{AP}$PP,$_l$-modified TFO, see Section 6.2) with a $\Delta T_m$ value of 3.0 °C. It is also reasonable to suggest that due to this destabilisation, replacing the second T with pdU has less stabilising effect than expected.

Finally, data for $^{Ac}$PP and $^UP$PP confirmed that the $T_m$s for furano-dT and $N$-methylpyrrolo-dC modified TFOs were approximately the same, only differing by 0.7 and 0.1 °C respectively. These results led into a large study, using $N$-methylpyrrolo-dC monomers, the results of which are discussed in Chapter 6.

### 4.3 Fluorescence Melting Mismatch Experiment

Experiments were conducted to test if triplex-mediated recognition of a base pair mismatch can occur using the $^{X}$PP,$_r$ modifications tested above. This may also provide information on binding motif of the X.CG triplet. All four modifications were assessed against a hairpin duplex containing a CC mismatch. Fluorescence melting was conducted using LC Programme 4 (95-30-95 °C at 0.25 and 0.2 °C/min) at pH 6.0 (20
Experiments were run once in duplicate and a 10:1 ratio of TFO:duplex (5:0.5 µM) was used as before (Table 4.4).

\[
\begin{align*}
5'\text{-Q-PMM TPM TXP TPT PTM PT} \\
5'\text{-F-GT GTT AGG AAG ACA AAA AAG AAC TGG T-H} \\
\text{CA CAA TCC TTC TCT TTT TTC TTG ACC A-H}
\end{align*}
\]

Figure 4.6. Fluorescence triplex melting mismatch experiment. TFO shown in bold. (M = 5-Me\textit{dC}, P = 5-(3-aminoprop-1-ynyl)-dU (pdU), X = \textit{MP} (OL10), \textit{AP} (OL11), \textit{AP} (OL12), \textit{UP} (OL13), Q = DABCYL (quencher), F = FAM (fluorophore), H = HEG (hexaethylene glycol), mismatched hairpin duplex (CC, OL17).

These results show a drop in \(T_m\) of 4.5 °C for \textit{MP} when CG is replaced by a CC mismatch. This is expected as even though \textit{MP} is proposed to hydrogen-bond only to one C of the CC base pair as is the case for CG, distortions due to the CC mismatch would cause destabilisation of the triplex as well.

<table>
<thead>
<tr>
<th>(T_m/°C,) melt/anneal</th>
<th>(\textit{MP.CG})</th>
<th>(\textit{MP.CC})</th>
<th>(\textit{AP.PP.CC})</th>
<th>(\textit{UP.PP.CC})</th>
</tr>
</thead>
<tbody>
<tr>
<td>53.1/50.8</td>
<td>48.6/47.1</td>
<td>48.9/45.9</td>
<td>49.3/46.2</td>
<td>50.0/48.4</td>
</tr>
</tbody>
</table>

Table 4.4. Fluorescence triplex melting mismatch experiment: \(\textit{PP}\) against CC mismatch hairpin duplex (OL17) at pH 6.0 (20 mM NaOAc, 200 mM NaCl) using LC Programme 4 (Section 9.2). Average \(T_m\) values given in °C for TFO melt/anneal. \textit{MP.CG} data from this experiment is shown for comparison.

The CC mismatch also reduces the duplex melting temperature by ~8 °C compared to that of the CG hairpin duplex. In addition, the derivative curve is markedly different to the duplexes containing matched base pairs, as it curves towards an apparent melting transition (~74 °C) just below duplex melting. It is possible that other structures may be present, or that this is an unexplained feature of the melting profile of a mismatch-containing hairpin duplex, the latter of which appears more likely.
Figure 4.7. Fluorescence melting curves/derivatives for $^{3}$P (OL10) against CG (red, OL6) and CC (black, OL17) at pH 6.0 (20 mM NaOAc, 200 mM NaCl). Data is smoothed and un-normalised.

Melting temperatures for all modifications were very similar; the urea performing marginally better than the rest. This is consistent with the proposed binding model, in which differentiation between modifications is achieved through additional hydrogen-bonds to G of the CG base pair. Altering the hydrogen-bond pattern, in conjunction with triplex distortion due to the mismatch, reduces these differences.

4.4 Conclusion

All melting studies demonstrated the effectiveness of the $^{X}$PP modifications at recognising CG with similar or better binding affinity/selectivity than $^{3}$P, which itself is a large improvement on T. Although it was unknown why order of stability differed between UV and fluorescence melting studies, this was investigated in further studies (Chapter 6). At this stage it was presumed to be caused by change in sequence (particularly of neighbouring nucleotides), buffer and pH, but this was/could not be confirmed.

The significant effect of neighbouring groups on the modification under investigation, was also identified. Results indicated that the modified nucleoside 5-(3-aminoprop-1-ynyl)-dU 14 (pdU, P), interacts differently with $^{X}$PP depending on which side it is located, giving rise to a difference in $T_{m}$. This neighbouring group interaction introduces important implications for design of TFOs, particularly for use as
oligotherapeutics. Understanding the interactions between modified nucleosides is vital, so that negative interactions can be minimised.

Melting experiments were also conducted against a CC mismatch in the same hairpin duplex motif. It was demonstrated that differences in $T_m$s between TFOs is reduced as key hydrogen-bonding residues are removed when G is replaced with C. The reduction in overall duplex and triplex $T_m$ is expected, due to the CC mismatch.

It would also be useful to test the CT and CA mismatches to identify if any differentiation could be made between modifications. Both CT and CA contain potential hydrogen-bonding acceptors on T and A, therefore some differentiation or stabilisation may be observed.

Assessing recognition of common base pair mismatches/mutations could also provide valuable information when designing TFOs for therapeutic use. Depending on location of mismatches and on which strand the mutation is located, it may be possible to bind to both desired and undesired sequences. As binding primarily occurs to one side of the base pair, only mutations to the other base should be tolerated.
Chapter 5

Amine-Modified $N$-Methylpyrrolo-dC Nucleosides for CG Recognition
5. **Amine-Modified N-Methylpyrrolo-dC Nucleosides for CG Recognition**

5.1 **Introduction**

In previous studies (Sections 3-4), N-methylpyrrolo-dC modifications were incorporated into TFOs as the furano-dT analogues then post-synthetically converted. Although the methylamine-insertion reaction was quantitative for each oligonucleotide, the acid-catalysed cyclisation reaction using acidic DOWEX 50WX8 ion-exchange resin, to reform the bicyclic core, did not proceed to completion. The major problem with this method, in addition to reduced yield of TFO, is difficulty in purification when labeled with DABCYL. For this reason, TFOs for fluorescence melting studies were used as the inseparable mixture containing approx. 20-40% uncyclised modification. This did not affect recognition of the CG base pair, although the concentration of correct TFO was lowered as a result. However, in fluorescence melting studies against GC, a second minor melting transition was observed at a similar or substantially higher temperature than against CG. This was proposed to be due to the small proportion of the uncyclised modification, which, as a C-analogue, exhibits GC recognition properties (also Sections 4.2 and 6.2). The very high temperature of one of these transitions could not be rationalised, however, unless the ring-opened modification is exceptionally strong at binding, which is very unlikely.

To overcome this problem, it was proposed, in house, to synthesise monomers as the N-methylpyrrolo-dC derivatives. Assuming this bicyclic structure was stable to deprotection conditions (i.e. could not be ring-opened by NH₃ or MeNH₂, in aqueous solution), no post-synthetic modification would be required. Ranasinghe synthesised, in house, the protected 6-aminoethyl and 6-aminopropyl- monomers 166,167 as the N-methylpyrrolo-dC derivatives (\(^{\text{\text{AE}}P_p}\) and \(^{\text{AP}}P_p\)), via methylamine insertion and acid-catalysed cyclisation, on the untritylated furano-dT nucleosides (c.f. post-synthetic modification of TFO – Section 3.3) (Scheme 5.1).
therefore this modification was deemed ideal for testing this synthetic route. The urea moiety increases substrate polarity, which hydrogen-bonding interaction with G of the CG base pair, in addition to the binding motif previously described. The first target was the ureido-modified monomer 5.1.1.

Although this route was successful for one monomer, the route needed to be robust; applicable to synthesis of all derivatives required for biophysical studies. The method therefore, went through optimisation to afford a general synthetic route applicable to all desired analogues (Section 5.2).

5.1.1 Synthesis of 6-(3-Ureidophenyl)-N-methylpyrrolo-dC monomer 179

The first target was the ureido-modified monomer 179, which should exhibit favourable hydrogen-bonding interaction with G of the CG base pair, in addition to the binding motif previously described. The urea moiety increases substrate polarity, which affected purification of the corresponding furano-dT monomer 146 and intermediates, therefore this modification was deemed ideal for testing this synthetic route.
The first method closely followed the synthetic routes used by Ranasinghe\textsuperscript{122} and Vadhia.\textsuperscript{206} This route proceeds via the ‘free’ (unprotected) furano-dT nucleoside 180, which was synthesised via the Sonogashira and cyclisation reactions discussed in Section 3.2.2.

Following Cu(I)-catalysed cyclisation, Ranasinghe and Vadhia ring-opened the ‘free’ nucleoside 180 with 33\% ethanolic methylamine, before acid-catalysed re-cyclisation. Ranasinghe also, successfully ring-closed a 6-butyl-derivative, by refluxing in acetonitrile with 1 mol\% toluene-sulfonic acid, to obtain an 88\% yield over these two steps. Vadhia used 80\% aqueous acetic acid, at 80 °C to afford the re-cyclised aniline-derivative 143 in 21\% yield. Subsequent tritylation proceeded with difficulty in 9\% yield. In both methods the acid was difficult to remove, and for the latter case, acetic acid may have also affected column purification and certainly tritylation, although poor to moderate solubility of the product 143 may also have played a role. Vadhia could not re-cyclise the ureido-derivative using acetic acid, most likely due to poor solubility.

In an attempt optimise this route, the above synthesis was first repeated (Scheme 5.2). 5-Iodo-2’-deoxyuridine 148 was coupled three times with 3-ureidophenyl acetylene 156 via the previously described Sonogashira reaction, finally yielding a ~1:1 mixture of cyclised 180 and uncyclised nucleoside 181, containing some alkyne dimer.

\[ \text{Scheme 5.2. First route to } \text{U} \text{PP}_x \text{ monomer 179.} \]
The product mixture 180,181 was refluxed with CuI in dry methanol and triethylamine. However, the mixture was poorly soluble, even at 80 °C under argon pressure, and NMR analysis of the crude material confirmed no change had occurred from the 1:1 mixture.

Addition of DMF solubilised the substrate, but failed to illicit cyclisation, even at 90 °C. Tritylation of the nucleoside mixture was also problematic due to poor solubility in pyridine. Excess DMTCl and some heating were necessary to afford some reaction, but a mixture of mono- and bis-DMT nucleosides was afforded.

It was noted that leaving the Sonogashira cross-coupling reaction for longer may have resulted in complete cyclisation. However, due to solubility issues, an alternative synthetic route was sought.

5.1.1.2 Synthesis via tritylated furano-dT derivative

In order to overcome problems with solubility, the DMT-protected furano-dT nucleoside 160 was used, synthesised as described in Section 3.2.2.

Scheme 5.3. Second revised route to 1PPp monomer 179.

Alternative sources of acid to p-TsOH and acetic acid were sought, to prevent detritylation if possible, but mainly for ease of removal after reaction. Cyclised nucleoside 160 was dissolved in 33 wt% methylamine/ethanol at rt, to afford the ring-
opened product 183 in near quantitative conversion. The crude material was dissolved in anhydrous DMF, divided into two, and agitated with DOWEX-50WX8 resin (pyridinium form) or silica gel/Et₃N, at 30 °C for 1 day, then 50 °C for 1 day and 70 °C for 12 hours. No change was observed using silica gel, and the DOWEX caused some detritylation, despite release of pyridine from the resin. The ring-opened compound 183 was also isolated (61% yield) in a larger-scale reaction.

In order to avoid detritylation by exposure to acid, heat alone was assessed as a method of cyclisation. After quantitative ring-opening with methylamine, strong heating of the resulting solution caused significant degradation. Refluxing the crude material in acetonitrile and triethylamine at 90 °C under argon pressure overnight afforded an unidentified product, less polar than the starting material, plus some degradation. This was non-fluorescent on TLC and was presumed to be the 4N-methyl-imino-dC derivative. Heating the mixture in ethanolic methylamine at 70 °C overnight also afforded ~50% conversion to the same unidentified, and undesired product, in one step.

Successful cyclisation was achieved using DOWEX 50WX8 resin, in the protonated form. Following ring-opening, the residue was heated at 60 °C overnight in DMF/H₂O (2:1 v/v) with DOWEX 50 (H⁺-form) affording the cyclised, detritylated nucleoside 182. Clearly, protonated DOWEX resin is a suitable, easily removable source of acid, however, it was decided this route was not practical for synthesis of this monomer. The high polarity/poor solubility would cause problems for subsequent purification and tritylation, as observed by Vadhia. Removal then replacement of the DMT protecting group is also not atom-efficient.

5.1.1.3 Synthesis via derivatisation of 5-iodo-4N-methyl-dC 185

The third revision to the original route involved initial conversion of tritylated 5-iodo-2’-deoxyuridine substrate 97 to the 4N-methyl-2’-deoxycytidine derivative 185. Although 5-iodo-2’-deoxycytidine is commercially available, the 4N-methylated analogue is not, but several methods of uridine to cytidine conversion are presented in the literature.
The 4-carbonyl group is first activated and converted into a good leaving group by a variety of reagents, before reaction with a suitable nucleophile (e.g. NH$_3$, MeNH$_2$). This conversion was conducted using N-methylimidazole (NMI) in presence of POCl$_3$, conditions published by Herdewijn et al. The reaction proceeds via the 4-N-methylimidazoloyl-dC intermediate 186, which is not isolated from the reaction (Scheme 5.4).

Scheme 5.4. Third revised route to $^{31}$PP$_p$ monomer 179.

After acetylation of the 3'-OH group in 91% yield (Scheme 5.4), the protected nucleoside 187 was treated with phosphorous oxychloride and NMI in pyridine to form the yellow intermediate 186 over 30-60 minutes. Treatment with 40 wt% aqueous methylamine for several hours afforded the partially deacetylated 4N-methyl-dC nucleoside. Further treatment with 33 wt% methylamine/ethanol, before or after aqueous workup completed the deacetylation.

On a 1.4 g scale, the product 185 was obtained in a 90% yield, after column chromatography using CH$_2$Cl$_2$/CH$_3$OH as eluent. Reaction on a 6.8 g scale afforded the compound in 80% yield after difficult removal of a significant quantity of NMI. This was removed by 4 columns (different eluents) and recrystallisation from toluene. On an 8.3 g scale, NMI was removed with difficulty by 2 columns and successive extraction with diethyl ether, followed by recrystallisation from ethyl acetate (46% yield).
When later resynthesised in house, by Dr. Mastoura Edrees-Abdou, stirring the reaction at rt overnight after addition of 40 wt% aqueous methylamine was sufficient to completely deacetylated, therefore no further treatment with 33 wt% ethanolic methylamine was required. Also, careful column chromatography using methanol/CH₂Cl₂ as eluent afforded the product in 84% yield.

Commonly, conversion via the 4-(1,2,4-triazol-1-yl)- intermediate is used, using a large excess of 1,2,4-triazole in presence of excess POCl₃. The intermediate was often isolated, although Sanghvi et al. reported an increased yield if this intermediate was not isolated, and have conducted this reaction up to kilo scale, in yields of 65-85%.

Other reagent systems used for this conversion include 3-nitro-1,2,4-triazole/diphenyl phosphoro chloridate, l-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-1H-triazole (MSNT)/cat. Ph₂PO₄ (forms the 3-nitro-1,2,4-triazolyl- intermediate), 2,4,6-mesitylenesulfonfonyl chloride/DMAP, 2,4,6-triisopropylbenzenesulfonfonyl chloride/DMAP, triisopropylsilyl chloride/DMAP, and 2- and 4-nitrophenol/POCl₃.

Sonogashira cross-coupling with 3-ureidophenyl acetylene gave the desired alkynyl product with a large quantity of the alkyne dimer impurity, and a minor unknown impurity. The alkyne dimer was partially removed by recrystallisation from methanol. Following cross-coupling, the nucleoside/alkyne dimer mixture was treated with copper iodide in dry, refluxing CH₃OH/Et₃N at 80 °C under McGuigan’s conditions, but only partial cyclisation was observed over 2 hours.

One of few literature examples of cyclisation of 5-alkynyl-dC derivatives was published by Inoue et al. A protected 4N-acetyl-5-ethynyl-dC nucleoside was cyclised by heating at 125 °C in DMF for 10 minutes, in presence of CuI (0.55 eq).

The ureido-modified Sonogashira product containing the alkyne dimer impurity, was successfully cyclised under similar conditions, to give the desired product in 55% then 79% yield, over two steps, finally using a slight excess of CuI (1.1 eq) and longer reaction time (40 minutes). The alkyne dimer impurity from the previous reaction was then easily removed by column chromatography.
The cyclised nucleoside 184 was subsequently phosphitylated to afford the desired $^4$PP$_r$ monomer 179 for incorporation into TFOs. During the first small-scale attempt, using the chloro-phosphitylating reagent 70, a larger quantity was added in error affording 41% yield of the bis-phosphoramide (reaction on primary urea) plus 19% yield of desired phosphoramide 179. Once the reactivity of the urea NH$_2$ was highlighted, the less reactive bis-aminophosphitylating reagent 189 (Figure 5.1) was used, in order to minimise secondary reaction. This necessitated 2.6 eq of reagent, 1.0 eq of activator diisopropylamine hydrotetrazolide (DIHT) 190 and very long reaction time. During this time, significant degradation occurred, with formation of a viscous yellow syrup, from which the product was extracted in 29% yield. No bis-phosphitylation was observed.

![Figure 5.1. Bis-amine phosphitylating reagent 189 and activator, DIHT 190.](image)

DIHT 190 is used in preference to tetrazole due to greater stability and solubility, and lower acidity and hygroscopicity.\(^{224}\)

Phosphitylation using the chloro-reagent 70 (1.1 eq) was, however, chosen finally, and the product was obtained in 51% yield after column chromatography and precipitation. The reaction was stopped after 2 hrs due to appearance of some bis-phosphitylated material. In all phosphitylation reactions, anhydrous DMF was added to solubilise the polar starting material. In total, 6 bottles of monomer (100-120 mg each) were obtained for DNA synthesis.

5.1.2 Synthesis of 6-(3-Acetamidophenyl)-$^N$-methylpyrrolo-dC monomer 191

The acetamide-modified monomer 191 was synthesised by the same route as described above. After Sonogashira cross-coupling of 3-acetamidophenyl acetylene 161 and 5-iodo-4-$^N$-methyl-dC nucleoside 185, the resultant product 192/alkyne-dimer mixture was treated under Inoue’s conditions\(^{222,223}\) and the cyclised acetamido nucleoside 193 was isolated in excellent 84% yield over the two steps (Scheme 5.5).
Scheme 5.5. Synthesis of \(^{3}^{2}P_{3}\) monomer 191.

Phosphitylation was carried out using the chloro-reagent 70 in anhydrous THF/DMF to afford the phosphoramidite 191 in excellent 86% yield. This substrate had no competing reaction sites and the reaction proceeded to completion. Despite the high polarity once cyclised, purification proved easier than for the ureido nucleoside 179. In total, 13 bottles of monomer (70-120 mg each) were obtained for DNA synthesis.

5.1.3 Synthesis of 6-(3-Trifluoroacetamidophenyl)-N-methylpyrrolo-dC monomer 178

Synthesis of the trifluoroacetamido-modified monomer 178 was achieved via a slightly altered route. Following Sonogashira coupling of 3-ethynylaniline (3-aminophenyl acetylene) 152 with 5-iodo-4\(N\)-methyl-dC nucleoside 185 (93-95% yield), cyclisation was carried out under Inoue’s conditions.\(^{222,223}\)

Scheme 5.6. Synthesis of \(^{3}^{2}P_{3}\) monomer 178.
The first cyclisation attempt afforded only 32% yield of product 195, plus 12% yield of a bis-tritylated product 196 and some untritylated (free) nucleoside 143. During workup, addition of acetone, methanol or dichloromethane (to precipitate impurities) had caused precipitation of what was thought at that stage to be a mixture of product and copper salts. The mixture was therefore re-dissolved in DMF and filtered, which unfortunately proved unsuccessful in removing significant impurities. After removing the resulting large volume of DMF under high vacuum at elevated temperature (40-50 °C), the dark brown residue was dissolved/suspended in dichloromethane and filtered, successfully removing impurities as originally intended. It was discovered, however, that during DMF removal, some detritylation (through prolonged heating) and retritylation of the product was observed. Retritylation was shown by NMR analysis to have occurred on the less hindered, though less reactive aniline NH$_2$, not the 3'-OH group. This is possible as the DMT$^+$ cation is capable of reacting further in absence of water (removed under heat/high vacuum). On repeating this reaction, DCM was used to precipitate impurities, and a yield of 80% was achieved with no DMT migration.

The aniline-NH$_2$ of 195 was trifluoroacetylated using trifluoroacetic anhydride with transient protection of the 3'-OH with a trimethylsilyl group. This method was used in house, by Dr. Hong Li, to protect the 4-NH$_2$ group of the C-nucleoside and GC recognition monomer, 3-methyl-4-aminopyridine (MAP), during an attempt to optimise the original synthesis. In previously described synthesis (Section 3.2.1), the aniline-NH$_2$ of the furano-dT analogue 154 was reacted with ethyl trifluoroacetate in only 35% yield with significant degradation due to essential prolonged heating (Section 3.2.1). This alternative method obviated this poor yield but introduced other problems.

During the procedure, the 3'-OH group is protected using trimethylsilyl chloride (TMSCl) in pyridine. Upon completion, trifluoroacetic anhydride is added, followed by water. Addition of water, in presence of pyridine/trifluoroacetic acid/HCl cleaves the TMS group affording the product. On the first attempt, despite adding further equivalents of TMSCl (upto 3.5 eq), some trifluoroacetylation at the 3'-position had occurred; this bis-trifluoroacetylated compound 198 proving very difficult to separate by column chromatography. Although the conversion to products was very good, only a 32% yield of the desired product 197 could be obtained pure. Some triethylamine was added after reaction, before removal of solvent, to prevent detritylation.
In the second attempt, in efforts to prevent further reaction, a larger excess of TMSCl (5.0 eq) was added at the start. Although the 3’-protection occurred more quickly than before, after addition of trifluoroacetic anhydride, white fumes were observed and the reaction mixture darkened slightly due to partial detritylation from HCl exposure. Triethylamine was added immediately to prevent further degradation, however, this subsequently prevented complete TMS cleavage by addition of water, as the solution was now too basic. Aqueous workup with 15% aq NH₃ removed excess HCl, affording a 1:1 mixture of TMS-protected product and unprotected nucleoside 197. Conveniently, the TMS group was cleaved during column chromatography on silica, and the product was afforded in 53% yield. Little bis-trifluoroacetylated compound 198 was observed, but the yield was compromised due to detritylation.

Dr. Edrees-Abdou later repeated this reaction, in house, using 20 eq of ethyl trifluoroacetate, 22 eq of Et₃N and 1 eq of DMAP in CH₂Cl₂ at 50 °C overnight. The product 197 was afforded in a maximum yield of 65%, despite some degradation. These conditions again highlight the low reactivity of this aniline due in part, to extended conjugation with the N-methylpyrrolopyrimidin-2-one bicycle.

For phosphitylation, the bis-amino phosphitylating reagent 189 was tested first. However, poor conversion to products combined with significant degradation on the column led to only a 24% yield of desired monomer 178. This material was incorporated into an oligoT DNA strand for deprotection tests (Section 7), however, the monomer coupled very poorly (~20%) due to the small quantity obtained (39 mg).

Phosphitylation was most successful using the chloro-phosphitylating reagent 70 in 70% yield. Despite an extended reaction time (4¾ hours), excess reagent (2.6 eq) and prolonged purification time, little degradation was observed and all hydrolysed excess reagent was removed. Two bottles (90 mg each) were obtained for incorporation into TFOs. The monomer contained a significant quantity of Et₃N.HCl, which did not affect coupling efficiency during oligonucleotide synthesis. Presence of the free amine would have inhibited coupling (hinders acid-catalysed detritylation step).
5.1.4 Synthesis of 6-(3-Guanidinylphenyl)-N-methylpyrrolo-dC monomer

The guanidinium moiety was expected to exhibit the same binding pattern as the urea, yet contribute additional stability by charge-stabilisation; the guanidine is protonated at physiological pH. The guanidinyl-modified monomer (\(\text{GPP}_p\)) proved difficult to synthesise, however, possessing both acid- and base-sensitive groups, necessitating careful manipulation, especially during phosphitylation.

Several methods have been employed for introduction of guanidinyl groups into DNA, both during synthesis\(^{41,225}\) or post-synthetically on the oligonucleotide.\(^{43,44}\) Although much easier to introduce post-synthetically, this method is not selective as all exposed primary amines capable of reacting, can react. The main issue with the former approach lies in compatibility with DNA synthesis. Protecting groups must be stable to the acidic (TCA or DCA) and oxidising (iodine) conditions encountered during oligonucleotide synthesis (excludes using BOC and Cbz for example). Using the free-guanidinium moiety significantly reduces the monomer’s solubility and therefore the coupling efficiency. The protecting group 2-cyanoethoxycarbonyl- (CEOC) is used as it is stable to oligonucleotide synthesis. However, the groups are base-labile, and removal of just one of these groups from the guanidinyl moiety (most labile on C=N) compromises oligonucleotide synthesis (investigated in house).

5.1.4.1 Improved Synthesis of Guanidinylating Reagent

The first step was synthesis of the base-sensitive guanidinylating reagent, \(N,N'-\text{bis-}[\text{2-cyanoethoxy} \text{carbonyl}]\)-S-methyl-isothiourea \(200\).

Scheme 5.7. Synthesis of guanidinylating reagent \(200\). (lit.\(^{42,225,226}\) yield in parentheses).
The first step in the route to this reagent was synthesis of the NHS-carbonate, (2-cyanoethyl)-N-succinimidyl carbonate (CEOC-succinimide) using di-succinimidyl carbonate (DSC) under anhydrous conditions. The literature protocol requires that DSC is added, followed by pyridine, to a solution of 2-cyanoethanol (3-hydroxypropionitrile, 3-HPN) upon which the suspension solubilises and a final yield of 94%, or 87% after further purification (column chromatography) is obtained. In order to reduce reaction time (7 hours), the concentration was increased almost three-fold, however, DSC did not dissolve after 1 hour as expected although the reaction proceeded to completion after 4 hours. After isolation (93%), NMR analysis in d6-DMSO (much greater solubility than in CDCl3) indicated a large degree of decomposition had occurred. It was hypothesised that the initially high concentration of 3-HPN in the reaction had caused further reaction with the product, due to poor solubility of DSC. The corresponding by-product could decarboxylate in presence of base, producing acrylonitrile and re-forming 3-HPN (Scheme 5.8). This appeared substantiated by significant gas evolution during aqueous workup with aq NaHCO3 (sat. soln:water, 1:1 v/v), and signals for NHS and acrylonitrile in the NMR spectrum. Following later recrystallisation and column chromatography, the compound was isolated in 75% yield, despite fears over loss due to degradation.

Scheme 5.8. Hypothesised mechanism for by-product formation.

The reaction was repeated on a smaller scale at a similar concentration to the literature, and the order of addition was reversed. DSC was stirred in anhydrous CH3CN for 10 minutes, pyridine was added, followed by 3-HPN dropwise 10 minutes later. The suspension dissolved after 20 minutes and the reaction was complete after 1 hour. Following column chromatography, even after 3 weeks in storage, the pure compound was afforded in 86% yield. MS analysis of both reaction products indicated formation of the correct product with a small quantity of NHS present. NMR analysis in CDCl3 confirmed this also. CEOC-succinimide was discovered to be unstable in

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DMSO due in part to presence of water. Also, changing the order of addition of reagents increased the yield by 11% on the previous attempt. On scaling up the reaction to 200 mmol, at twice the concentration in the literature, the reaction was complete within 1.5 hours and a 94% yield was obtained.

The literature protocol for formation of the guanidinylating reagent is a two-phase reaction (CH$_2$Cl$_2$:conc. aq NaHCO$_3$, 1:1 v/v), coupling the organic-soluble NHS-carbonate with water-soluble S-methylisothiourea hemisulfate. The literature yield of 35% is poor and not cost-effective on a large-scale due to the price of DSC.

Due to base-sensitivity of the product and in order to increase the yield, the reaction was tested in CH$_2$Cl$_2$:H$_2$O and THF:H$_2$O (4:1 v/v), with only 6 equivalents of NaHCO$_3$, 2.5 eq of CEOC-succinimide, and using argon-degassed solvents. After workup, TLC indicated a ~3:2 mixture of mono- to bis-modified S-methylisothiourea (Figure 5.1).

The sp$^2$ nitrogen is more difficult to functionalise due to higher protecting group lability under basic conditions. To push the reaction to completion, each reaction was dried, dissolved in CH$_2$Cl$_2$ or THF, split into two and treated with 1.5 eq of CEOC-succinimide with or without 1 eq of pyridine. Only reaction in CH$_2$Cl$_2$, especially without pyridine, achieved significant further reaction over 26 hours even under non-anhydrous conditions.

Using this knowledge, the reaction was scaled up (87 mmol), at a higher concentration than in the literature under the best conditions from the tests. The two-step procedure worked very efficiently, benefitted by using argon-degassed/distilled solvents, 2 eq then 1 eq of CEOC-succinimide and only 3 eq of NaHCO$_3$ in the first step. A yield of 89% was achieved, a significant improvement on the literature method, and could be optimised further, using less CEOC-succinimide in the second step. A yield greater than 35% appears extremely difficult to achieve without modifying the literature protocol.
5.1.4.2 Final Steps towards $^{G_{PP}}$ Monomer 199

The anilino-$N$-methylpyrrolo-dC intermediate 195 (synthesised following the synthetic route for the trifluoroacetamidophenyl monomer 178, see Section 5.1.3) was guanidinylated using guanidinylating reagent 200 under slightly different conditions to the literature, based on experiments in house.

![Scheme 5.9. Synthesis of $^{G_{PP}}$ monomer 199.](image)

The reaction was conducted in CH$_2$Cl$_2$ with pyridine (instead of DMF with triethylamine) at 35 °C instead of room temperature due to the previously demonstrated low reactivity of the aniline NH$_2$. Only 1.1 eq of guanidinylating reagent 200 was added initially, using only 0.5 eq of pyridine due to base-sensitivity. Guanidinylation of a primary alkylamine-modified nucleoside, in house, required only 0.5 eq of pyridine. Over more than 1½ days, further reagent and pyridine were added (upto 2.5 eq and 5.2 eq respectively) and the temperature was increased to 50 °C. Although prolonged reaction time and temperature caused some degradation/cleavage of CEOC-groups, these conditions were necessary to ensure adequate reaction occurred. The product was purified by column chromatography, on pyridine-neutralised silica gel, using eluent containing no base, affording a 42% yield. Neutralising the silica gel is important to prevent cleavage of the acid-sensitive DMT group, yet excess base is washed from the column and base is omitted from the eluent to prevent base-mediated cleavage of the CEOC protecting groups from the product 207.

Phosphitylation was not straight-forward as during storage, some CEOC-cleavage was observed, likely to be caused due to presence of byproduct CH$_3$SH. The reaction was tested on this material, using 1.1 eq and 1.5 eq of the chloro-phosphitylating reagent 70 and 1.5 eq and 2.0 eq of DIPEA. Literature methods$^{41,225,226}$ use the *bis*-amino reagent
DIPEA can cause CEOC-cleavage. However, the low aniline reactivity necessitated these conditions, and less DIPEA was used. The products of both reactions (better conversion for the latter) were combined and purified twice by column chromatography and precipitation. However, due to phosphitylation of the single unprotected guanidinyl nitrogen, the product (208) of which eluted similarly to the desired product 199, the monomer could not be obtained pure. $^{31}$P-NMR indicated peaks for both phosphoramidites at ~150 ppm, and signals at ~ 71 ppm corresponding to the N-phosphoramidyl group (Figure 5.2).

**Figure 5.3.** Expansions of $^{31}$P-NMR of $^{6}$PP$_{p}$ phosphoramidite mixture 199,208, and structure of bis-phosphoramidite 208. Bis-phosphoramidyl peaks labelled with an asterisk.

Although the $^{1}$H-NMR spectrum was too complicated to assign, both the desired phosphoramidite 199 and bis-phosphoramidite 208 were also detected by MS.

When the monomer was re-synthesised in house, by Dr. Edrees-Abdou, guanidinylation was achieved using 2.5 eq of GR 200, 5 eq of pyridine in refluxing CH$_2$Cl$_2$ under argon pressure (50 °C) for 2 days, in 44% yield. Phosphitylation using 1.8 eq of chlorophosphitylating reagent 70 with 2 eq of DIPEA at rt afforded the desired $^{6}$PP$_{p}$ monomer after 2 hours reaction time, in 58% yield. The monomer precursor 207 was precipitated from DCM into hexane to remove residual pyridine and methanethiol and no degradation was encountered during storage.
5.1.5 Synthesis of 6-(3-Thioureidophenyl)-N-methylpyrrolo-dC monomer 158

The final modification investigated was a thioureido moiety. The greater acidity of thiourea N-H bonds compared to the analogous urea, should result in strengthening of the proposed hydrogen-bonds with G of the CG base pair.

The initial synthetic route to the thiourea-modified monomer (TU\(^{\text{PP}_p}\)) 158 closely followed the procedure for the \(^{\text{UP}}\text{PP}_p\) monomer 179 (Scheme 5.10). After synthesis of the modified alkyne 208, Sonogashira cross-coupling, cyclisation and phosphitylation were expected to afford the desired monomer 158.

Scheme 5.10. Initial synthetic route to \(^{\text{TU}}\text{PP}_p\) monomer 158.

Synthesis of the alkyne for Sonogashira cross-coupling, 3-thioureidophenyl acetylene 209, was first attempted using one of two methods published by Saxena et al.\(^{227}\) for synthesis of N-arylthioureas. Refluxing 3-ethynylaniline 152 in 15% aq HCl with ammonium thiocyanate, however, afforded only 6% yield of product 209 with some recovery of starting material. Poor solubility despite strong heat and vigorous stirring may have hindered the reaction (Scheme 5.11).

Scheme 5.11. Synthetic routes to 3-thioureidophenyl acetylene 209.
After noting that isothiocyanates could be used for formation of aryl-thioureas, 227,228 3-EA 152 was treated with trimethylsilyl isothiocyanate (TMS-NCS) in dry Et₂O, with and without base (DIPEA). No desired thiourea could be observed; only thiocyanic acid salts of DIPEA or 3-EA were formed, which were broken down during workup. Although potentially a very convenient route to primary ureas due to facile deprotection of the TMS-thiourea product, TMS-NCS appears to hydrolyse before the poorly reactive aniline can react.

The other method published by Saxena 227 was used successfully to afford the desired alkyne 209 in 59% yield over two steps. 3-Ethynylaniline 152 was reacted with benzoyl isothiocyanate 210 cleanly in 75% yield. Cleavage of the benzoyl group of 211 was achieved by heating at 90 °C in 5% aq NaOH for 15 minutes in 79% yield. Neither step required chromatography and little benzamide byproduct 212 was produced (removed by filtration) (Scheme 5.11).

Sonogashira cross-coupling reactions throughout this work have used the catalyst, Pd(PPh₃)₄. This posed a problem for this step, as due to the high affinity of sulphur for palladium, it was likely that the thiourea moiety may bind to palladium, either poisoning or consuming the catalyst.

Small-scale test reactions demonstrated that much higher quantities of Pd catalyst and CuI were required to elicit any reaction other than minimal alkyne dimerisation. Two other products were also detected by TLC, which appeared to correspond to Pd-thiourea complexes of the alkynylated product 213 and alkyne 209.

This reaction was subsequently conducted on a 1.3 g and 0.3 g scale using 0.5-0.7 eq of Pd(PPh₃)₄, 1.1 eq of CuI and 2.0-2.5 eq of alkyne, during which, both starting material and alkyne were consumed. After column chromatography, the product from the larger-scale reaction was divided. Aqueous workup was unsuccessful removing inorganic impurities, however, passing through a benzyl-thiol StratoSpheres™ SPE cartridge (PL-BnSH MP Resin) successfully cleaved the residual thiourea complexed-Pd and removed excess Pd from the mixture.

After combining all batches, a portion of the Sonogashira product 213 was treated under Inoue’s conditions for cyclisation.222,223 Unexpectedly, after 40 minutes, nothing had
occurred except precipitation of a black solid. The mixture was filtered, concentrated in vacuo, dried and treated again under the same conditions, after confirming the starting material had remained unchanged. The starting material was mostly consumed, as determined by TLC analysis using several solvent systems, over 1½ hours hence the reaction was worked up and purified twice by column chromatography. TLC analysis of the resulting seven fractions indicated a complex mixture of products, some fluorescent and all UV active. The desired cyclised product 214 could not be detected by MS; only the analogous ureido compound 184 was identified. It was subsequently discovered that in recent work by Shibahara et al., a variety of thiocarbonyl compounds were oxidatively desulfurised in presence of copper (I) chloride (20 mol%), oxygen and heat (80 °C) in excellent yield under the right conditions. Under the conditions of Inoue, it is possible that traces of oxygen, combined with strong heating (125 °C) and CuI (1.1 eq), may have induced oxidative desulfurisation and other degradation products. This explains the presence of the urea “oxidation product” 183 in the mixture.

The remaining uncyclised starting material was treated, without CuI, under the otherwise same conditions affording only complete detritylation of the starting material. This was recovered and treated under Inoue’s conditions, at 50 °C this time, but no change was observed after 24 hours. It was decided to abandon this synthetic route and a new route was proposed.

Scheme 5.12. Revised route to $^\text{TUP}_p$ monomer 158.
The new proposed route proceeds via modification of the cyclised anilino-nucleoside 195, with benzoyl isothiocyanate 210, following suitable protection of the 3'-OH (Scheme 5.12). This synthesis was subsequently investigated by Dr. Edrees-Abdou with success after some further modifications to the route. The phosphoramidite 158, however, coupled very poorly when incorporated into DNA and MS analysis proved inconclusive for the desired oligonucleotides. The thiourea-derivative ($^{(TU)}$PP$_p$) was therefore put on hold. It was proposed that deprotection conditions and/or the iodine oxidation step during oligonucleotide synthesis may have caused degradation/oxidation but this could not be confirmed.

### 5.2 Future Work

Following this work, other modifications were suggested, which could enhance binding and recognition. The first was substituting the 3-aminophenyl- modification for a 3,5-diaminophenyl- moiety. Due to free rotation around the aryl-bicycle bond, it is possible that the 3-amino group may not always be correctly aligned for binding to G. Employing two groups should present the correct hydrogen-bonding for both rotamers. Any one of the bis-acetamide, bis-urea, bis-trifluoroacetamide or bis-guanidinium could be synthesised. However, the bis-acetamide and bis-urea are likely to be too polar to handle, and the bis-guanidinium might be difficult to form, due both to low reactivity and steric hindrance.

![Figure 5.4](image-url)

**Figure 5.4.** Alternative CG recognition monomer synthetic targets 217-224 and binding motif.
It may also be beneficial to change the aromatic ring to a heterocycle such as pyridine, triazine, pyrrole or imidazole. Altering hydrophilicity and electronic properties may have beneficial effects on binding affinity.

The first target, however, is 6-phenyl-\textit{N}-methylpyrrolo-dC phosphoramidite 220. It is important to confirm what role the aromatic ring plays in triplex binding and recognition, and whether the modifications do benefit binding or could even be omitted. In addition, it would be necessary therefore to investigate how changing the ring, whether aromatic (phenyl, napthyl), heteroaromatic (pyridine, triazine, pyrrole, thiazole) or non-aromatic (cyclohexyl, cyclopentyl), affects binding, before further derivatisation. The phenyl monomer 221 has been synthesised in house, in excellent overall yield by Dr. Edrees-Abdou, and biophysical studies are in progress.

\section{5.3 Conclusion}

Three \textit{N}-methylypyrrolo-dC monomers (\textsuperscript{\textit{A}}PP\textsubscript{p} 178, \textsuperscript{\textit{Ac}}PP\textsubscript{p} 191, \textsuperscript{\textit{U}}PP\textsubscript{p} 179), were synthesised for incorporation into TFOs for biophysical studies. All were synthesised from the key intermediate, 5\textprime-(4,4\textprime-dimethoxytrityl)-5-iodo-4\textit{N}-methyl-2\textprime-deoxy-cytidine 185, made from the 2\textprime-deoxyuridine analogue 97 in two steps. Sonogashira cross-coupling\textsuperscript{124} and a modified copper (I)-catalysed cyclisation\textsuperscript{222,223} were used to form the derivatised bicyclic base in typically good to excellent yield.

Problems were again encountered with the low reactivity of the aniline NH\textsubscript{2} (c.f. Section 3), necessitating prolonged reaction times, a higher reagent excess, and raised temperature (e.g. trifluoroacetylation, guanidinylation). Care was also taken for the guanidinium monomer (\textsuperscript{\textit{G}}PP\textsubscript{p}) and precursor, to avoid cleavage of the base-labile protecting groups, whilst preventing cleavage/activation of the acid-sensitive DMT and phosphoramidite moieties. A revised method for synthesis of the guanidinylation reagent 200 from CEOC-succinimide 201 was also evaluated, affording a final semi-optimised yield of 89\%, compared to the literature yield of 35\%.\textsuperscript{41,225,226} This allows for a much more accessible synthesis, owing to cost of DSC.

The fourth monomer, \textsuperscript{\textit{G}}PP\textsubscript{p} 199, was synthesised later in house, after synthetic investigation (see above), and a fifth monomer, \textsuperscript{\textit{TU}}PP\textsubscript{p} 158, although eventually
synthesised later in house, could not be incorporated and studied as desired. Although the thiourea moiety should have imparted extra stability over the urea in the triplex, it is unlikely this modification will be continued. Instability during oligonucleotide synthesis and/or deprotection introduced too many problems. However, there are several new directions for future work, and the syntheses and biophysical studies (Section 6) already undertaken are promising.

The precursors to the four monomers that were incorporated into TFOs, were also deprotected and fluorescence measurements were taken. This is discussed in detail in Section 6.3.
Chapter 6

Biophysical Studies of
*N*-Methylpyrrolo-dC Modified
TFOs for CG Recognition
6. Biophysical Studies of N-Methylpyrrolo-dC Modified TFOs for CG Recognition

Four modified N-methylpyrrolo-dC phosphoramidite monomers (\(^{i}\text{PP}_p 178, \ ^{1}\text{PP}_p 191, \ ^{\text{o}}\text{PP}_p 179, \ ^{\text{o}}\text{PP}_p 199\)) were incorporated into unlabelled/labelled TFOs for UV and fluorescence melting studies. The initial study (from Section 4.2.2) was designed to compare both post-synthetically modified furano-dT, and \(N\)-methylpyrrolo-dC nucleosides at CG recognition in the four sequences introduced in Chapter 4. Following this work, the main biophysical studies were carried out. These were again designed to test binding affinity for CG and selectivity against the other base pairs (GC, AT, TA) at several pHS. In addition to determination of the best modification for CG recognition, the other aim was to compare UV and fluorescence melting data (collected using the same buffer conditions), which appeared not to correlate in previous studies.

6.1 Initial Fluorescence Melting Study

This section discusses the \(N\)-methylpyrrolo-dC results from the ‘Sequence Study’ introduced in Section 4.2.2.

![Figure 6.1. Fluorescence triplex melting experiment. TFO shown in bold. \(M = ^{5\text{Me}}\text{dC},\ P = 5\text{-}(3\text{-aminoprop-1-ynyl})\text{-dU (pdU)}, \ X = ^{\text{N}}\text{PP} & \text{VXW} = \text{TXT (OL18), PXT (OL19), TXP (OL20), PXP (OL21), or } X = ^{i}\text{PP} \& \text{VXW} = \text{TXT (OL22), PXT (OL23), TXP (OL24), PXP (OL25), } Q = \text{DABCYL (quencher), F = FAM (fluorophore), H = HEG (hexaethylene glycol), hairpin duplex (CG, OL6).}

As previously described, these fluorescence melting experiments (Figure 6.1) were conducted using LC Programmes 1 and 2 (95-30-95 °C at 0.2 °C/min) at pH 6.2 (10 mM sodium phosphate, 200 mM NaCl). Experiments were run once in triplicate and a 10:1 ratio of TFO:duplex (5:0.5 \(\mu\)M) was used.
Table 6.1. Fluorescence triplex melting experiment: $^{\text{Ac}}$PP$_p$ and $^{\text{U}}$PP$_p$ against CG, each in four TFOs ($^{\text{Ac}}$PP – OL18-OL21 & $^{\text{U}}$PP – OL22-OL25) at pH 6.2 (10 mM sodium phosphate, 200 mM NaCl) using LC Programme 1 and 2 (Section 9.2). Average $T_m$ values for TFO melt/anneal given in °C. Data obtained for XPP$_f$-modified TFOs is shown for comparison, in parentheses (Section 4.2.2).

These results confirm the previous findings, that placing pdU (P) on the 5'-side of the modification X (XPP) has a slightly destabilising effect compared to the 3'-side, notably for the urea ($^{\text{U}}$PP). The results for the TXP TFOs for both furano-dT (in parentheses) and N-methylpyrrolo-dC modifications were similar (within experimental error), hence previous furano-dT experimental data can be corroborated.

The trends identified when examining the combined XPP$_f$ and XPP$_p$ results, correlate with those in the main fluorescence melting studies, despite differences in buffer composition and pH.

In addition, hysteresis was on average 1.5 °C lower for XPP$_p$ than for XPP$_f$ modifications. This may rise from the higher TFO purity when no post-synthetic modification is required (Sections 3.3, 5.1).

6.2 Primary UV Melting Study

A comprehensive UV melting study was conducted in cooperation with Dr. Edrees-Abdou, to examine the binding affinity/selectivity of the four monomers, in comparison also with the natural base T. Data was collected at pH 6.2, 6.6 and 7.0, and at pH 7.0 with 2 mM spermine added. Spermine is a general/non-specific DNA stabilising agent and component in all eukaryotic cells, which is used in this context, to increase the stability of a duplex/triplex if the $T_m$ is too low to be easily measured. Prior to this
work, large quantities of the pdU phosphoramidite monomer were synthesised by Dr. Edrees-Abdou and Dr. Imenne Bouamaied for synthesis of the TFOs used in this both studies.

\[
5'-\text{TTT TTM VXW MTM TMT} \\
5'-\text{GCT AAA AAG AYA GAG AGA TCG} \\
\text{CGA TTT TTC TTT CTC TCT AGC-5'}
\]

Figure 6.2. UV triplex melting experiment. TFO shown in bold. \( M = 5\text{-Me}dC, \ P = 5\text{-}(3\text{-aminoprop-1-ynyl})dU \) (pdU), \( \text{VXW} = \text{TTT (OL32), TTP (OL33), PTT (OL34), PTP (OL35)} \); \( X = ^4\text{PP} \) \& \( \text{VXW} = \text{TXT (OL36), TXP (OL37), PXT (OL38), PXp (OL39)} \); \( X = ^6\text{PP} \) \& \( \text{VXW} = \text{TXT (OL44), TXP (OL45), PXT (OL46), PXp (OL47)} \); \( X = ^\text{Ac}PP \) \& \( \text{VXW} = \text{TXT (OL40), TXP (OL41), PXT (OL42), PXp (OL43)} \); \( X = ^\text{U}PP \) \& \( \text{VXW} = \text{TXT (OL40), TXP (OL41), PXT (OL42), PXp (OL43)} \); \( X = ^\text{G}PP \) \& \( \text{VXW} = \text{TXT (OL40), TXP (OL41), PXT (OL42), PXp (OL43)} \); purine strand \( Y = \text{C (OL1), G (OL26), A (OL27), T (OL28)} \), pyrimidine strand \( Z = \text{G (OL2), C (OL23), T (OL30), A (OL31)} \).

UV melting experiments were performed (as described previously, and in detail in Section 9.2), using a 5:1 ratio of TFO:duplex (5:1 \( \mu \)M). Experiments were conducted at pH 6.2, 6.6, 7.0 and pH 7.0 + 2 mM spermine (10 mM sodium phosphate, 200 mM NaCl, 1 mM Na\(_2\)EDTA). All oligonucleotides for this study were synthesised without capping to avoid undesired acetylation of TFOs.

Standard melt programme No. 3 was used (15-50-80 °C at 0.25-0.5 °C/min) with 5 heat/ anneal cycles in total (Section 9.2). Condensation was not a problem at low temperature (15 °C).

There are several trends, which can be drawn from the data given below in Table 6.2. Firstly, at pH 6.2, against CG in sequence TXT, (Table 6.2, Lines 1, 6, 19, 28, 37) the aniline/amine (\(^4\text{PP}, \text{OL36}\)) outperforms all other monomers, and the poorest monomer (\(^\text{Ac}PP, \text{OL40}\)) gives a \( T_m \) that is 1.7 °C greater than T. This result appears to contradict the original order of stability as derived from the proposed hydrogen-bonding triplet motif. The proposed motif places the urea (\(^\text{U}PP\)) and guanidinium (\(^\text{G}PP\)) as the strongest binders, as they can form two hydrogen-bonds and the latter also contributes additional charge-stabilisation. However, these results suggest steric or electronic factors may play a role, where such groups cause destabilisation relative to the sterically non-hindering amino group.
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**Table 6.2.** UV triplex melting experiment: X against YZ at pH 6.2, 6.6, 7.0 and 7.0 + 2 mM spermine (10 mM sodium phosphate, 200 mM NaCl, 1 mM Na₂EDTA) using UV melting programme No. 3 (Section 9.2). Average $T_m$ values for TFO melt/anneal and duplex (average of melt and anneal $T_m$), and hysteresis given in °C. n.d. = not determined ($T_m < 17$ °C).
At pH 6.6 (Lines 2, 10, 23, 32, 41), the urea and aniline (A_PP) show similar binding affinity, greater than the guanidinium, which should be the strongest binder.

At pH 6.6, against CG for all the pdU-containing TFOs, the aniline is again the strongest binder, followed by the urea. The order of stability for the other modifications varies, such that for PXT (OL50) and PXP (OL51), the guanidinium is of equal or lower stability than T. This may be attributed to disfavourable steric interaction and also charge-repulsion with the pdU on the 5’-side as previously discussed.

All monomers demonstrated a very high selectivity for CG at pH 6.2, over the other base pairs, by 6.9-8.6 °C. For all monomers except U_PP, the second highest affinity was for AT not TA, the latter of which has often been observed for CG recognition monomers (Section 1.2.2). A putative X.AT triplet can be drawn to support this observation, with hydrogen bonds between N^3 and anilino NH of X_PP and the NH_2 of A and C=O of T. Only U_PP and G_PP show significant differences (> 1 °C) between the T_m's against the other base pairs. In addition, the aniline demonstrates very high selectivity for CG, by 10.2 °C at pH 7.0 with 2 mM spermine, where the second highest affinity was for GC (Lines 15-18).

Examination of the data for the pdU-containing TFO series, obtained at pH 6.6, identifies significant differences from the initial fluorescence melting data discussed in Sections 4.2 and 6.1. The main difference is order of stability, where higher T_m's are observed for PXT- rather than TXP-oligonucleotides. It was deduced from previous fluorescence melting data, that disfavourable interaction between the pdU and X_PP amino-modified side-chains occurred when pdU was located on the 5’-side. However, the reverse is observed here, and the difference between TXP and PXT is pronounced for the aniline (1.7 °C) and acetamide (1.4 °C). Otherwise increase in T_m with each T-pdU substitution varies from 0.1 to 1.3 °C. Interestingly, the TXT TFO (X = G_PP, OL48) is 0.2 °C more stable than the corresponding TXP oligonucleotide (OL49), but this may simply be due to experimental error. The results for T at pH 6.6, appear spurious, where the order of stability (TXP < TXT < PXP < PXT) cannot be easily explained.

In summary, this study highlights the high selectivity of all monomers for CG over the other base pairs. The order of binding affinity, however, contradicts the proposed triplet
binding motif, although the urea remains a strong binder. These observations suggest the aniline or urea would be highly suitable candidates for CG recognition, the latter perhaps particularly at higher pH. Selectivity at pH 7.0 with 2 mM spermine should be carried out for the urea to confirm whether the urea does indeed outperform the aniline at higher pH. Without spermine, no $T_m$ could be measured for any monomer at pH 7.0. The acetamide and guanidinium should also be assessed against CG at pH 7.0 + 2 mM spermine to determine whether the effect of the positive-charge of the guanidinium is beneficial to binding at higher pH, where little protonation of other amine functionalities should occur. The acetamide performed poorest throughout and the guanidinium proved less effective than expected.

6.3. Primary Fluorescence Melting Study

The main fluorescence melting study was conducted in cooperation with Dr. Edrees-Abdou, to confirm the previous results and make more accurate comparisons between monomer recognition capabilities. Data was collected using the same buffer systems as for the UV melting study so that direct comparisons between the studies could be made.

\begin{figure}[h]
\centering
\begin{tabular}{cccccccc}
5’ & -Q-PMM & TPM & VXW & TPT & PTM & PT \\
5’ & -F-GT & GTT & AGG & AAG & AYA & AAA & AAG & AAC & TGG & T-H \\
& CA & CAA & TCC & TTC & TTT & TTC & TTG & ACC & A-H \\
\end{tabular}
\caption{Fluorescence triplex melting experiment. TFO shown in bold. M = $^{5}$Me-dC, P = 5-(3-aminoprop-1-ynyl)-dU (pdU), VXW = TTT (OL60), TTT (OL61), TTP (OL62), PTP (OL63); X = $^{4}$PP & VXW = TXT (OL52), PXT (OL53), TXP (OL54), PXP (OL55); X = $^{\Lambda}$PP & VXW = TXT (OL18), PXT (OL19), TXP (OL20), PXP (OL21); X = $^{6}$PP & VXW = TXT (OL22), PXT (OL23), TXP (OL24), PXP (OL25); X = $^{5}$PP & VXW = TXT (OL56), PXT (OL57), TXP (OL58), PXP (OL59); Q = DABCYL (quencher), F = FAM (fluorophore), H = HEG (hexaethylene glycol), hairpin duplex YZ = CG (OL6), GC (OL7), AT (OL8), TA (OL9).}
\end{figure}
Table 6.3. Fluorescence triplex melting experiment: X against YZ at pH 6.2, 6.6, 7.0 and 7.0 + 2 mM spermine (10 mM sodium phosphate, 200 mM NaCl, 1 mM Na₂EDTA) using LC Programme 3 (Section 9.2). Average $T_m$ values for TFO melt/anneal and values for hysteresis given in °C. n.d. = not determined ($T_m < 33$ °C). Hysteresis: 0.7–9.3 °C, average hysteresis: 4.0 °C. Continued on following page.
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Table 6.3 cont. Fluorescence triplex melting experiment. Continued from previous page.

Fluorescence melting studies were performed (as described previously, and in detail in Section 9.2), on a Roche LightCycler®. Experiments were performed at pH 6.2, 6.6 and 7.0, and at pH 7.0 + 2 mM spermine (10 mM sodium phosphate, 200 mM NaCl, 1 mM Na$_2$EDTA), and a 10:1 ratio of TFO:duplex (5:0.5 μM) was used. Runs in these experiments were performed up to three times in triplicate, using LC Programme 3 (95-27-95 °C at 0.2 °C/min, see Section 9.2).
At pH 6.2, against CG in sequence TXT, (Table 6.3, Lines 1, 5, 23, 43, 63), GPP outperforms the other monomers by 2.9 °C, however, the $T_m$ of the poorest monomer (AcPP) is 0.4 °C below T. The aniline, urea and T have similar $T_m$s. At pH 6.6 also (Lines 13, 33, 53, 72), the guanidinium monomer has the strongest binding affinity by 2.3 °C, followed by the urea, aniline then acetamide. In the PXP sequence at pH 6.2 (Lines 9, 27, 47, 68), the urea has the highest $T_m$ by 1.3 °C, followed surprisingly by the aniline then guanidinium and acetamide, which is 4.1 °C above T. A similar pattern was observed for TXT and PXP at pH 7.0 and 7.0 with spermine.

The guanidinium monomer performed best, as expected, in the TXT sequence but not for PXP. It is possible that the presence of a protonated amine species either side of the positively-charged guanidinium may have destabilised it significantly, below that of the aniline as observed. Otherwise, apart from the acetamide performing slightly worse than the aniline, the order of stability can be rationalised.

A different situation was encountered for the PXT and TXP sequences. At pH 6.6, for TXP (Lines 17, 37, 57, 76), the guanidinium and urea performed best, whereas for PXT (Lines 18, 38, 58, 77), these appear destabilised such that the acetamide has a similar $T_m$ to GPP. This indicates that both the guanidinium and urea have a negative interaction with pdU when they are located on the 5'-side (PXT) as originally noted (Section 4.2.2). It is also perhaps surprising, that changing from PXT to PXP has a significant effect on the order of binding affinity, whereas changing from TXT to TXP makes little difference. It was originally thought that the proposed negative interaction was due to steric hindrance between the aminopropynyl group of pdU and modified phenyl group of the monomer. However, charge-repulsion may also play an important role.

All monomers demonstrate good to high selectivity for CG over the other base pairs, at all pHs tested in both TXT and PXP sequences. In all cases, the second most stable binding is to TA as originally expected. The least stable binding occurs to GC then AT, or to AT then GC for GPP. For TXT at pH 6.2, the guanidinium and aniline demonstrate the highest selectivity, by 7.5 °C, and the acetamide the least, by 2.0 °C. For TXT at pH 6.6, the guanidinium is most selective ($\Delta T_m$ 7.4 °C) and the acetamide the least ($\Delta T_m$ 2.4 °C), and for PXP at pH 6.2, the aniline is most selective ($\Delta T_m$ 9.6 °C), followed by the urea ($\Delta T_m$ 8.4 °C), the guanidinium ($\Delta T_m$ 5.0 °C) then the acetamide ($\Delta T_m$ 4.7 °C).
The high selectivity of $^{G}\text{PP}$ and $^{U}\text{PP}$ compared to $^{\Lambda}\text{PP}$ can be rationalised by the extra potential hydrogen-bond, which can effect differentiation between the binding motifs of CG and the other base pairs more effectively. It does not explain the good selectivity for the aniline possessing only one potential hydrogen-bond donor, although in the PXP sequence, general destabilisation of $^{G}\text{PP}$ and $^{U}\text{PP}$, due to neighbouring pdU nucleotides, may reduce their selectivity relative to the aniline.

Data for the pdU-containing TFOs demonstrates greater stability for TXP over PXT for aniline, urea and guanidinium, and the reverse for the acetamide, in all cases (not tested at pH 6.2 for $^{A}\text{PP}$ and $^{G}\text{PP}$). The greatest difference between TXP and PXT occurs for $^{G}\text{PP}$ ($\Delta T_m$ 2.5 °C), is moderate for $^{U}\text{PP}$ ($\Delta T_m$ 1.2 °C) and $^{\Lambda}\text{PP}$ ($\Delta T_m$ 1.1 °C) and small for $^{\Lambda}\text{PP}$ (av. $\Delta T_m$ 0.4 °C).

Charge-repulsion may explain this difference for the guanidinium monomer, and steric hindrance for the urea, but there must be alternative reasons for the observed differences for the aniline and acetamide.

The fluorescence melting study results suggest the aniline, urea or guanidinium would prove suitable candidates. The guanidinium has the greatest binding affinity in most of the conditions tested and the aniline proved the most selective, followed by the guanidinium and urea. Due to sensitivity of intermediates in the synthesis of the guanidinium monomer, either the urea or aniline are probably more suitable.

6.4. Melting Study Comparison

The fluorescence melting study data (Section 6.3) correlates with previous fluorescence melting data (Sections 4.2.2, 6.1), but not with the UV melting study data (Section 6.2). Importantly, the order of binding affinity has stark differences between the two studies.

It is possible that the sequence composition may be partly responsible for the observed differences. Examining the neighbouring triplets to the central three-triplet triplex core of the UV and fluorescence melting motifs, only the triplet on the 5’-side is the same (M’.GC) (Figure 6.4).
It is difficult to explain all the differences between the two studies, however, by analysing the differences in the sequence around the central core, without extensive and time-consuming melting and NMR experiments. It is likely that the general triplex shape, general TFO composition and whether the duplex part is a hairpin or comprises separate strands, contributes to the differences in trends between systems.

In order to rule out the possibility that the method of analysis is responsible, although unlikely, UV melting experiments should be performed using the fluorescence melting triplex motif. The fluorophore and quencher should not affect the trends, only causing a slight general increase in $T_m$. The oligonucleotides could also be resynthesised without DABCYL and FAM, although this would create significant work.

If the method of analysis is proven not to be a factor, the differences must be sequence-dependent. This would have considerable implications when designing TFOs for gene-targeting and oligo-therapeutics. Depending on the sequence and also interaction with other modified/un-natural nucleotides and recognition monomers, the same monomer may have significant differences in selectivity and binding affinity. Optimising both of these properties is vitally important for achieving mixed-sequence recognition under physiological conditions.

6.5. **Fluorescence Properties of CG Recognition Monomers**

The synthesis and fluorescent properties of pyrrolo-C nucleobases, nucleosides and PNA monomers have been studied in detail by Robert Hudson *et al.*\(^{120,230-235}\) and others\(^ {119}\) as they can be used as fluorescent reporter groups in SNP analysis\(^ {236}\) and for fluorimetric detection of guanosine.\(^ {120,233}\) Fluorescence decreases significantly only on duplex hybridisation with G, such that the change is visible to the naked eye on irradiation of the solution with long-wave UV light (365 nm).
These pyrrolo-dC compounds have not, however, been evaluated as probes or fluorophores in triplexes, and only the triplex-forming properties of \(N\)-methylpyrrolo-dC derivatives have been studied thus far.\textsuperscript{67,122,207} For this purpose, the fluorescence properties of the four deprotected nucleosides, \(^{\text{A}}\text{PP 143}, \text{AcPP 225}, \text{UPP 182}\) and \(\text{GPP 226}\) were evaluated to afford an indication of their usefulness in this application. Their deprotection procedures are described in Section 9.2.6 (Scheme 6.1).

![Scheme 6.1. Deprotection of \(N\)-methylpyrrolo-dC nucleosides 184,193,195,207.](attachment:image.png)

The excitation and emission wavelengths were measured in HPLC grade methanol (\(^{\text{A}}\text{PP 143}, \text{AcPP 225}\)) or anhydrous DMF (\(^{\text{U}}\text{PP 182}, \text{GPP 226}\)) using a Varian Cary 400 Scan UV-Visible Spectrophotometer and a Varian Cary Eclipse Fluorescence Spectrophotometer respectively (Section 9.2). The extinction coefficient was calculated for each excitation maximum using the Beer-Lambert Law (\(A = \varepsilon c L\); \(A\) = absorbance, \(\varepsilon\) = molar extinction coefficient, \(c\) = molar concentration, \(L\) = pathlength/cm – see Section 9.2) and all data is detailed in Table 6.4 below.

![Table 6.4. UV/Fluorescence data for \(N\)-methylpyrrolo-dC nucleosides 143,225,182,226:](attachment:image.png)

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<th>(\lambda_{abs2})</th>
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<td>276.4</td>
<td>13410</td>
<td>458.0</td>
<td>91.4</td>
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</table>

Absorption and emission spectra are displayed in Figure 6.5. Absorption data has been truncated for samples run in DMF due to noise caused by loss of solvent transparency.
below ~270 nm. Emission data has been scaled for easy visual comparisons between curves.

**Figure 6.5.** UV absorption (solid line) and fluorescence emission (dashed line) spectra for \(N\)-methylpyrrolo-dC nucleosides. Properties measured at different concentrations. \(^\text{\textsuperscript{\textit{U}}\text{PP}}_p\) and \(^\text{\textsuperscript{\textit{G}}\text{PP}}_p\) absorption data were truncated below ~260 nm. Emission data scaled by scale factor = 1 (\(^\text{\textsuperscript{\textit{A}}\text{PP}}_p\)), 0.1 (\(^\text{\textsuperscript{\textit{Ac}}\text{PP}}_p\)), 10 (\(^\text{\textsuperscript{\textit{U}}\text{PP}}_p\)), 0.6 (\(^\text{\textsuperscript{\textit{G}}\text{PP}}_p\)). Fluorescence spectra obtained by excitation at \(\lambda_{abs}^\text{\textit{maxima}}\) (350-370 nm).

The urea, which demonstrated good binding affinity and selectivity, has the highest extinction coefficient (15140 L mol\(^{-1}\) cm\(^{-1}\)) by greater than a factor of 2. The Stokes shift, although lowest at 71.0 nm, is still very good. The acetamide, despite performing poorest in melting studies, has the highest Stokes Shift (102.5 nm), comparable with that of the 6-arylpyrrolo-C nucleobases 227-229 and PNA monomers 230,231 of Hudson et al.230,231 The guanidine demonstrated a good Stokes Shift (91.4 nm) and has the second highest extinction coefficient of 6690 L mol\(^{-1}\) cm\(^{-1}\), thus may show most potential as a triplex probe. Additionally, all emission spectra maxima have minimal spectral overlap with their corresponding excitation maxima.
All nucleosides emit close to the excitation wavelength of DABCYL (471 nm), and therefore could be used instead of fluorescein (FAM) in fluorescence melting studies, or in combination with other acceptors for FRET analysis. There may also be a significant change in fluorescence emission on triplex hybridisation, analogous to that observed by Hudson in the duplex, on pairing with G.120,233 3’-Pyrene-modified dT has also found use as a probe, showing marked increase in fluorescence on triplex formation, depending on position within the triplex.237 Fluorescence was strongly dependent on correct hybridisation, which offered the potential for detection of duplex mutations.

The quantum yields should be determined next, for absolute confirmation of the best N-methylpyrrolo-dC probe candidate.

### 6.6 Conclusions & Future Work

The final melting studies confirm the efficacy of these monomers to specifically recognise a CG inversion in a homopurine tract. Comparing the results from both studies, the monomers of choice should be the urea and the aniline. The aniline outperformed the other monomers in the UV melting study for both binding affinity and selectivity, and the urea also demonstrated its efficacy. The guanidinium monomer demonstrated the highest binding affinity in the fluorescence melting study, followed by the urea. However, the aniline proved slightly more selective and the urea less so on average, and these are easier to synthesise than the guanidinium. The acetamide performed relatively poorly throughout both studies, comparibly with T, except where placing pdU on the 5’-side caused destabilisation of the other monomers.

It is difficult to rationalise the differences between the two studies, which should be well correlated. Differences such as the change in order of stability of the pdU-
containing triplexes, and relative binding affinities and selectivity have not yet been explained, but several factors may contribute and there are some studies, which can be carried out to examine this. The three best monomers have been tested against real gene targets, and are currently being re-synthesised for further tests, in conjunction with other recognition monomers. Such mixed-sequence triplex-based recognition has already been evaluated, and gene targeting studies are in progress.

The 6-phenyl-\(N\)-methylpyrrolo-dC phosphoramidite monomer \(221\) was synthesised by Dr. Edrees-Abdou, as detailed in Section 5.2, in excellent yield and melting studies are underway. Further modifications for assessment are described also in Section 5.2.

Finally, the fluorescence properties of the four CG recognition monomer nucleosides show potential for their use as probes within triple helices and in fluorescence studies, including melting. The Stokes Shifts and extinction coefficients are comparable with the NH-pyrrolo-C analogues of Hudson et al.,\(^\text{230,231}\) which have already found use in fluorimetric detection of G.\(^\text{120,233}\) Quantum yields must be determined, however, so the most efficient substrate can be chosen.
Chapter 7

Oligonucleotide Deprotection Study
7. Oligonucleotide Deprotection Study

7.1 Introduction

The industry standard method for custom chemical oligonucleotide synthesis is the automated phosphoramidite or ‘phosphite triester’ cycle on solid-support, first developed by Prof. Marvin Caruthers et al. in 1981. Modern automated DNA/RNA synthesisers can synthesise oligonucleotides on solid-support, from 2-250 nucleotides long, with stepwise coupling efficiencies of 98.0-99.5% for the standard DNA phosphoramidites, dA, dC, dG and dT. Oligonucleotides may be synthesised on a 40 n mole up to multi-millimole scale for therapeutic applications. Syntheses of up to 0.2 and 1.0 µmole are typical for research applications. The method, to be successful, requires a robust protecting group strategy due to the conditions, which the developing oligonucleotide is exposed to during synthesis. Equally, protecting groups must be labile under easily accessible deprotection conditions after synthesis, and ideally cleavage from the support should be facile and as near to quantitative as possible.

Standard solid-supports include controlled pore glass (CPG) and polystyrene-based resin/beads, to which the oligonucleotide is attached by a base-labile linker. The exocyclic amines in the standard nucleotides are protected with base-labile protecting groups, such as benzoyl and isobutyryl, which are stable during synthesis but easily removed afterwards (Figure 7.1).

![Figure 7.1](image-url) Protected oligodeoxynucleotide (ODN) on solid-support and standard DNA nucleobases, illustrating base-labile functionalities (succinyl linker – red; 2-cyanoethyl phosphate protecting group – blue; acyl protecting groups – violet). dT nucleobase requires no protection.
Non-standard nucleosides, either natural or synthetically modified, are incorporated by the same method, appropriately protected with base-labile or other labile groups. Alternative compatible protecting groups for primary/exocyclic amines include phthalimide, trifluoroacetyl and phenoxyacetyl. Hydroxyl groups may be protected as the TBDMS- or allyl-ether for example. The protecting group lability can be altered depending on the oligonucleotide sensitivity and desired deprotection conditions.

7.2 Deprotection Conditions for Oligonucleotides

The standard reagents for deprotection of non-modified oligodeoxynucleotides (ODNs), are concentrated (~33 wt%) aqueous ammonia and concentrated (40 wt%) aqueous methylamine. The oligonucleotide may be cleaved from the solid-support under mild conditions (conc. aq ammonia, rt, 1 hour) before heating or further treatment, or the support may be removed from the synthesis column and all deprotections carried out in one step. Treatment at rt overnight or at 55 °C for 4-6 hours is usually sufficient to ensure full deprotection of shorter ODNs. Using a 1:1 mixture of conc. aq ammonia and conc. aq methylamine (AMA) may also be used to significantly reduce deprotection time for nucleosides such as Ac\text{dC}, Br\text{dA} and iBu\text{dG}. AMA can also be used for quick cleavage of DNA from the solid-support (5-15 minutes). Methylamine and AMA should not be used for deprotection of Br\text{dC}, however, as there is precedent for attack of the C4-position by alkylamines, to form the 4N-alkyl-dC derivative. Oligonucleotides may also be deprotected using pressurised ammonia or methylamine gas, following cleavage from the support.

Standard deprotection conditions are also usually sufficient for deprotection of other base-labile groups. Phthalimide may be cleaved using methylamine or hydrazine, and allyl groups can be removed by automated treatment with palladium (0), such as a solution of Pd(PPh3)4 in chloroform, containing 5% acetic acid and 2.5% N-methylmorpholine, before removal from the synthesiser for cleavage/deprotection. Oligonucleotides may also be treated with diethylamine before cleavage from the resin, or by addition of nitromethane during deprotection. These reagents cleave 2-cyanoethyl groups from the phosphate esters and scavenge acrylonitrile, a by-product of oligonucleotide synthesis. Nitromethane acts predominantly as a scavenger of acrylonitrile. Without such treatments, a small proportion of dT nucleotides are
cyanoethylated at the \(N^3\)-position. Such adducts however, do not form readily when deprotecting using methylamine, which also acts as a scavenger. Ammonia is not nucleophilic enough to act as an effective scavenger.

Washing of the solid-support with 20% diethylamine/acetonitrile is incorporated into the automated synthesis procedure on some new DNA/RNA synthesisers. 2-Cyanoethyl groups are cleaved and acrylonitrile scavenged before cleavage from the solid-support.\(^{246}\) Diethylamine will only cause cleavage from solid-support upon prolonged exposure (>5 hours).

### 7.3 Oligonucleotide Deprotection Study

#### 7.3.1 Background

Oligonucleotides synthesised for the initial UV and fluorescence melting studies (Chapters 4, 6) were analysed by analytical HPLC and either electrospray or MALDI-TOF MS. Analysis of the non-labelled furano-dT modified TFOs for UV melting, showed that after deprotection with 30 wt% aqueous methylamine (rt, 6-24 hours), and treatment with DOWEX-H\(^+\) resin, cyclisation of the methylamine ring-opened intermediate was not quantitative (Figure 7.2). However, HPLC purification of these oligonucleotides was successful as resolution was obtained.

![Figure 7.2. HPLC traces of UV melting TFOs (OL4,5) following first HPLC purification and treatment with DOWEX-H\(^+\) resin.](image)
HPLC analysis of the DABCYL-labelled TFOs for fluorescence melting, however, could not resolve the components. The large lipophilic DABCYL moiety causes the labelled DNA to adhere strongly to the stationary phase, thus causing reduction in resolution (“streaking”). MS analysis of several of the first DABCYL-labelled N-methylpyrrolo-dC modified TFOs (OL18-21, \(^{\text{Ac}}\text{PP}_p\); OL23, \(^{\text{Up}}\text{PP}_p\)), indicated they were clean following HPLC purification, and they were subsequently used in melting studies.

HPLC analysis of some of the crude mixtures before purification (OL18-21), identified a normal distribution of minor impurities around the major peak. Analytical ion-exchange (IONEX) chromatography of HPLC-purified TFO OL19, resolved a small possible failure sequence peak, and due to the long retention time was deemed unsuitable as a method of purification (Figure 7.3).

![Figure 7.3. HPLC traces of \(^{\text{Ac}}\text{PP}_p\)-modified TFOs (OL18-21) before purification and IONEX trace of OL19 after HPLC purification.](image)

Following this work, a Capillary Electrophoresis system was acquired, from which very high resolution chromatograms could be obtained. CE analysis of several of the DABCYL-modified TFOs, resolved each into three peaks for both furano-dT (OL14, \(^{\text{Ap}}\text{PP}_p\)) and \(N\)-methylpyrrolo-dC modified TFOs (OL20, \(^{\text{Ac}}\text{PP}_p\); OL23, \(^{\text{Up}}\text{PP}_p\)) (Figure 7.4). This result could be rationalised for the furano-dT modified TFO, as a mixture of cyclised (highest retention time), uncyclised/ring-opened TFOs and a sequence where one phosphoramidite coupling did not occur (failure sequence, lowest retention time). The observation is harder to explain for the \(N\)-methylpyrrolo-dC modified TFOs
(deprotected using conc. aq NH₃), where only ring-opening by ammonia or failure sequences could be held accountable. Failure sequences are usually capped by treatment with acetic anhydride, before the oxidation step in each cycle of oligonucleotide synthesis. If synthesised without capping, failure sequences, which are usually minor, may become more prominent, especially when using synthetically-modified nucleosides. However, this may account only for the smaller peak of lowest retention time, and ring-opening by ammonia appears unlikely. Secondary structures may also provide an explanation but are even more unlikely.

Concurrently there were problems with TFOs containing the GC-recognition monomer, bis-amino-U (BAU). Following incorporation into the TFO, the oligonucleotide was deprotected using 40 wt% aqueous methylamine. Methylamine was also required for cleavage of the phthalimide protecting group on the 2’-moiety (Scheme 7.1). Despite previous successful deprotections and melting studies, problems were later encountered. Although HPLC analysis of the TFOs indicated they were pure, melting studies afforded poor results, i.e. little or no triplex formation. On receipt of the CE machine, analysis of some of these TFOs revealed a mixture of peaks, which could only be accounted for by excessive degradation of the TFO, not by failure sequences alone. This suggested that the deprotection conditions must have changed since the previous TFOs containing this monomer were synthesised, deprotected and purified.

**Figure 7.4.** CE traces of DABCYL-modified TFOs (OL14, OL20, OL23).
After these results, it was decided to investigate oligonucleotide deprotection conditions to determine the optimum conditions for monomers used within the research group.

7.3.2 Deprotection Study

A series of oligonucleotides were synthesised on a 1.0 μmole scale (with capping) based on oligoT (T₁₂), containing the monomers under examination (Figure 7.5).

After each oligonucleotide synthesis, the solid-support was removed from the column. Small portions of the resin (5-8 mg) were deprotected for varying lengths of time, at different temperatures using conc. aq ammonia, 20 wt% aq MeNH₂ and 40 wt% aq MeNH₂. A list of all deprotections for each oligonucleotide is given in Table 7.1.

Following deprotection, the resin was removed and solvent evaporated, the oligonucleotide residue was dissolved in distilled water (1.0 mL) and desalted using a disposable Sephadex NAP™ 10 column. All samples were then analysed, without HPLC purification, by CE. ES and MALDI-TOF MS analyses were taken as appropriate. Analytical HPLC and IONEX were also used for several samples.
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**Table 7.1.** Oligonucleotides, deprotection conditions and MS analysis. Optimum conditions evaluated are in bold. **Column washed with 20% Et₂NH/MeCN for 10 minutes before removal of resin for deprotection.**
7.3.2.1 Control T₁₂ Oligonucleotide

Control oligonucleotide T₁₂ (OL64) was deprotected successfully by three methods. No degradation was observed by CE, hence even the strongest deprotection conditions (40 wt% aq MeNH₂) caused no degradation of the DNA backbone (Figure 7.6).

![CE traces for deprotections of T₁₂ (OL64).](image)

It was deduced therefore, that any impurities observed for other oligonucleotides must be due to failure sequences (n-1, n-2 etc.), degradation of the modified nucleotide, or fragmentation of the oligonucleotide due to the modification.

7.3.2.2 Test Oligonucleotides – 5-(3-Aminopropynyl)-dU (pdU)

The first modified monomer to be tested, pdU, was incorporated into three sequences with one, three and seven insertions.
The best conditions for T₈XT₃ (X = pdU, OL65), giving the least degradation, are conc. aq ammonia at rt for 5 hours (Figure 7.7). A longer time does not significantly affect the impurity profile. Heating at 55 °C for 5 hours is also suitable, but heating overnight (15 hrs) caused further appearance of impurities. A 20 wt% aqueous solution of methylamine also deprotected causing little degradation overnight.

However, it is very clear that using conc. aq methylamine (40 wt%) causes formation of a significant impurity (2581 Da) plus other minor peaks. This major impurity can be explained by cleavage of the oligonucleotide at the 3'-side of pdU, and displacement of the alkynyl-U nucleobase by methylamine (Scheme 7.2, Figure 7.8).
Figure 7.8. MALDI-TOF MS spectra for two deprotections of T₃XT₃ (X = pdU, 3627.4 Da, OL65). * Internal T₁₀ and T₁₅ references.

Similar observations were made for three additions of pdU (T₄XTXTXT₃, OL66). The mildest successful deprotection conditions were treatment with conc. aq ammonia at rt for 1 hour only, after washing the resin with 20% Et₂NH/CH₃CN for 10 minutes. Without the diethylamine wash, impurities were present. For this sample, this was due either to poor coupling of pdU, perhaps caused by presence of pyridine in phosphoramidite, or too little time for complete deprotection, not the lack of a diethylamine wash. Under these conditions, 1 hour is the shortest possible time for cleavage from the resin, but may not be long enough for consistent deprotection of all protecting groups (Figures 7.9, 7.10).

Figure 7.9. CE traces for deprotections of T₄XTXTXT₃ (X = pdU, OL66).
Heating at 55 °C for only 4 hours with conc. aq ammonia formed several small impurities, and treatment overnight at rt with 40 wt% methylamine caused extensive oligonucleotide degradation. Treatment with 20 wt% methylamine at rt could be used for 5 hours, but impurities formed over 15 hours.

Figure 7.10. ES MS spectra for two deprotections of T,X,T,X,T,X,T (X = pdU, 3705.5 Da, OL66). Data deconvoluted to resolution of 1 Da.

Oligonucleotide T,X,T,X,T,X,T (X = pdU, OL67) was successfully deprotected under mild conditions (conc. aq ammonia, 55 °C, 1 hr) with and without a prior diethylamine wash. The small impurities were slightly greater without washing and failure sequences became slightly more prominent. A slightly longer deprotection time (~2 hours) may have removed some of these trace impurities. HPLC and IONEX traces were similar for both (Figure 7.11).

A small impurity was present in these oligonucleotides (3651 Da in OL66), due to incorporation of a single dU nucleoside instead of pdU. The dU impurity was formed during the Sonogashira cross-coupling reaction in synthesis of the pdU phosphoramidite monomer 164 and was present at approximately 2-3%. When used in synthesis of the deprotection study oligonucleotides, it only became noticeable on multiple additions. When oligonucleotide OL66 was re-synthesised using a different batch of monomer, with and without capping for comparison, only trace baseline impurities were noticeable. Synthesis was as effective with capping as without capping indicating absence of failure sequences.
7.3.2.3 Test Oligonucleotides – Bis-amino-U (BAU)

The next monomer under test was bis-amino-U (BAU). The best conditions for deprotection were discovered to be 20 wt% aq methylamine, at rt for 5 hours.

Little change was noted after 8 hours, but after 15 and 30 hours notable impurities had formed. Heating at 55 °C in conc. aq ammonia for 15 hours, and treating with AMA (conc. aq NH3/40 wt% aq MeNH2, 1:1 v/v) at rt for 15 hours produced a similar significant pattern of impurities (Figure 7.12).
Figure 7.13. MALDI-TOF/ES MS spectra for two deprotections of T₈X₃T₃ (X = BAU, 3686.5 Da, OL68). ES data deconvoluted to resolution of 1 Da. * Internal T₁₀ and T₁₅ references.

Only a ‘minus T’ failure sequence (3382.3 Da) was identified by MS. The other MS peaks could not be assigned (Figure 7.13). Methylamine is required for phthalimide cleavage, but a high concentration causes significant degradation.

7.3.2.4 Test Oligonucleotides – 6-(3-Acetamidophenyl)-N-methylpyrrolo-dC (ACP₃)

The acetamide monomer for CG recognition was incorporated into a ‘T₁₂ oligonucleotide’ (T₈XT₃, X = ACP₃, OL69) and proved stable to conc. aq ammonia at rt for 5 hours, and 55 °C for 4 hours, and also to 20 wt% aq methylamine at rt for 5 hours (Figure 7.14). Heating at 55 °C in conc. aq ammonia for 15 hours caused more significant degradation than AMA at rt for the same time.
Concentrated aq methylamine, however, primarily caused major conversion to the cleavage product (2581 Da) shown in Scheme 7.2 (Figure 7.15).

This was originally presumed to be the deacetylated oligonucleotide, but was confirmed otherwise by ES and MALDI-TOF MS. No ring-opening of the N-methylpyrrolopyrimidine nucleobase by methylamine was observed.
7.3.2.5  Test Oligonucleotides – S base (TA recognition monomer, S)

The ‘S base’ monomer for TA recognition (Section 1.2.2.1), was incorporated into oligo OL70 (T₈XT₃, X = S) for deprotection studies. The main aim for this modification was to find conditions, to which the acetamide moiety would be stable. CE analysis produced only a single peak for each sample, but was unable to resolve acetylated and deacetylated products.

![CE trace and MS spectra](image)

Figure 7.16. CE trace for strongest deprotection conditions (40% aq methylamine, rt, 15 hrs), and ES/MALDI-TOF MS spectra for two deprotections of T₈XT₃ (X = S, 3737.6 Da, OL70). Deacetylated oligonucleotide mass (3695.6 Da). ES data deconvoluted to resolution of 1 Da. * Internal T₁₀ and T₁₅ references. Structure of ‘S’ is shown.

MS analysis highlighted stability in conc. aq ammonia at rt for up to 15 hrs. Heating at 55 °C in conc. ammonia for only 5 hours caused some deacetylation, and in 40 wt% aq methylamine at rt, a significant amount (Figure 7.16). CE analysis of the sample deprotected with 20% aq methylamine (rt, 5 hrs) showed a very sharp peak, but conc. ammonia was concluded as the best deprotection medium.

7.3.2.6  Test Oligonucleotides – 5-(3-aminopropynyl)-2′-O-methoxyethyl-U (MOEpU)

AT recognition monomer, MOEpU, was incorporated into sequence T₈XT₃ (OL71) for comparison with its 2′-unmodified analogue, pdU. Poor coupling of the monomer, however, resulted in a major failure sequence (T₁₁) of similar proportion to product by
CE. Of the three conditions tested (Table 7.1), heating at 55 °C in conc. aq ammonia for 5 hrs caused an increase in the apparent failure sequence peak and a small increase in the general impurity profile over the other two.

7.3.2.7 Test Oligonucleotides – Other AT recognition monomers

AT recognition monomer, 2’-aminoethoxy-T (AE-T) was incorporated into T₈XT₃ (OL72) and T₄XTTXTXT₃ (OL73) and both were deprotected at 55 °C in conc. aq ammonia for 4 hours. Both samples produced very clean HPLC traces hence this method was deemed suitable, and this should be applicable also at rt.

5-(3-Guanidinylprop-1-ynyl)-dU (GpdU) was incorporated once (T₈XT₃, OL74) and treated under two deprotection conditions. Deprotection with conc. aq ammonia (rt, 5 hrs) caused formation of two major peaks plus impurities by CE (Figure 7.17).

![Figure 7.17. CE traces for deprotections of T₈XT₃ (X = GpdU, OL74).](image)

One major peak (~22.8 minutes) appears to correspond to the oligonucleotide with a fully-protected guanidinyl moiety. The other (~21.9 minutes) may correspond to a stable triazinedione derivative, caused by cyclisation due to attack by ammonia. This was observed by Prakash et al. when deprotecting oligonucleotides, containing 2-cyanoethyloxycarbonyl- (CEOC) protected guanidinyl moieties, with ammonia (Figure 7.18). Prakash et al. cleaved the CEOC groups in 50% aq piperidine at rt for 24 hours, prior to heating at 55 °C in conc. ammonia. Treating with 50% aq piperidine also
cleaved the 2-cyanoethyl phosphate protecting groups and cleaved the oligonucleotide from the resin.

![Figure 7.18. Possible mechanism of formation of triazinedione derivative.](image)

It was later discovered that another secondary amine, diethylamine, could be used instead of piperidine. Washing the column with 20% Et$_2$NH/CH$_3$CN for at least 30 minutes prior to deprotection, was sufficient to remove the most labile CEOC group thus preventing triazine formation. It is likely that methylamine would attack the CEOC groups in the same manner but the steric bulk of the methyl group might prevent cyclisation after attack.

The second deprotection of OL74 was carried out in 'BuNH$_2$:CH$_3$OH:H$_2$O (1:1:2 v/v/v) at rt for 21 hours. Tert-butylamine was used to cleave all protecting groups at the same time. It was presumed that the sterically-hindering tert-butyl group would prevent attack of the CEOC groups and cyclisation. These conditions, however, proved too mild, producing a mixture of bis-CEO, mono-CEO and fully deprotected oligonucleotides in an approximate ratio of 9:5:1 (Figure 7.17).

### 7.4 Conclusion

The results of this study highlight the need for suitable/compatible deprotection conditions for each modified nucleotide within the oligonucleotide. It cannot be assumed that the conditions used for one modification, will be safe to use for another, as proven by the compatibility of most modifications to deprotection with conc. ammonia at rt, except the CEOC-protected guanidinyl-modified nucleotide.
Concentrated (40 wt%) aq methylamine was proven too strong for deprotection of all oligonucleotides containing modified non-natural nucleotides. It is likely that previous deprotections of BAU-containing oligonucleotides using conc. aq methylamine were successful, due to evaporation of methylamine over time from the bottle in use, thus reducing the effective concentration.

In general, conc. aq ammonia can be used to deprotect most modified oligonucleotides, either at rt or sometimes with heating. Methylamine is needed for cleaving phthalimide groups, and may be required for cleavage of more stable protecting groups.

Washing the column with 20% Et₂NH/CH₃CN solution for 10-20 minutes before removal from the synthesiser, was incorporated into the standard oligonucleotide synthesis protocol, on all synthesisers where it was not already included and could be easily achieved. It cleanly cleaves 2-cyanoethyl groups and scavenges acrylonitrile, and proves highly effective at removing CEOC-protecting groups on guanidinyl-modified nucleotides.

These results were used for choosing deprotection conditions for oligonucleotides used within the research group, including for the TFOs used in the primary melting studies (Sections 6.2, 6.3). For all TFOs used in these melting studies, following synthesis, the solid-support column was washed with 20% Et₂NH/CH₃CN for 20 minutes, or 3 hours for PP₄-containing TFOs (OL48-51, OL56-59). Each oligonucleotide was then deprotected in conc. aq ammonia at rt for 12 hours, with or without 2 hours at 50 °C, or at rt for 24 hours followed by heating at 50 °C for 1 hour to ensure complete deprotection. CE analysis indicated good to high purity for most TFOs after one HPLC purification. Where impurities constituted a significant proportion, the TFO was carefully repurified, collecting only the centre of the peak. Impurities did not, however, hinder performance in melting studies.
Chapter 8

Conclusion
8. Conclusion

8.1. Modified 6-Oxocytidine Nucleosides for GC Recognition

Synthesis of a bis-amino-modified 6-oxocytidine monomer 64, was re-investigated as a potential GC recognition monomer, which exhibits pH-independent binding within a triplex. Previous published studies\textsuperscript{70,71} reported pH-independent triplex formation using 6-oxoC nucleotides up to pH 8, and enhanced binding to GC at higher pH compared to C. Modifications to this nucleoside,\textsuperscript{11,70,76} however, produced varied or negative results, hence further modifications were proposed to introduce protonated amine functionalities, which should enhance triplex stability.

Several synthetic investigations were carried out, in order to assess reaction feasibility. Palladium-catalysed alkynylation was ruled out as a method of introduction of amine functionality at the 5-position due to inactivity of the halogenated substrate or degradation. Modification by amine- or thiol-halide exchange was investigated with limited success, but this requires extensive optimisation if a robust synthetic route is to be found. Feasibility of 2’-modification was also investigated, although this requires further work. Optimised synthetic routes to some key intermediates have been developed.

8.2. Amino-Modified Bicyclic Nucleosides for CG Recognition

Recognition of pyrimidine.purine base pairs is a greater challenge than purine.pyrimidine base pairs as fewer hydrogen-bonds are presented for binding in the major groove. Previous published research describes the development of recognition monomers from T, based around the bicyclic C analogue, \textit{N}-methylpyrrolo-dC.\textsuperscript{122} This nucleobase retains the C\textsuperscript{2} carbonyl group, vital for CG recognition, whilst blocking GC recognition by replacing an H for a methyl group at the 4\textit{N} position.

The latest design had employed an aminoalkyl group for targeting of hydrogen-bonding residues on G across the base pair. This research examined various meta-aminophenyl groups; rigidified linkers better placed for binding.
Furano-dT 145-147 and N-methylpyrrolo-dC phosphoramidite monomers 178,179,191 (199 – synthesis repeated later in house) were synthesised for assessment. All were synthesised by a common synthetic route (Sonogashira cross-coupling, Cu(I)-catalysed cyclisation and phosphorylation) with some modifications, starting from DMT-protected 5-iodo-2’-deoxyuridine 97, and analogue 5-iodo-N-methyl-2’-deoxycytidine 185 respectively. Yields ranged from moderate to excellent and most steps were optimised despite high substrate polarity.

Furano-dT monomers underwent post-synthetic modification to the N-methylpyrrolo-dC nucleotide after incorporation into DNA. N-Methylpyrrolo-dC monomers could be incorporated with no further modification necessary. Although post-synthetic modification was not complete, and HPLC purification was unable to resolve the mixture for DABCYL-labelled oligonucleotides, both performed equally in melting studies.

Thiourea-modified monomer 158 (synthesis finished later in house) could not be incorporated into TFOs due to issues with stability to oligonucleotide synthesis or deprotection, hence could not be assessed in biophysical studies.

Extensive melting studies demonstrated the effectiveness of these monomers at recognising CG with high selectivity and good binding affinity compared to previous monomers (T, 4H T, M P). The order of stability was altered, however, between UV and fluorescence melting studies. This was proposed to be due to changes in target sequence and local environment around the CG inversion target, but requires further investigation.

The effect of neighbouring groups on the modification was also highlighted. The CG recognition monomers appear to interact differently with 5-(3-aminoprop-1-ynyl)-dU (pdU, P), depending on which side it is located. This neighbouring group interaction introduces important implications when using TFOs as oligotherapeutics, as all interactions must be taken into account for successful development.
8.3 Oligonucleotide Deprotection

A study was conducted to determine the optimum deprotection conditions for monomers used within the research group, after experiencing several problems.

Concentrated aq ammonia was determined the best medium for deprotection at rt or sometimes with heating. Most monomers also tolerated 20% aq methylamine for approximately 5 hours. Deprotection times of 15 hours or greater caused some degradation for several monomers, and the standard deprotection media, conc. aq methylamine and AMA, as used for non-modified DNA, caused significant to extensive degradation and strand cleavage for everything except the oligonucleotide T12.

A column wash with 20% Et2NH/CH3CN solution was introduced on to DNA synthesisers where not already incorporated, after demonstrating efficient cleavage of 2-cyanoethyl phosphate protecting groups and prevention of cyanoethylation by scavenging of acrylonitrile. This wash also proved very effective at partial deprotection of the guanidine moiety of two monomers, thus avoiding subsequent side-reactions during deprotection.
Chapter 9

Experimental
HPLC grade acetonitrile, methanol and water were purchased from Fisher Scientific. Pyridine, dichloromethane, triethylamine and diisopropylethylamine were distilled over calcium hydride; methanol, ethanol and isopropanol over magnesium and iodine; phosphorous oxychloride over sodium wire, and tetrahydrofuran over sodium wire and benzophenone before use. Anhydrous $N,N$-dimethylformamide and 1,2-dichloroethane were purchased from Aldrich. Diethyl ether was purchased from Fisher Scientific and dried by passing through a column of dry alumina then degassed at low temperature, or argon-degassed over 4Å molecular sieves. Molecular sieves were oven-dried at greater than 120 °C over 2 days or microwave-activated. Acetone was purchased from Fisher Scientific and dried over Drierite (anhydrous CaSO$_4$). 6-Aminouracil was purchased from Aldrich, recrystallised from boiling water and dried over P$_2$O$_5$ before use. Iodine/pyridine/THF/water solution (0.02 M), phosphitylating reagents and DIHT were purchased from Link Technologies. $N$-Iodosuccinimide was purchased from Aldrich and recrystallised from 1,4-dioxane/carbon tetrachloride before use. Deuterated NMR solvents were purchased from Apollo Scientific Ltd. DOWEX 50WX8 ion-exchange resin was purchased from Aldrich and washed with 0.2 M HCl before treatment. All other reagents were purchased from Aldrich, Lancaster, Avocado, Alfa Aesar, BDH, Fisher Scientific, Acros and Fluka, and used without purification. All reactions requiring absence of oxygen were carried out under an atmosphere of argon. Glassware was oven-dried overnight before use, for reactions requiring exclusion of moisture.

Reactions were monitored by TLC, using Merck Kieselgel 60 F$_{254}$ or Machenary-Nagel Alugram Sil G/UV$_{254}$ silica gel plates (0.22 mm thickness, aluminium backed). Compounds were visualised by irradiation at 254/365 nm, or staining with $p$-anisaldehyde (A’), potassium permanganate (B’), ninhydrin (C’, D’), 10% H$_2$SO$_4$/ethanol (E’) or ceric sulfate (F’), followed by heating.
A’ – *p*-anisaldehyde / glacial AcOH / conc. H$_2$SO$_4$ / ethanol (9.2:3.8:12.5:338 v/v)
B’ – KMnO$_4$ / K$_2$CO$_3$ / 5% aq NaOH / water (3:20 g/5:300 mL)
C’ – ninhydrin (5 g) / acetone (100 mL)
D’ – 0.3% w/v ninhydrin / butan-1-ol / 3% v/v glacial AcOH
E’ – 10% v/v conc. sulfuric acid / ethanol
F’ – 8% w/v Ce(SO$_4$)$_2$.2H$_2$SO$_4$ hydrate / 15% v/v sulphuric acid

Column chromatography was carried out under air or argon pressure using Fisher Scientific DAVISIL 60Å (35-70 micron) silica gel. Silica gel was pre-equilibrated with triethylamine or pyridine for purification of acid-sensitive compounds.

Proton and carbon NMR spectra were recorded at 300/400 MHz and 75/100 MHz, respectively, using either a Bruker AC300 or Bruker DPX400 spectrometer. Fluorine and phosphorous NMR spectra were recorded at 282 MHz and 121 MHz respectively, using a Bruker AC300 spectrometer. NMR spectra were recorded in deuterated chloroform or dimethylsulfoxide. Chemical shifts are given in ppm and spectra are calibrated to the residual solvent peak.$^{247}$ J values are correct to within 0.5 Hz. Assignment was aided by DEPT-135, $^1$H–$^1$H COSY, HMQC and HMBC experiments and Lorentz-Gauss resolution enhancement data reprocessing (Win-NMR Lite).

Low-resolution mass spectra were recorded using electrospray ionisation (ES) on a Fisons VG platform instrument, or on a Waters ZMD quadrupole mass spectrometer in HPLC grade acetonitrile, methanol or water, or using electron ionisation (EI) on a ThermoQuest TraceMS single quadrupole GC-MS instrument in dichloromethane. High-resolution mass spectra were recorded in HPLC grade acetonitrile, methanol or water using electrospray ionisation on a Bruker APEX III FT-ICR mass spectrometer.

IR spectra were recorded on a Satellite FT-IR instrument using a ‘Golden Gate’ or ‘Smart Orbit’ adapter, and visualised using Win First-lite or OMNIC software respectively. All absorptions are measured in cm$^{-1}$ and are described as broad (br), weak (w), medium (m), strong (s) or very strong (vs).

Melting points were recorded on a Gallenkamp Electrothermal melting point apparatus and uncalibrated.
Elemental (CHN) Thermal Combustion Analysis was carried out by MEDAC Ltd., Egham, Surrey, UK.
9.1.2 Experimental Procedure

1,3,5-Tri-O-acetyl-2-O-(2-phthalimidoethyl)-D-ribofuranose, 65<sup>37</sup>

![Chemical Structure of 1,3,5-Tri-O-acetyl-2-O-(2-phthalimidoethyl)-D-ribofuranose, 65](image)

To a stirred solution of 89 (3.31 g, 5.71 mmol) in glacial acetic acid/acetic anhydride (1:1 v/v, 30 mL), under an argon atmosphere, conc. sulfuric acid (0.35 mL, 6.57 mmol, 1.1 eq) was added slowly. The reaction mixture was stirred at rt for 1 hour. Saturated aq sodium bicarbonate (250 mL) was added carefully to the dark green reaction mixture, which was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 100 mL). The combined organic fraction was washed with sat. aq sodium bicarbonate and sat. aq KCl, dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated <i>in vacuo</i> to yield an orange-brown oil/solid. Following purification by column chromatography (1:1, hexane/ethyl acetate), and drying under high vacuum, the product 65 was isolated, as an anomic mixture (α:β ca 2:1), as a very pale yellow oil (2.11 g, 4.70 mmol, 82% yield).

R<sub>f</sub> 0.17 (1:1, hexane/ethyl acetate, A'); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ signals due to α anomer 7.86-7.80 (2H, m, H<sub>5</sub><sup>6</sup>), 7.74-7.68 (2H, m, H<sub>6</sub><sup>6</sup>), 6.07 (1H, s, H<sub>4</sub><sup>1</sup>), 5.00 (1H, dd, J = 4.8, 6.8 Hz, H<sub>4</sub><sup>2</sup>), 4.35-4.30 (1H, m, H<sub>4</sub><sup>3</sup>), 4.29 (1H, t, J = 4.8 Hz, H<sub>4</sub><sup>4</sup>), 4.14-4.08 (1H, m, H<sub>4</sub><sup>5</sup>), 4.12-4.07 (1H, m, H<sub>4</sub><sup>6</sup>), 3.94-3.83 (2H, m, H<sub>5</sub><sup>7</sup>), 3.83-3.72 (2H, m, H<sub>5</sub><sup>8</sup>), 2.05, 2.04 (3H, s, COCH<sub>3</sub>), 2.00 (3H, s, COCH<sub>3</sub>), signals due to β anomer 7.86-7.80 (2H, m, H<sub>5</sub><sup>6</sup>), 7.74-7.68 (2H, m, H<sub>6</sub><sup>6</sup>), 6.29 (1H, d, J = 4.5 Hz, H<sub>4</sub><sup>1</sup>), 5.13 (1H, dd, J = 2.5, 6.5 Hz, H<sub>4</sub><sup>2</sup>), 4.38 (1H, br dd, J = 3.3, 7.3 Hz, H<sub>4</sub><sup>3</sup>), 4.24 (1H, dd, J = 3.5, 12.6 Hz, H<sub>4</sub><sup>4</sup>), 4.06 (1H, dd, J = 5.5, 12.1 Hz, H<sub>4</sub><sup>5</sup>), 3.96 (1H, dd, J = 4.5, 6.5 Hz, H<sub>5</sub><sup>2</sup>), 3.94-3.83 (2H, m, H<sub>5</sub><sup>3</sup>), 3.83-3.72 (2H, m, H<sub>5</sub><sup>4</sup>), 2.07, 2.03, 1.96 (3H, s, COCH<sub>3</sub>); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ signals due to α anomer 170.6, 170.4, 169.5 (COCH<sub>3</sub>), 168.2 (C<sub>3</sub><sup>3</sup>), 134.2 (C<sub>6</sub><sup>6</sup>), 132.2 (C<sub>4</sub><sup>4</sup>), 123.4 (C<sub>5</sub><sup>5</sup>), 98.9 (C<sub>1</sub><sup>1</sup>), 80.8 (C<sub>2</sub><sup>2</sup>), 79.5 (C<sub>4</sub><sup>4</sup>), 71.9 (C<sub>5</sub><sup>5</sup>), 67.8 (C<sub>1</sub><sup>1</sup>), 63.8 (C<sub>5</sub><sup>5</sup>), 37.6 (C<sub>2</sub><sup>2</sup>), 21.2, 20.8, 20.5 (COCH<sub>3</sub>), signals due to β anomer 171.2, 170.5, 170.0 (COCH<sub>3</sub>), 168.2 (C<sub>3</sub><sup>3</sup>), 134.1 (C<sub>6</sub><sup>6</sup>), 123.2 (C<sub>4</sub><sup>4</sup>), 123.3 (C<sub>5</sub><sup>5</sup>), 94.8 (C<sub>1</sub><sup>1</sup>), 81.4 (C<sub>4</sub><sup>4</sup>), 77.9 (C<sub>5</sub><sup>5</sup>), 69.7 (C<sub>3</sub><sup>3</sup>), 68.6 (C<sub>1</sub><sup>1</sup>), 63.4 (C<sub>5</sub><sup>5</sup>), 37.6 (C<sub>2</sub><sup>2</sup>), 21.1, 20.9, 20.7 (COCH<sub>3</sub>); LRMS (ES<sup>+</sup>): m/z (%): 546 (38), 472 ([M + Na]<sup>+</sup>, 100).
Analytical results consistent with reported data.\textsuperscript{37}

\textbf{N-Trifluoroacetyl propargylamine, 68}\textsuperscript{117,170}

\begin{center}
\begin{lrbox}{0}
\begin{tikzpicture}
\node (text) at (0,0) {
\begin{minipage}{0.8\textwidth}
Propargylamine (3.71 g, 67.3 mmol) 98 was added dropwise to an ice-cooled, stirred solution of ethyl trifluoroacetate (10.4 mL, 87.6 mmol, 1.3 eq) in distilled methanol (50 mL) under an argon atmosphere. After addition, the solution was allowed to warm to rt and stirred for 15 hrs. The reaction mixture was concentrated \textit{in vacuo} and redissolved in CH$_2$Cl$_2$ (200 mL). The solution was washed with 10\% w/v aq citric acid (2 × 100 mL) and sat aq KCl (100 mL), dried (Na$_2$SO$_4$) and concentrated \textit{in vacuo} to give an orange/brown oil. Following column chromatography (CH$_2$Cl$_2$), and drying \textit{in vacuo}, the product 68 was isolated as a very pale yellow oil (5.81 g, 38.4 mmol, 57\%).
\end{minipage}
\end{tikzpicture}
\end{lrbox}
\end{center}

\begin{itemize}
\item \textbf{R$^f$}: 0.27, 0.33 (CH$_2$Cl$_2$, B', D'), 0.53, 0.66 (1:9, methanol/CH$_2$Cl$_2$, B', D'); \textbf{IR} (film): ν 3300 (br m, CC–H), 3092 (br w, N–H), 1703 (vs, C=O), 1551 (m, N–H), 1428, 1363, 1335 (w), 1207 (s), 1152 (br vs, C–F), 1046 (w), 997 (m), 926, 826 (w), 724 (m, C–F), 667 (m), 638 (br s), 602, 556 (m) cm$^{-1}$; \textbf{\textsuperscript{19}F NMR} (282 MHz, CDCl$_3$) δ -76.2 (CF$_3$); \textbf{\textsuperscript{1}H NMR} (300 MHz, CDCl$_3$) δ 6.73 (1H, br s, NH), 4.15 (2H, dd, $J = 2.6$, 5.3 Hz, CH$_2$), 2.33 (1H, t, $J = 2.6$ Hz, C≡CH); \textbf{\textsuperscript{13}C NMR} (75 MHz, CDCl$_3$) δ 157.2 (q, $J = 37.6$ Hz, COCF$_3$), 115.7 (q, $J = 287.5$ Hz, COCF$_3$), 77.2 (C≡CH), 73.3 (C≡CH), 29.8 (CH$_2$); \textbf{LRMS} (ES\textsuperscript{+}): m/z : 206 ([M + CH$_3$OH + Na]$^+$, 100), 174 ([M + Na]$^+$, 45); (ES): m/z : 150 ([M – H]$^-$, 100), 80 (57).
\end{itemize}

Analytical results consistent with reported data.\textsuperscript{117,170}
Sodium iodide (6.63 g, 44.2 mmol, 1.0 eq) was dissolved in conc. aq HCl (15.5 mL) and cooled in an ice bath. To this, an 8.8% w/v aqueous solution of NaClO (37.4 mL, 44.1 mmol) was added slowly, with swirling, so that the temperature was kept below 20 °C. Effervescence and a brown residue were observed. Towards the end of addition, the bleach solution was added dropwise until all brown colouration had disappeared. A 10% aq solution of NaI was added dropwise (20 mL) until a trace amount of iodine reappeared. The resulting yellow/orange solution was added portionwise to a solution of benzyltriethylammonium chloride (10.0 g, 44.1 mmol) in water (14 mL) to give a brilliant yellow precipitate. The mixture was filtered, and the precipitate was washed with water and dried under high vacuum over P₂O₅, to give the product salt as a fine, bright yellow powder (16.5 g, 42.2 mmol, 96% yield).

Mp 89-98 °C, lit. 84-86 °C; IR (solid): 3053 (w, C–H), 2984 (m, C–H), 1479 (s, C–H, Aryl C=C), 1390 (m, C–H), 1360 (m), 1155 (m), 1004 (m), 903 (w), 796 (m), 757 (s, Aryl C–H), 708 (vs Aryl C–H), 605 (w) cm⁻¹; H NMR (300 MHz, d₆-DMSO): δ 7.52 (5H, app. t, J = 1.8 Hz, CH₅), 4.48 (2H, s, H₃), 3.17 (6H, q, J = 7.3 Hz, H₂), 1.31 (9H, t, J = 7.3 Hz, H₁); C NMR (75 MHz, d₆-DMSO): δ 132.5 (C₅), 130.2 (C₆), 129.0 (C₇), 127.8 (C₄), 59.5 (C₃), 52.0 (C₂), 7.5 (C¹); LRMS (ES⁺): m/z (%): 192 (M⁺, 100); (ES⁻): m/z (%): 380 (I⁺, 16), 197/199/201 (ICl⁻, 4, 11:3:1), 126 (I⁻, 100).

Analytical results are consistent with the reported data.
To a stirred solution of nucleoside 73 (0.66 g, 1.18 mmol) in distilled CH₂Cl₂ (20 mL) and distilled methanol (10 mL), was added anhydrous sodium bicarbonate (0.30 g, 3.56 mmol, 3.0 eq), followed by a solution of benzytriethylammonium dichloroiodate 71 (0.69 g, 1.77 mmol, 1.5 eq) in distilled CH₂Cl₂ (15 mL), and the reaction mixture was stirred vigorously at rt for 50 minutes. The crude reaction mixture was filtered and concentrated in vacuo to give an orange/brown gum. The residue was redissolved in CH₂Cl₂ (50 mL) and washed with water (3 × 50 mL). This was further washed with sat. aq sodium thiosulfate (50 mL), water (2 × 50 mL) and brine (2 × 50 mL), dried (Na₂SO₄) and concentrated in vacuo to yield a pink foam. The compound was twice purified by column chromatography (0-2% methanol/CH₂Cl₂) to yield after drying in vacuo, the chlorinated product 74 as a white crystalline solid (0.37 g, 0.54 mmol, 46%).

Rf 0.50 (14:1, CH₂Cl₂/methanol, A’); Mp 94-99 °C (methanol/CH₂Cl₂); IR (solid): ν 3485 (w), 2942, 2891, 2864 (m, C–H), 1714 (m, C=O), 1637 (s, C=O, C=C), 1578 (vs, C=O), 1296, 1246 (m), 1122 (s), 1074 (s, C–O), 1029 (vs, Si–O), 917 (m), 882, 861, 761 (s), 690 (vs, C–Cl), 587 (s) cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ 8.16 (1H, s, H¹), 6.24 (1H, d, J = 1.5 Hz, H¹'), 4.98 (1H, t, J = 7.0 Hz, H¹''), 4.60 (1H, dd, J = 1.5, 6.2 Hz, H²'), 4.01 (2H, d, J = 5.5 Hz, H³'), 3.84 (1H, td, J = 5.3, 7.3 Hz, H⁴), 3.33 (1H, v br s, 2'-OH), 3.19, 3.12 (3H, s, NCH₃), 1.15-1.02 (28H, m, CH(CH₃)₂); ¹³C NMR (75 MHz, CDCl₃) δ 160.0 (C⁶), 156.8 (N=C⁷–N), 151.4 (C², C⁴), 94.2 (C⁵–Cl), 89.4 (C¹'), 82.8 (C⁴'), 73.7 (C²'), 73.1 (C³'), 64.1 (C⁸'), 41.6, 35.2 (N–CH₃), 17.6, 17.5, 17.5, 17.4, 17.3, 17.3, 17.2 (Si–CH(CH₃)₂), 13.4, 13.3, 12.9, 12.8 (Si–CH(CH₃)₂); LRMS (ES⁺): m/z (%): 1203 ([2M + Na]⁺, 44), 613 ([M + Na]⁺, 100); HRMS (ES⁺): calcd for C₂₄H₄₀O₈NaClSi₂ (M), [M + Na]⁺ = 613.2251, found 613.2267.
A suspension of dry 6-aminouracil 62 (8.00 g, 62.9 mmol, 1.1 eq) and ammonium sulfate (1.67 mg, 12.6 mmol, 0.2 eq) in HMDS (131.5 mL, 631 mmol, 10.5 eq) was heated to reflux, under an argon atmosphere, whilst stirring vigorously, for 24 hours, during which the starting material dissolved. The reaction was allowed to cool to rt and the solvent was removed under high vacuum. The cream-coloured solid was redissolved in anhydrous DCE (150 mL) under an argon atmosphere, to which was added acetylated sugar 65 (19.0 g, 59.8 mmol). Trimethylsilyl triflate (11.4 mL, 63.0 mmol, 1.1 eq) was carefully added dropwise and the reaction was stirred at rt, under an argon atmosphere, for 43 hours. After concentrating in vacuo the residue was redissolved in CH$_2$Cl$_2$ (250 mL), and washed with water (150 mL) and dilute aq sodium bicarbonate (150 mL), during which a white solid precipitated out of solution. The mixture was filtered, and following drying under high vacuum at 50 °C for 2 days, the product β-anomer 75 was afforded as a fine white solid (16.9 g, 43.8 mmol, 73% yield). The aqueous layer was re-extracted (CH$_2$Cl$_2$, 100 mL) and the combined, filtered organic fractions were washed with sat. aq KCl (200 mL), dried (Na$_2$SO$_4$) and concentrated in vacuo to yield a peach-coloured foam/solid (3.52 g) containing the sugar 65 and α- 76 and β-anomers 75 of the product nucleoside.

R$_f$ 0.28 (1:9 methanol/CH$_2$Cl$_2$, A'); Mp 197-201 °C (CH$_2$Cl$_2$), lit.$^{71}$ 228-229 °C (ethanol/diethyl ether); IR (solid): v 3447, 3324 (w, N–H), 3211, 2992 (w), 2911, 2789 (w, C–H), 1736 (m, C=O), 1718 (s, C=O), 1652, 1586 (br m), 1537, 1427 (m), 1372 (m, C–H), 1252, 1215 (s, C–O), 1071, 1044 (s), 973, 796, 750 (m), 641 (s), 623, 582 (m), 547 (vs) cm$^{-1}$; $^1$H NMR (400 MHz, $d_6$-DMSO) δ 10.64 (1H, br s, N$_2$H$_3$), 6.47 (2H, br s, N$_2$H$_2$), 6.15 (1H, br s, H$_1'$), 5.64 (1H, dd, J = 2.8, 6.8 Hz, H$_2'$), 5.47 (1H, app. t, J = 7.0 Hz, H$_3'$), 4.55 (1H, s, H$_5'$), 4.31 (1H, dd, J = 3.0, 11.5 Hz, H$_5''$), 4.08 (1H, ddd, J = 3.1, 6.4, 8.0 Hz, H$_4'$), 4.02 (1H, dd, J = 6.5, 11.0 Hz, H$_4''$), 2.06, 2.03, 2.00 (3H, s, COCH$_3$); $^{13}$C NMR (100 MHz, $d_6$-DMSO) δ 170.1, 169.5, 169.3 (COCH$_3$), 161.9 (C$_6$),
154.5 \((C^4-\text{NH}_2)\), 150.4 \((C^2)\), 84.4 \((C^1)\), 77.7 \((C^4)\), 73.6 \((C^3)\), 72.6 \((C^2)\), 69.9 \((C^3)\), 63.1 \((C^5)\), 20.5, 20.4, 20.2 \((\text{COCH}_3)\); \text{LRMS} \((\text{ES}^+)\): \(m/z\) (%): 793 \([2M + \text{Na}]^+\), 12, 430 (32), 408 \([M + \text{Na}]^+\), 100; \((\text{ES}^-): m/z\) (%): 769 \([2M - \text{H}]^-\), 32, 384 \([M - \text{H}]^-\), 100).

Analytical results consistent with reported data.\textsuperscript{71,248}

\textbf{5-Bromo-2',3',5'-tri-O-acetyl-6-oxocytidine, 79}

To a stirred suspension of 2',3',5'-tri-O-acetyl-6-oxocytidine \textbf{75} (3.05 g, 7.91 mmol) and NBS (1.41 g, 7.92 mmol, 1.0 eq) in distilled THF (30.0 mL) was added AIBN (27.0 mg, 0.16 mmol, 2.1 mol %), and the reaction was stirred in absence of light, under an argon atmosphere at rt for 30 minutes. The white slurry was concentrated \textit{in vacuo} to yield a white solid. Following purification by column chromatography (25-80% acetone/toluene), and drying \textit{in vacuo}, the product \textbf{79} was afforded as a powdery white solid (3.47 g, 7.48 mmol, 95% yield) containing 2.0 mol % succinimide.

**\(R_f\) 0.35 (1:1, acetone/toluene, A’), 0.41 (1:9 methanol/CH\(_2\)Cl\(_2\), A’); \textbf{IR} \text{ (solid): \nu} 3412 (w, N–H), 3301 (br w, N–H), 3240, 3194, 3137 (w), 3089 (w, N–H), 3020, 2909 (w, CH\(_2\)), 1739 (s, C=O), 1718 (vs, C=O), 1628, 1557 (s, N–H), 1426 (m), 1371 (s, CH\(_3\)), 1225 (vs, C–O), 1156, 1128, 1091 (s), 1040 (vs), 1013 (s), 941, 930 (m), 897, 765, 727 (s), 678 (m), 617 (s), 538 (s, C–Br) cm\(^{-1}\); \textbf{\(1^H\) NMR} (300 MHz, d\(_6\)-DMSO) \(\delta\) 11.00 (1H, br s, NH\(^3\)), 6.70 (2H, s, NH\(_2\)), 6.16 (1H, br s, H\(^4\)), 5.62 (1H, dd, \(J = 2.5, 6.5\) Hz, H\(^2\)), 5.49 (1H, app. t, \(J = 7.3\) Hz, H\(^3\)), 4.33 (1H, dd, \(J = 3.3, 11.8\) Hz, H\(^5\)), 4.11 (1H, ddd, \(J = 3.0, 6.5, 8.0\) Hz, H\(^4\)), 4.02 (1H, dd, \(J = 6.3, 11.8\) Hz, H\(^5\)), 2.07, 2.03, 2.00 (3H, s, COCH\(_3\)); \textbf{\(1^C\) NMR} (75 MHz, d\(_6\)-DMSO) \(\delta\) 170.0, 170.0, 169.3 \((\text{COCH}_3)\), 158.1 \((C^6)\), 151.4 \((C^4-\text{NH}_2)\), 149.2 \((C^2)\), 85.9 \((C^1)\), 77.9 \((C^4)\), 72.6 \((C^3)\), 69.7 \((C^3)\), 69.1 \((C^5)\), 62.9 \((C^5)\), 20.5, 20.3, 20.2 \((\text{COCH}_3)\); \textbf{LRMS} \((\text{ES}^+)\): \(m/z\) : 949/951/953 \([2M + \text{Na}]^+\), 1:2:1, 52), 527/529 \([M + \text{CH}_3\text{CN} + \text{Na}]^+\), 1:1, 40), 495/497 (1:1, 100), 486/488 \([M + \text{Na}]^+\), 1:1, 99); \textbf{HRMS} \((\text{ES}^+)\): calcd for C\(_{15}\)H\(_{19}\)N\(_2\)O\(_6\)Br (M), [M + Na]\(^+\) = 486.0124/488.0104,
found 486.0114/488.0097 (1:1), [2M + Na]$^+ = 949.0351/951.0330/953.0310$, found 951.0254 (1:2:1).

5-Iodo-2',3',5'-tri-O-acetyl-6-oxocytidine, 83

![Chemical Structure](image)

To a stirred suspension of 2',3',5'-tri-O-acetyl-6-oxocytidine 75 (5.00 g, 13.0 mmol) and AIBN (46.3 mg, 0.28 mmol, 2.2 mol %) in distilled THF (50.0 mL) was added NIS (2.95 g, 13.1 mmol, 1.0 eq), and the reaction was stirred in absence of light, under an argon atmosphere, at rt for 15 minutes. The orange/brown slurry was concentrated *in vacuo* to yield an orange/brown solid. A portion of the solid (~0.5 g) was dissolved in ethyl acetate (250 mL) and washed with dilute aq Na$_2$S$_2$O$_3$ (100 mL, adjusted to pH 7 using NaHCO$_3$) until decolourised then separated immediately. The solution was further washed with sat. aq KCl (2 × 100 mL), dried (Na$_2$SO$_4$) and concentrated *in vacuo* to give an off-white solid (0.34 g). The remainder was suspended in ethyl acetate (550 mL) and allowed to stand in the fridge for 2 hours. The mixture was vacuum filtered, and the residue was washed with ice-cold ethyl acetate (3 × 150 mL) and dried under high vacuum over P$_2$O$_5$, to yield the product 83 as a white, powdery solid (5.47 g) containing 0.4 mol % succinimide (recalculated yield - 10.7 mmol, 82%).

R$_f$ 0.35 (1:1, acetone/toluene, A’), 0.54 (tetrahydrofuran, A’); IR (solid): ν 3400, 3295 (w, N–H), 3207 (br w, N–H), 1740, 1719 (s, C=O), 1624 (s, C=C), 1551 (s, N–H), 1422 (m), 1371 (s, CH$_3$), 1228 (vs), 1096, 1040 (s, C–O), 942, 931, 898 (m), 763 (s), 736 (m), 722 (m, CH$_2$), 677, 647, 630 (m), 602, 571, 537 (s), 512 (m, C–I) cm$^{-1}$; $^1$H NMR (300 MHz, d$_6$-DMSO) δ 10.91 (1H, br s, NH$_3$), 6.54 (2H, s, NH$_2$), 6.17 (1H, s, H$_1''), 5.61 (1H, dd, J = 1.8, 6.4 Hz, H$_4''$), 5.49 (1H, app. t, J = 7.2 Hz, H$_3''$), 4.33 (1H, dd, J = 2.7, 11.5 Hz, H$_5''$), 4.10 (1H, ddd, J = 2.9, 6.4, 8.0 Hz, H$_4'$), 4.02 (1H, dd, J = 6.2, 11.5 Hz, H$_5'$), 2.07, 2.03, 2.00 (3H, s, COCH$_3$); $^1$C NMR (100 MHz, d$_6$-DMSO) δ 170.0, 169.5, 169.3 (COCH$_3$), 159.3 (C$^6$), 153.3 (C$^4$–NH$_2$), 149.5 (C$^3$), 86.1 (C$'''$), 77.8 (C$^5''$), 72.7
(C²), 69.6 (C⁰), 62.8 (C⁵), 41.7 (C⁴-I), 20.5 (2'-OCOCH₃), 20.3 (3'-OCOCH₃), 20.2 (5'-OCOCH₃); LRMS (ES⁺): m/z: 1061 ([2M + K]⁺, 3), 1045 ([2M + Na]⁺, 8), 1023 ([M + K]⁺, 2), 550 ([M + K]⁺, 24), 534 ([M + Na]⁺, 100), 512 ([M + H]⁺, 77); (ES⁻): m/z (%): 1021 ([2M – H]⁻, 2), 608 ([M + H₃PO₄ – H]⁻, 4), 510 ([M – H]⁻, 100);


1-O-Methyl-3,5-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-D-ribofuranose, 86

Acetyl chloride (0.91 mL, 12.8 mmol, 0.1 eq) in distilled methanol (50 mL) was added portionwise to a stirred solution of D-ribose 84 (30.0 g, 200 mmol) in distilled methanol (200 mL). The reaction mixture was stirred at rt, under an argon atmosphere, for 22 hours. Sodium bicarbonate (10.1 g, 120 mmol, 0.6 eq) was added and the mixture was stirred for 10 minutes. The mixture was filtered and concentrated in vacuo to yield the crude product, 1'-O-methylribofuranoside 85 as an anomeric mixture (α:β ca 1:1) as a viscous, orange/red oil (38.3 g), which was used without further purification.

Rf 0.47, 0.32 (4:1, ethyl acetate/methanol, A’); LRMS (ES⁺): m/z (%): 187 ([M + Na]⁺, 100).

The crude product 85 (5.63 g) was co-evaporated with distilled pyridine and dried under high vacuum before re-dissolving in distilled pyridine (40 mL). The solution was cooled to 0 °C, under an argon atmosphere, and 1,3-dichloro-1,1,3,3-tetraisopropyl-disiloxane (13.2 mL, 41.3 mmol, 1.4 eq) was added dropwise. The reaction mixture was allowed to warm to rt and stirred for 15 hours, then concentrated under high vacuum, co-evaporating with toluene. The resulting cream slurry was partitioned between ethyl acetate (400 mL) and water (200 mL). The organic layer was washed with water (2 × 200 mL), 2 M aq HCl (200 mL) then water (200 mL), dried (Na₂SO₄) and concentrated in vacuo to yield a yellow oil. Following partial purification by
column chromatography (4:1, hexane/ethyl acetate), and drying under high vacuum the product 86 was afforded, as an anomeric mixture (α:β ca 6:1), as a very pale yellow viscous oil (15.1 g, 37.1 mmol, quant.).

R_f 0.47, 0.30 (4:1, hexane/ethyl acetate, A’), 0.74 (4:1, ethyl acetate/methanol, A’); 'H NMR (300 MHz, CDCl_3) δ signals due to α anomer 4.85 (1H, s, H^1), 4.70 (1H, br t, J = 5.3 Hz, H^3), 4.13-4.02 (1H, m, H^2), 3.99 (1H, dd, J = 4.9, 8.9 Hz, H^4), 3.85 (1H, dd, J = 2.5, 12.3 Hz, H^5), 3.69 (1H, dd, J = 2.2, 12.3 Hz, H^5), 3.40 (3H, s, Me), 2.75 (1H, br s, 2-OH), 1.11-0.80 (28H, m, CH(CH_3)_2), signals due to β anomer 4.87 (1H, d, J = 4.5 Hz, H^1), 4.50 (1H, br s, H^3), 4.29-4.16 (1H, m, H^4), 4.13-4.01 (2H, m, H^2, H^5), 3.78 (1H, br d, J = 10.6 Hz, H^5), 3.32 (3H, s, Me), 2.75 (1H, br s, 2-OH), 1.10-0.80 (28H, m, CH(CH_3)_2); LRMS (ES^+): m/z (%): 447 (100), 429 ([M + Na]^+, 50).

Analytical results consistent with reported data.37

1-O-Methyl-2-O-methylethanoyl-3,5-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-d-ribofuranose, 8737

![Diagram of the reaction](image)

Compound 86 (15.1 g, 37.1 mmol) was dissolved in anhydrous DMF, and cooled to −5 °C (ice/methanol) under an argon atmosphere. Methyl bromoacetate (18.0 mL, 190.2 mmol, 5.13 eq) was added followed by sodium hydride (60% w/w dispersion in mineral oil) (6.48 g, 162 mmol, 4.4 eq) portionwise. The reaction was stirred under an argon atmosphere at −5 °C for 15 minutes, then allowed to warm and stirred vigorously at rt for 17 hours. Saturated aq KCl (100 mL) was added carefully to the stirred reaction, and the mixture was extracted with diethyl ether (3 × 150 mL). The combined organic fraction was dried (Na_2SO_4) and concentrated under high vacuum, co-evaporating with toluene to yield a two-layered liquid (yellow/colourless). The crude compound was twice-purified by column chromatography (5-20% ethyl acetate/hexane) to yield, after drying under high vacuum, the product 87, as an anomeric mixture (α:β ca. 5:1), as a white solid (10.6 g, 22.1 mmol, 60% yield).
R, 0.33, 0.21 (4:1, hexane/ethyl acetate, A'); ¹H NMR (300 MHz, CDCl₃) δ signals due to α anomer 4.86 (1H, s, H¹), 4.48 (1H, dd, J = 4.2, 7.9 Hz, H³), 4.45 (1H, d, J = 16.7 Hz, H¹'), 4.32 (1H, d, J = 16.7 Hz, H¹'), 4.04 (1H, td, J = 2.9, 7.9 Hz, H⁴), 3.98 (1H, dd, J = 2.9, 12.1 Hz, H⁵), 3.86 (1H, dd, J = 5.9, 12.1 Hz, H⁵'), 3.83 (1H, d, J = 4.0 Hz, H²), 3.74 (2H, m, O-CH₃), 1.10-0.93 (28H, m, C₆H₁₃(CH₃)₂); ¹³C NMR (75 MHz, CDCl₃) δ signals due to α anomer 170.5 (C²'), 106.0 (C¹'), 83.3 (C²), 81.0 (C⁴), 74.2 (C³), 68.4 (C¹'), 63.9 (C⁵), 54.9 (1-OCH₃), 51.9 (CO₂CH₃), signals due to β anomer 171.0 (C²') other signals not visible, indistinguishable signals due to both anomers 17.6, 17.5, 17.5, 17.4, 17.2, 17.1 (Si–CH(CH₃)₂), 13.5, 13.4, 12.9, 12.8 (Si–CH(CH₃)₂); LRMS (ES⁺): m/z (%): 501 ([M + Na⁺], 100).

Analytical results consistent with reported data.³⁷

1-O-Methyl-2-O-(2-hydroxyethyl)-3,5-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-D-ribofuranose, 88³³³

![Reaction Scheme]

To a solution of compound 87 (9.13 g, 19.1 mmol) in distilled THF (80 mL), under an argon atmosphere, LiBH₄ (0.84 g, 38.5 mmol, 2.0 eq) was added portionwise and the reaction was stirred at rt. Further LiBH₄ (0.21 g, 9.6 mmol, 0.5 eq) was added after 55 minutes and the reaction was stirred for a further 20 minutes. The reaction mixture was poured into a conical flask, cooled in an ice bath and stirred vigorously. A solution of methanol (7 mL) in THF (50 mL) was added with extreme caution and the reaction mixture was left to stir for 20 minutes. Methanol (30 mL) was added with care, and the mixture was diluted with diethyl ether (150 mL). The solution was washed with water, dried (Na₂SO₄) and concentrated in vacuo to yield an orange oil. Following purification by column chromatography (14-20% ethyl acetate/hexane), and drying under high
vacuum, the product 88 was isolated as an anomeric mixture ($\alpha$:$\beta$ ca 1:3), as a very pale yellow to colourless oil (3.10 g, 6.87 mmol, 36% yield).

**Rt** 0.09 (4:1, hexane/ethyl acetate, A'); $^1$H NMR (300 MHz, CDCl$_3$) δ signals due to $\alpha$ anomer 4.77 (1H, s, H$^1$), 4.50 (1H, dd, $J = 4.5$, 7.8 Hz, H$^3$), 4.05-3.96 (1H, m, H$^4$), 4.00 (1H, dd, $J = 5.4$, 11.3 Hz, H$^5$), 3.91-3.79 (3H, m, H$^6$, H$^7$), 3.73-3.67 (2H, m, H$^8$), 3.54 (1H, d, $J = 7.7$ Hz, H$^9$), 3.32 (3H, s, OCH$_3$), 2.06 (1H, br s, OH), 1.10-1.00 (28H, m, CH(CH$_3$)$_2$), signals due to $\beta$ anomer 4.63 (1H, d, $J = 7.5$ Hz, H$^1$), 4.12 (1H, app. t, $J = 3.6$ Hz, H$^3$), 3.91-3.79 (1H, m, H$^5$), 3.91-3.73 (2H, m, H$^7$), 3.78-3.73 (2H, m, H$^8$), 3.75 (1H, t, $J = 4.8$ Hz, H$^9$), 3.73 (1H, dd, $J = 4.8$, 11.2 Hz, H$^9$), 3.66 (1H, dd, $J = 2.6$, 11.2 Hz, H$^9$), 3.47 (3H, s, OCH$_3$), 2.06 (1H, br s, OH), 1.10-1.00 (28H, m, CH(CH$_3$)$_2$); $^{13}$C NMR (75 MHz, CDCl$_3$) δ signals due to $\alpha$ anomer 106.1 (C$^1$), 83.8 (C$^2$), 79.4 (C$^4$), 73.2 (C$^5$), 71.8 (C$^3$), 66.2 (C$^4$), 62.7 (C$^5$), 56.5 (OCH$_3$), signals due to $\beta$ anomer 100.5 (C$^1$), 81.2 (C$^2$), 73.7 (C$^4$), 71.3 (C$^3$), 65.0 (C$^4$), 63.8 (C$^5$), 61.8 (C$^5$), 54.8 (OCH$_3$), indistinguishable signals due to both anomers 17.6, 17.6, 17.5, 17.4, 17.4, 17.1, 17.0 (Si–CH(CH$_3$)$_2$), 13.6, 13.4, 12.8, 12.7 (Si–CH(CH$_3$)$_2$); LRMS (ES$^+$): m/z (%): 473 ([M + Na]$^+$, 100).

Analytical results consistent with reported data. 37

1-O-Methyl-2-O-(2-phthalimidoethyl)-3,5-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-$\alpha$-D-ribofuranose, 89 37

To a solution of compound 88 (2.90 g, 6.43 mmol) in distilled THF (30 mL), under an argon atmosphere, triphenylphosphine (1.86 g, 7.07 mmol, 1.1 eq) and phthalimide (1.04 g, 7.07 mmol, 1.1 eq) were added, followed by DEAD (1.1 mL, 6.99 mmol, 1.1 eq) dropwise, whilst cooling in a water bath. The reaction mixture was then stirred under an argon atmosphere, at rt for 2 hours. The solvent was removed in vacuo and the crude material was eluted through a short silica plug (4:1, hexane/ethyl acetate). Following further purification by column chromatography (4:1, hexane/ethyl acetate),
and drying under high vacuum, the product α-anomer 89 was isolated as a cream-coloured solid (3.49 g, 6.0 mmol, 94% yield).

Rf 0.26 (89, α), 0.10 (β) (4:1, hexane/ethyl acetate, A'); 1H NMR (400 MHz, CDCl3) δ signals for α anomer 89 only 7.84 (2H, dd, J = 3.0, 5.5 Hz, H5'), 7.70 (2H, dd, J = 4.3, 7.8 Hz, H3'), 4.09 (1H, d, J = 4.7, 9.3 Hz, H1'), 3.98-3.90 (1H, m, H1'), 3.95-3.90 (1H, m, H4), 3.94 (1H, dd, J = 3.0, 12.0 Hz, H5), 3.87-3.80 (2H, m, H2'), 3.86 (1H, dd, J = 5.8, 12.3 Hz, H5), 3.73 (1H, d, J = 4.3 Hz, H2), 3.28 (3H, s, OCH3), 1.09-0.93 (28H, m, CH(CH3)2); 13C NMR (100 MHz, CDCl3) δ signals for α anomer 89 only 168.3 (C3'), 134.0 (C6'), 132.3 (C4'), 123.4 (C5'), 106.2 (C1'), 83.0 (C3'), 81.0 (C4'), 73.8 (C2'), 68.2 (C5'), 63.7 (OCH3), 54.8 (OCH3), 38.0 (C2'), 17.6, 17.5, 17.5, 17.5, 17.4, 17.2, 17.1 (Si–CH(CH3)2), 13.5, 13.4, 12.8, 12.8 (Si–CH(CH3)2); LRMS (ES+): m/z (%): 602 ([M + Na]+, 100).
Analytical results consistent with reported data.37

1,2,3,5-Tetra-O-acetyl-β-D-ribofuranose, 91249,250

1-O-Methylribofuranoside 85 (30.0 g, 183 mmol) was dissolved in acetic anhydride (105 mL) and glacial acetic acid (80 mL), to which was added conc. sulfuric acid (3.00 mL, 56.3 mmol, 0.3 eq) dropwise with ice-cooling. The deep red-brown solution was stirred at rt for 1 hour after which further conc. sulfuric acid (5.00 mL, 93.8 mmol, 0.5 eq) was added dropwise with ice-cooling. After stirring the solution for a further 2 hours at rt, sodium acetate (22.6 g, 275 mmol, 1.5 eq) was added, and the solution was stirred vigorously for 10 minutes. The solvent was removed under high vacuum, co-evaporating with ethanol/isopropanol, to yield a brown, opaque syrup. The syrup was dissolved in chloroform (1.5 L) and washed with water (2 × 400 mL) and sat. aq KCl (2 × 400 mL), dried (Na2SO4) and concentrated under high vacuum to afford a clear, brown syrup. To the syrup was added ice-cold ethanol (100 mL) and the mixture was cooled, from which crystallised the product. Following washing with ice-cold ethanol
(2 × 50 mL) and ice-cold diethyl ether (3 × 100 mL), and drying under high vacuum over KOH, the product β-anomer 91 was afforded, as a white, crystalline solid (22.6 g, 71.2 mmol, 39% yield). The remaining fractions and washings were dried under high vacuum to give a brown syrup, as an anomic mixture (α:β ca 65:35) (37.4 g) from which further product could not be crystallised.

Rf 0.47 (91, β), 0.40 (α) (2:1, ethyl acetate/hexane, A'), 0.30 (91, β), 0.26 (α) (1:1, ethyl acetate/hexane, A'); Mp 79-81 °C (ethanol), lit.249,250 81-83 °C (ethanol); IR (solid): ν 3025, 2992, 2961, 2932 (w, C–H), 1751 (s, C=O), 1740 (vs, C=O), 1445 (m), 1374 (m, C–H), 1215 (br vs, C–O), 1095, 1072, 1030 (m, C–O), 956, 913, 893, 883 (s), 815, 729, 679, 643, 621, 603, 589, 552, 536 (m) cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ signals for β anomer 91 only 6.15 (1H, s, H₁), 5.34 (1H, dd, J = 4.8, 8.1 Hz, H₃), 5.33 (1H, app. s, H₂), 4.40-4.33 (1H, m, H₄), 4.32 (1H, dd, J = 3.3, 12.1 Hz, H₅), 4.14 (1H, dd, J = 5.3, 11.9 Hz, H₅), 2.11 (3H, s, COC₃H₃), 2.08 (3H, s, COC₃H₃), 2.06 (3H, s, COC₃H₃); ¹³C NMR (75 MHz, CDCl₃) δ 170.5, 169.8, 169.5, 169.1 (C₂O, C=O), 98.3 (C₁), 79.4 (C₄), 74.3 (C₂), 70.7 (C₂), 63.8 (C₅), 21.1, 20.8, 20.6, 20.6 (COCH₃); LRMS (ES⁺): m/z (%): 659 ([2M + Na]⁺, 22), 382 ([M + CH₃CN + Na]⁺, 87), 341 ([M + Na]⁺, 100).

Analytical results consistent with reported data.250

5′-O-(4,4′-Dimethoxytrityl)-5-iodo-2′-deoxyuridine, 97²⁰⁹

5-Iodo-2′-deoxyuridine 148 (25.0 g, 70.6 mmol) was dried by co-evaporation with distilled pyridine (2 × 50 mL) under high vacuum, then dissolved in distilled pyridine (120 mL). 4,4′-Dimethoxytrityl chloride 112 (25.1 g, 74.1 mol, 1.1 eq) was added in five portions over 4 hours, under an argon atmosphere, whilst stirring vigorously, at rt. The reaction mixture was stirred for a further 3.5 hours cooling in ice, then quenched with methanol/water (1:1 v/v, 20 mL) stirring for 10 minutes at rt. The reaction mixture
was concentrated in vacuo and by co-evaporation with methanol/toluene. The foam/syrup was dissolved in CH$_2$Cl$_2$ (400 mL), washed with water (2 × 200 mL) during which unreacted material 148 precipitated. The aqueous was filtered and the precipitate was washed with water (50 mL), CH$_2$Cl$_2$ (150 mL), ethanol (30 mL) and diethyl ether (2 × 60 mL) and dried over P$_2$O$_5$ to give 148 as a white powder (2.41 g, 6.82 mmol, 10%). The combined aqueous fraction was re-extracted (CH$_2$Cl$_2$, 150 mL) and combined CH$_2$Cl$_2$ layers were washed with sat aq KCl (3 × 150 mL), dried (Na$_2$SO$_4$), and concentrated in vacuo and dried under high vacuum to give a yellow foam. Following purification by column chromatography (50-95% ethyl acetate/hexane + 0.5% Et$_3$N → 0.5-1.0% methanol/ethyl acetate + 0.5% Et$_3$N → 10-15% methanol/ethyl acetate + 0.5-1.0% Et$_3$N), and drying under high vacuum, the desired mono-tritylated product 97, the bis-tritylated by-product, and a mixture thereof, were afforded as a white foam (37.1 g, 56.5 mmol, 80%), a yellow foam/solid (3.41 g, 3.56 mmol, 5%) and a pale yellow foam (2.19 g) respectively.

R$_f$ 0.19 (97), 0.58 (bis) (5:95 methanol/CH$_2$Cl$_2$ + 0.3% Et$_3$N, A'); 0.39 (97), 0.66 (bis) (1:9 methanol/CH$_2$Cl$_2$ + 0.3% Et$_3$N, A'); 0.29 (1:9 acetone/CH$_2$Cl$_2$ + 0.7% pyridine, A'); $^1$H NMR (400 MHz, $d_6$-DMSO) $\delta$ 11.67 (1H, NH$_3$), 8.01 (1H, s, H$_6$), 7.40 (2H, d, $J$ = 7.6 Hz, H$_{13}$), 7.32 (2H, t, $J$ = 7.3 Hz, H$_{14}$), 7.29 (4H, d, $J$ = 9.0 Hz, H$_9$), 7.23 (1H, t, $J$ = 7.2 Hz, H$_{18}$), 6.90 (4H, d, $J$ = 8.8 Hz, H$_{10}$), 6.10 (1H, t, $J$ = 6.8 Hz, H$_{1'}$), 5.29 (1H, br s, 3'-OH), 4.23 (1H, br s, H$_3'$), 3.90 (1H, dd, $J$ = 3.6, 7.5 Hz, H$_4'$), 3.74 (6H, s, OCH$_3$), 3.20 (1H, dd, $J$ = 4.8, 10.8 Hz, H$_5'$), 3.16 (1H, dd, $J$ = 3.3, 10.6 Hz, H$_5''$), 2.25 (1H, td, $J$ = 6.8, 13.6 Hz, H$_{2'}$), 2.17 (1H, ddd, $J$ = 3.4, 6.2, 13.3 Hz H$_2''$); $^{13}$C NMR (100 MHz, $d_6$-DMSO) $\delta$ 160.5 (C$_4$), 158.1 (C$_{11}$-OCH$_3$), 150.1 (C$_2$), 144.7 (C$_{12}$), 144.2 (C$_6$), 135.5, 135.4 (C$_8$), 129.7 (C$_9$), 127.9 (C$_{14}$), 127.6 (C$_{13}$), 126.7 (C$_{15}$), 113.3 (C$_{10}$), 85.8 (C$_7$Ar$_3$), 85.8 (C$_4$), 84.9 (C$_1'$), 70.5 (C$_3'$), 69.8 (C$_8'$-I), 63.7 (C$_8$), 55.0 (OCH$_3$), 39.8 (C$_2'$); LRMS (ES$^+$) m/z: 679 ([M + Na]$^+$, 100).

Analytical results consistent with reported data.$^{51,170,251}$
Nucleoside 79 (1.57 g, 3.37 mmol) was dissolved with heating in a 0.4 M solution of K$_2$CO$_3$ in methanol/water (4:1, v/v) (34.0 mL, 13.6 mmol K$_2$CO$_3$, 4.0 eq) over 2 minutes then stirred at rt. After 10 minutes, DOWEX 50Wx8-400 (pyridinium form) was added with stirring until the solution was neutralised (pH 12.0→7.0). The mixture was filtered, the DOWEX washed with methanol (2×20 mL), water (2×20 mL), and water/methanol (1:1 v/v, 40 mL) and the filtrate was concentrated *in vacuo* to yield a gum. The gum was suspended in ethanol (60 mL) and allowed to stand for 2 days in the fridge. The powder obtained after filtration, washing and drying was hygroscopic and was therefore treated again with DOWEX in methanol/water (1:1 v/v, 25 mL) solution to acidify (pH 8.0→6.0). The DOWEX was washed and filtrate was concentrated *in vacuo* as previously described. The solid was suspended in isopropanol (70 mL) and allowed to stand overnight in the fridge. Following vacuum filtration, the solid was washed with ice-cold isopropanol (10 mL) and ice-cold diethyl ether (2×25 mL) and dried under high vacuum over P$_2$O$_5$, to afford the desired product 99, as an off-white chalky solid (1.04 g, 3.06 mmol, 91%).

**R$_f$** 0.49 (3:1:1, n-BuOH:AcOH:water, A’); **IR** (solid): v 3306 (m, N–H), 3207 (m, O–H), 2979 (w, O–H), 2915, 2790 (w, C–H), 1716 (m, C=O), 1604 (vs, C=O), 1538 (s, C=C), 1468, 1444 (m, CH$_2$), 1372 (m, O–H), 1256, 1172 (w), 1107 (m, C–O), 1037 (s, C–O), 957, 902, 760, 741, 680 (m), 624 (s), 554 (s, C–Br) cm$^{-1}$; **$^1$H NMR** (400 MHz, $d_6$-DMSO) $\delta$ 10.80 (1H, br s, N$_2$H), 6.58 (2H, s, N$_2$H$_2$), 6.02 (1H, d, $J = 3.8$ Hz, H$_{1'}$), 4.96 (1H, br d, $J = 4.8$ Hz, 2′-OH), 4.77 (1H, br d, $J = 5.5$ Hz, 3′-OH), 4.53 (1H, br t, $J = 5.5$ Hz, 5′-OH), 4.46 (1H, br dd, $J = 4.8$, 9.6 Hz, H$^4$), 4.07 (1H, br dd, $J = 5.5$, 11.1 Hz, H$^5$), 3.65 (1H, td, $J = 3.3$, 6.0 Hz, H$^4$), 3.58 (1H, br td, $J = 3.6$, 11.5 Hz, H$^5$), 3.40 (1H, td, $J = 6.0$, 12.0 Hz, H$^5$); **$^{13}$C NMR** (100 MHz, $d_6$-DMSO) $\delta$ 158.8 (C$^6$), 151.1 (C$^3$–NH$_2$), 149.5 (C$^2$), 88.1 (C$^1$), 84.2 (C$^4$), 71.0 (C$^2$), 70.1 (C$^3$), 69.6 (C$^5$), 62.4 (C$^8$); **LRMS** (ES$^+$): $m/z$: 360/362 ([M + Na]$^+$, 1:1, 100); (ES$^-$): $m/z$: 336/338 ([M – H]$^-$, 1:1,
Nucleoside 83 (1.01 g, 1.98 mmol) was dissolved with heating in a 0.4 M solution of K$_2$CO$_3$ in methanol/water (4:1, v/v) (17.0 mL, 6.8 mmol K$_2$CO$_3$, 3.4 eq) over 1 minute then stirred at rt. After 20 minutes, DOWEX 50WX8-400 (pyridinium form) was added with stirring until the solution was slightly acidified (pH 11.0→5.5). The mixture was filtered, the DOWEX was washed with methanol (8 mL), water (8 mL), then methanol again (20 mL), and the filtrate was concentrated in vacuo to yield a white solid. The solid was suspended in ethanol (100 mL) and allowed to stand in the fridge for 2.5 hours. Following vacuum filtration the residue was washed with the filtrate, ethanol (30 mL) and diethyl ether (2 × 20 mL), and dried under high vacuum over P$_2$O$_5$, to afford the product 100 as a white, chalky solid (0.59 g, 1.53 mmol, 77%).

R$_f$ 0.56 (3:1:1, n-BuOH:AcOH:water, A’); IR (solid): ν 3368 (br m, N–H, O–H), 3297, 3191 (br m, O–H), 2923 (w, C–H), 1703, 1614 (s, C=O), 1542 (s, C=C), 1422 (s), 1368 (m, O–H), 1102, 1037 (s, C=O), 982, 904, 850 (m), 758, 706, 676, 601 (s), 564 (vs), 524 (s, C–I) cm$^{-1}$; $^1$H NMR (300 MHz, d$_6$-DMSO) δ 10.73 (1H, br s, NH$_3$), 6.42 (2H, s, NH$_2$), 6.03 (1H, d, J = 3.8 Hz, H$^{1'}$), 4.96 (1H, br d, J = 4.9 Hz, 2’-OH), 4.77 (1H, br d, J = 5.7 Hz, 3’-OH), 4.53 (1H, br t, J = 5.4 Hz, 5’-OH), 4.44 (1H, br dd, J = 4.9, 9.8 Hz, H$^{3'}$), 4.06 (1H, br dd, J = 5.6, 11.3 Hz, H$^{3'}$), 3.65 (1H, td, J = 3.5, 6.2 Hz, H$^{4'}$), 3.58 (1H, br td, J = 3.7, 11.6 Hz, H$^{5'}$), 3.40 (1H, td, J = 5.9, 11.8 Hz, H$^{6'}$); $^{13}$C NMR (100 MHz, d$_6$-DMSO) δ 160.0 (C$^6$), 153.1 (C$^4$-NH$_2$), 150.0 (C$^2$), 88.3 (C$^1$), 84.2 (C$^4$), 71.0 (C$^2$), 70.1 (C$^3$), 62.4 (C$^5$), 42.3 (C$^6$); LRMS (ES$^+$): m/z : 430 ([M – H + 2Na]$^+$, 26), 408 ([M + Na]$^+$, 100); (ES$^-$): m/z : 384 ([M – H$^-$], 100); HRMS (ES$^+$): calcd for C$_9$H$_{12}$N$_3$O$_6$I (M),

6-Oxocytidine, 101

Nucleoside 75 (1.55 g, 4.03 mmol) was dissolved with heating in a 0.4M solution of K$_2$CO$_3$ in methanol/water (4:1 v/v) (40.0 mL, 16.0 mmol K$_2$CO$_3$, 4.0 eq) over 3 minutes, then stirred at rt for 5 minutes. DOWEX 50WX8-400 (pyridinium form) was added with stirring until the solution was slightly acidified (pH 12.0→5.5). The mixture was filtered, the DOWEX washed with the filtrate, methanol/water (1:1 v/v, 60 mL) then water (20 mL), and the combined filtrate was concentrated in vacuo, co-evaporating with methanol/toluene ($\times$5) to yield a sticky white foam. The residue was suspended in isopropanol (50 mL) and allowed to stand in the fridge overnight. The mixture was vacuum filtered, washed with ice-cold isopropanol (25 mL) and diethyl ether (2×50 mL), and dried under high vacuum over P$_2$O$_5$, to afford the product 101 as a powdery white solid (0.91 g) containing 37.7 mol % potassium acetate (recalculated yield - 2.52 mmol, 71%).

R$_f$ 0.13 (8:2:1, CH$_2$Cl$_2$/methanol/acetic acid, A'), 0.11 (1:4, methanol/CH$_2$Cl$_2$, A'); IR (solid): ν 3334 (br w, O–H, N–H), 3191 (br w, O–H), 1704 (m, C=O), 1603 (s, C=O), 1404 (br m, O–H), 1290 (m), 1200 (w), 1098 (m), 1020 (br s, C–O), 896, 781, 768 (m), 640 (br s), 533 (vs) cm$^{-1}$; $^1$H NMR (400 MHz, $d_6$-DMSO) δ 7.18 (2H, br s, NH$_2$), 6.06 (1H, d, $J = 4.0$ Hz, H$^1$), 4.47 (1H, dd, $J = 4.3$, 5.8 Hz, H$^2$), 4.44 (1H, s, H$^5$), 4.08 (1H, t, $J = 6.0$ Hz, H$^3$), 3.65 (1H, td, $J = 2.8$, 8.5 Hz, H$^4$), 3.56 (1H, dd, $J = 2.9$, 11.7 Hz, H$^5$), 3.40 (1H, dd, $J = 5.4$, 11.7 Hz, H$^6$); $^{13}$C NMR (100 MHz, $d_6$-DMSO) δ 163.3 (C$^6$), 156.8 (C$^4$–NH$_2$), 152.7 (C$^2$), 86.8 (C$^1$), 84.1 (C$^4$), 73.7 (C$^5$), 71.1 (C$^2$), 70.3 (C$^3$), 62.5 (C$^5$); LRMS (ES$^+$) m/z: 282 ([M + Na]$^+$, 100); (ES$^-$) m/z: 356 ([M + H$_3$PO$_4$ – H]$^-$, 53), 258 ([M – H]$^-$, 100); HRMS (ES$^+$): calcd for C$_9$H$_{13}$N$_3$O$_6$ (M), [3M + Na]$^+$ = 800.2311,
found 800.2285, [2M + Na]⁺ = 541.1506, found 541.1497, [M + Na]⁺ = 282.0697, found 282.0700.

Analytical results consistent with reported data.\(^{71,248}\)

5-Chloro-6-oxocytidine, 108

Nucleoside 109 (1.42 g, 3.38 mmol) was dissolved with heating in a 0.4 M solution of K₂CO₃ in methanol/water (4:1 v/v) (33.9 mL, 13.5 mmol K₂CO₃, 4.0 eq) over 3 minutes then stirred at rt. After 5 minutes, DOWEX 50WX8-400 (pyridinium form) was added with stirring, until the solution was slightly acidified (pH 12.0 → 5.5). The mixture was filtered, the DOWEX washed with methanol/water (1:1 v/v, 2 × 50 mL) then water (50 mL), and the combined filtrate was concentrated in vacuo to dryness. The residue was suspended in ethyl acetate (60 mL) and allowed to stand in the fridge overnight. The mixture was vacuum filtered, washed with ethyl acetate (30 mL), ethyl acetate/isopropanol (2:1 v/v, 30 mL) and diethyl ether (2 × 30 mL), and dried under high vacuum over P₂O₅, to afford a powdery white solid (1.24 g).

The solid was retreated with 0.4 M solution of K₂CO₃ in methanol/water (4:1 v/v) (12.7 mL, 5.08 mmol K₂CO₃, 1.5 eq) for 15 minutes, then DOWEX 50WX8-400 (pyridinium form), and concentrated in vacuo to dryness. Following suspension in isopropanol (35 mL) overnight, filtration, washing and drying under high vacuum, over P₂O₅, the product 108 was afforded as a white powdery solid (1.07 g) containing 35.6 mol % potassium acetate and 1.8 mol % succinimide (recalculated yield - 3.05 mmol, 90%).

**Rf** 0.52 (3:1:1, n-propanol/conc. aq NH₃/water, A’); **IR** (solid) ν 3306 (br w, O–H), 3213 (br w, O–H), 2980 (br w, C–H), 1714 (m, C=O), 1613 (br vs, C=O), 1434 (m), 1322, 1267 (w), 1113 (s), 1051, 1023 (s, C–O), 962, 897, 861 (m), 756 (s), 658 (s, C–Cl), 557 (s) cm⁻¹; **¹H NMR** (400 MHz, d₀-DMSO) δ 6.69 (2H, v br s, NH₂), 6.12 (1H, d,
$J = 4.3\ Hz, H^1$, $4.73\ (3H,\ extremely\ br\ s, OH), 4.53\ (1H,\ dd,\ J = 4.5, 6.0\ Hz, H^2), 4.13\ (1H, t, J = 5.8\ Hz, H^3), 3.67\ (1H,\ dt, J = 2.8, 4.9\ Hz, H^4), 3.56\ (1H,\ dd,\ J = 2.6, 11.7\ Hz, H^5), 3.41\ (1H,\ dd,\ J = 4.2, 11.6\ Hz); ^13C\ NMR\ (100\ MHz, d_6-DMSO) \delta\ 159.8\ (C^6), 159.2\ (C^4–NH_2), 154.0\ (C^2), 87.9\ (C^1'), 84.3\ (C^4'), 80.5\ (C^5), 71.1\ (C^3'), 70.4\ (C^2'), 62.5\ (C^5'); \ LRMS\ (ES^+\ m/z): 316/318\ ([M + Na]^+, 3:1, 100); (ES^-\ m/z): 585/587/589\ ([2M – H]^-, 3:2:1, 9). 292/294\ ([M – H]^-, 3:1, 100); \ HRMS\ (ES^+): calcd for C_{15}H_{18}N_3O_9Cl (M), [2M + Na]^+ = 609.0721/611.0692, found 609.0702 (2:1), [M + Na]^+ = 316.0307/318.0277, found 316.0298 (3:1).

5-Chloro-2',3',5'-tri-O-acetyl-6-oxocytidine, 109

**Method 1:** To a stirred solution of 2',3',5'-tri-O-acetyl-6-oxocytidine 75 (0.39 g, 1.00 mmol) in CH_2Cl_2 (7 mL) and methanol (5 mL), was added sodium bicarbonate (0.25 g, 3.02 mmol, 3.0 eq), followed by a solution of iodinating agent benzyltriethylammonium dichloroiodate 71 (0.59 g, 1.51 mmol, 1.5 eq) in CH_2Cl_2 (8 mL), and the reaction was stirred vigorously at rt for 30 minutes. The reaction mixture was filtered and concentrated in vacuo to yield a brown gum. The residue was redissolved in CH_2Cl_2 (40 mL), washed with sat. aq sodium thiosulfate (30 mL) and brine (2 x 40 mL), dried (Na_2SO_4) and concentrated in vacuo to yield a yellow foam (466 mg). The compound was twice purified by column chromatography (0-5% methanol/CH_2Cl_2) to afford, after drying under high vacuum, the undesired chlorinated product 109 as a white foam (0.11 g, 0.26 mmol, 26% yield).

**Method 2:** To a stirred suspension of 2',3',5'-tri-O-acetyl-6-oxocytidine 75 (1.52 g, 3.95 mmol) and AIBN (13.3 mg, 0.08 mmol, 2.1 mol %) in distilled THF (15.0 mL), was added NCS (0.56 g, 4.15 mmol, 1.1 eq), and the reaction was stirred in absence of light, under an argon atmosphere, at rt for 18 hours. The thick white slurry was dissolved in acetone/methanol (1:1 v/v, 100 mL), and concentrated in vacuo to dryness.
Following purification by column chromatography (40-60% acetone/toluene), the desired product **109** was afforded as a white powdery solid (1.64 g) containing 29.6 mol % succinimide (recalculated yield - 3.57 mmol, 90%).

**RF** 0.37 (5:95, methanol/CH2Cl2, A'), 0.36 (1:1, acetone/toluene, A'); **IR** (solid): v 3419, 3307 (w, N–H), 3198, 3140, 3087, 3024 (w), 2909 (w, C–H), 1741, 1718 (s, C=O), 1629 (s, N–H), 1559 (br s, N–H), 1430 (m), 1372 (s, C–H), 1228 (br vs, C–O), 1155 (m), 1044 (s, C–O), 939, 897, 766, 747 (m), 629 (C–Cl), 601 (m) cm⁻¹; **1H NMR** (400 MHz, d₆-DMSO) δ 10.96 (2H, br s, NH₂), 6.15 (1H, br s, H¹'), 5.64 (1H, dd, J = 2.5, 6.5 Hz, H²'), 4.03 (1H, dd, J = 6.3, 11.8 Hz, H⁵'), 2.07 (3H, s, CH₃), 2.03 (3H, s, CH₃), 2.00 (3H, s, CH₃); **13C NMR** (100 MHz, d₆-DMSO) δ 170.0, 169.5, 169.3 (C=OCH₃), 158.0 (C₆), 150.5 (C₄–NH₂), 148.7 (C²), 85.6 (C¹'), 80.3 (C₄–Cl), 77.9 (C²'), 72.5 (C³'), 69.7 (C³), 62.9 (C⁴'), 20.4, 20.3, 20.2 (COCH₃); **LRMS** (ES⁺) m/z: 861/863/865 ([2M + Na]+, 6:5:2, 11), 442/444 ([M + Na]+, 3:1, 100), 420/422 ([M + H]⁺, 3:1, 26); (ES⁻) m/z: 418/420 ([M – H]⁻, 3:1, 100); **HRMS** (ES⁺): calcld for C₁₅H₁₈N₃O₉Cl (M), [2M + Na]+ = 861.1355/863.1326/865.1296, found 861.1374 (6:4:1), [M + Na]+ = 442.0624/444.0594, found 442.0627 (3:1).

**N-(2-aminoethyl)-bis-N,N-[2-(trifluoroacetamido)ethyl]amine, 110**

To a stirred solution of tris(2-aminoethyl)amine **103** (0.50 mL, 3.34 mmol) in distilled methanol (4.0 mL) and distilled Et₃N (0.51 mL, 3.66 mmol, 1.1 eq), under an argon atmosphere, at −5 °C, was added ethyl trifluoroacetate (0.80 mL, 6.72 mmol, 2.0 eq) dropwise over 30 minutes. The mixture was stirred for a further 2.5 hours, then concentrated in vacuo and dried under high vacuum co-evaporating with methanol/toluene. Following purification by column chromatography (20-40% acetone/ethyl acetate + 0.5% Et₃N), bis-trifluoroacetamide **110** was afforded, following drying under high vacuum, as a viscous, pale yellow oil (422 mg, 1.25 mmol, 37%).
**Bis-N,N-[2-(trifluoroacetamido)ethyl]ammonium trifluoroacetate, 111<sup>182,192</sup>**

To a stirred solution of diethylene triamine 127 (3.20 mL, 29.6 mmol) in HPLC grade acetonitrile (50.0 mL), was added distilled water (0.64 mL, 35.5 mmol, 1.2 eq), followed by slow addition of ethyl trifluoroacetate (12.3 mL, 104 mmol, 3.5 eq). The solution was heated at reflux, under argon pressure at 105 °C for 16 hours. The reaction mixture was concentrated in vacuo and dried under high vacuum co-evaporating with methanol (2 × 50 mL). The syrup was soaked in CH<sub>2</sub>Cl<sub>2</sub> (100 mL) overnight, then suspended and the resulting ‘gel’ was filtered. The solid was washed with CH<sub>2</sub>Cl<sub>2</sub> (2 × 100 mL) and dried under high vacuum over P<sub>2</sub>O<sub>5</sub>, to afford the product salt 111 as an off-white, papery solid (11.3 g, 27.6 mmol, 93%).

**R:<sup>o</sup> 0.21 (2:3, acetone/ethyl acetate + 0.3% Et<sub>3</sub>N, D’), 0.15 (1:9, methanol/CH<sub>2</sub>Cl<sub>2</sub> + 0.3% Et<sub>3</sub>N, D’), <sup>1</sup>H NMR (300 MHz, <sup>δ</sup> 9.12 (2H, extremely br s, NH), 3.25 (4H, t, <sup>J</sup> = 6.4 Hz, H<sup>3</sup>), 3.18 (2H, app. t, <sup>J</sup> = 7.0 Hz, H<sup>2</sup>), 2.65 (4H, q, <sup>J</sup> = 6.6 Hz, H<sup>4</sup>), 2.59 (2H, t, <sup>J</sup> = 6.6 Hz, H<sup>1</sup>), 1.88 (2H, t, <sup>J</sup> = 1.3 Hz, NH<sub>2</sub>); LRMS (ES<sup>+</sup>) m/z: 379 (100), 339 ([M + H]<sup>+</sup>, 13); HRMS (ES<sup>+</sup>): calcd for C<sub>16</sub>H<sub>16</sub>N<sub>4</sub>O<sub>2</sub>F<sub>6</sub> (M), [M + H]<sup>+</sup> = 339.1250, found 339.1258. Found also 379.1568.**
To a stirred solution of tris(2-aminoethyl)amine (TAEA) 103 (1.79 mL, 11.9 mmol, 7.0 eq) in distilled CH₂Cl₂ (4.0 mL), under an argon atmosphere at 0 °C, was added very slowly dropwise in portions, a solution of MMTCl 115 (0.53 g, 1.70 mmol) in distilled pyridine (3.5 mL), over 5.5 hours. The reaction was quenched with methanol (2 mL), stirring for 5 minutes, then concentrated in vacuo, co-evaporating with CH₂Cl₂ (×5) then under high vacuum, co-evaporating with methanol/toluene (×3). The wet bottle-green powder was washed with hexane (2 × 50 mL) and CH₂Cl₂ (4 × 25 mL). The resulting blue solid was washed a third time with CH₂Cl₂ (4 × 25 mL) and all combined organic fractions were concentrated in vacuo and under high vacuum to give a brown/green oil. The oil was dissolved in CH₂Cl₂ (100 mL), washed with water (100 mL), the aqueous was re-extracted (CH₂Cl₂, 3 × 50 mL), and the organic fraction was dried (Na₂SO₄), and concentrated in vacuo and under high vacuum, to afford the crude mono-MMT protected polyamine, as a pale bottle green gum. (0.61 g).

Rᵣ 0.35 (5:1:1, ethyl acetate/methanol/aq NH₃, C’);

To a stirred solution of the green resin (0.40 g) in distilled CH₂Cl₂ (5.0 mL) and distilled Et₃N (5.00 mL), under an argon atmosphere, at rt, was added, ethyl trifluoroacetate (1.15 mL, 9.66 mmol, 10.0 eq) dropwise, and the reaction was stirred for 3 hours. The reaction was diluted with CH₂Cl₂ (100 mL), washed with water (2 × 50 mL), the aqueous was re-extracted (CH₂Cl₂, 50 mL). The combined organic fraction was washed with sat. aq KCl (50 mL), dried (Na₂SO₄) and concentrated in vacuo to give a
yellow/brown syrup. The compound was twice-purified by column chromatography (1% methanol/CH\textsubscript{2}Cl\textsubscript{2} + 0.5% Et\textsubscript{3}N; toluene + 0.5% Et\textsubscript{3}N → 1.5% methanol/CH\textsubscript{2}Cl\textsubscript{2} + 0.5% Et\textsubscript{3}N) to afford the product 116 as a pale yellow foam (0.14 g, 0.23 mmol, 20% over two steps).

R\textsubscript{f} 0.53 (3% methanol/CH\textsubscript{2}Cl\textsubscript{2} + 0.3% Et\textsubscript{3}N, C’, E’); IR (solid) ν 3303 (br w, water), 3057 (br w, N–H), 2952, 2837 (br w, C–H), 1703 (s, C=O), 1607, 1556 (w, Aryl C–H), 1508 (m, Aryl C–H), 1448 (m), 1297 (w), 1248 (m), 1206 (s, C–F), 1148 (br vs, C–F), 1032 (m), 903 (s, Aryl C–H), 703 (s, Aryl C–H), 583 (m) cm\textsuperscript{-1}; \textsuperscript{19}F NMR (282 MHz, d\textsubscript{6}-DMSO) δ -74.1 (COCF\textsubscript{3}); \textsuperscript{1}H NMR (400 MHz, d\textsubscript{6}-DMSO) δ 9.20 (1H, br s, NHCOCF\textsubscript{3}), 7.38 (4H, d, J = 8.0 Hz, H\textsubscript{4}), 7.29-7.23 (2H, m, H\textsubscript{3}), 7.27 (4H, d, J = 8.5 Hz, H\textsubscript{8}), 7.16 (2H, t, J = 7.3 Hz, H\textsubscript{11}), 6.83 (2H, d, J = 9.0 Hz, H\textsubscript{4}), 3.71 (3H, s, OCH\textsubscript{3}), 3.31 (1H, s, NHMMT), 3.19 (4H, br dd, J = 6.3, 10.8 Hz, H\textsubscript{2}), 2.58 (2H, t, J = 6.5 Hz, H\textsubscript{6}), 2.48 (4H, t, J = 6.5 Hz, H\textsubscript{1}), 2.07 (2H, dd, J = 6.0, 12.6 Hz, H\textsubscript{13}); \textsuperscript{13}C NMR (100 MHz, d\textsubscript{6}-DMSO) δ 157.3 (C\textsubscript{15}), 156.2 (q, J = 36.0 Hz, COCF\textsubscript{3}), 146.5 (C\textsubscript{8}), 138.1 (C\textsubscript{12}), 129.5 (C\textsubscript{13}), 128.2 (C\textsubscript{8}), 127.6 (C\textsubscript{10}), 125.9 (C\textsubscript{11}), 115.9 (q, J = 288.2 Hz, COCF\textsubscript{3}), 113.0 (C\textsubscript{14}), 69.7 (C\textsubscript{7}Ar\textsubscript{1}), 54.9 (OCH\textsubscript{3}), 54.0 (C\textsubscript{6}), 52.1 (C\textsubscript{1}), 41.1 (C\textsubscript{5}), 37.3 (C\textsubscript{2}); LRMS (ES\textsuperscript{+}) m/z: 723 ([M + F\textsubscript{3}CO\textsubscript{2}H – H\textsuperscript{+}], 38), 672 (21), 609 ([M – H\textsuperscript{+}], 13), 145 (100); HRMS (ES\textsuperscript{+}): calcd for C\textsubscript{30}H\textsubscript{32}N\textsubscript{6}O\textsubscript{5}F\textsubscript{8} (M), [M + Na\textsuperscript{+}] = 633.2271, found 633.2274.

Bis-N,N-[2-(trifluoroacetamido)ethyl]amine, 128\textsuperscript{104,232}

\[
\text{H}_2\text{N} \quad \text{H} \quad \text{NH}_2 \\
\text{C}_2\text{H}_8\text{N}_4\text{H}_2
\]

\[
\begin{array}{c}
\text{F}_2\text{C} \quad \text{O} \quad (2.3 \text{ eq}) \\
\text{CH}_2\text{Cl}_2 \text{(distilled), } 5^\circ\text{C, argon}
\end{array}
\]

To a stirred solution of diethylene triamine 127 (2.00 mL, 18.5 mmol) in distilled CH\textsubscript{2}Cl\textsubscript{2} (40.0 mL), and distilled Et\textsubscript{3}N (6.45 mL, 46.3 mmol, 2.5 eq), under an argon atmosphere, at \textasciitilde 5 °C, ethyl trifluoroacetate (5.07 mL, 42.6 mmol, 2.3 eq), was added dropwise over 1 hour. After stirring for a further 1¼ hours, the reaction mixture was concentrated in vacuo and redissolved in argon-degassed CH\textsubscript{2}Cl\textsubscript{2} (100 mL). The solution was washed with degassed sat. aq KCl (2 × 50 mL), and the combined aqueous fraction was re-extracted (degassed CH\textsubscript{2}Cl\textsubscript{2}, 2 × 30 mL). The combined CH\textsubscript{2}Cl\textsubscript{2};
fractions were dried (Na₂SO₄), concentrated in vacuo and dried under high vacuum, to afford the product bis-trifluoroacetamide 128, as an off-white, slightly air-sensitive solid (5.13 g, 17.4 mmol, 94%).

\[ \text{IR (solid): } \nu 3287 \text{ (w, N–H), 2945, 2850 (w, C–H), 1704 (s, C=O), 1563 (m, N–H, C=O), 1435 (m, C–H), 1349 (w), 1183, 1140 (br vs, C–F), 950 (w), 866, 843 (m), 724 (s, CH₂), 681 (br m, C–F) cm}^{-1}; \]

\[ \text{19F NMR (282 MHz, } d_6\text{-DMSO) } \delta -74.1 \text{ (COCF}_3 \text{)}; \]

\[ \text{1H NMR (400 MHz, } d_6\text{-DMSO) } \delta 9.25 \text{ (2H, v br s, N} \text{H} \text{COCF}_3 \text{)}, 3.24 \text{ (4H, t, } J = 6.4 \text{ Hz, } H_2 \text{)}, 2.64 \text{ (4H, t, } J = 6.5 \text{ Hz, } H_1 \text{);} \]

\[ \text{13C NMR (100 MHz, } d_6\text{-DMSO) } \delta 156.3 \text{ (q, } J = 36.0 \text{ Hz, NHCO} \text{C} \text{OCF}_3 \text{)}, 115.9 \text{ (q, } J = 287.7 \text{ Hz, NHCO} \text{C} \text{OF}_3 \text{)}, 47.1 \text{ (C}1 \text{)}, 39.3 \text{ (C}2 \text{);} \]

\[ \text{LRMS (ES}^+\text{) m/z: 296 ([M + H] }^+\text{, 100); (ES}^+\text{) m/z: 408 ([M + CF}_3\text{CO}_2\text{H – H} ]^+\text{, 20), 392 ([M + H}_2\text{PO}_4\text{ – H} ]^+\text{, 100), 294 ([M – H] }^+\text{, 77); HRMS (ES}^+\text{): calcd for } \text{C}_8\text{H}_11\text{N}_3\text{O}_2\text{F}_6 (M), [M + Na}^+ = 318.0648, \text{ found } 318.0672; [M + H}^+ = 296.0828, \text{ found } 296.0830. \]

Analytical results consistent with reported data.²⁵²

2,2’-Anhydro-6-oxocytidine and 6,2’-anhydro-6-oxocytidine, 141,142

To a stirred solution of 6-oxocytidine 101 (0.20 g, 0.60 mmol) in anhydrous DMF (2.50 mL), under an argon atmosphere, was added diphenyl carbonate (0.14 g, 0.66 mmol, 1.1 eq) and sodium bicarbonate (3.0 mg, 0.04 mmol, 6.0 mol %) and the mixture was stirred at 100 °C for 3 hours. The reaction mixture was allowed to cool to rt, then diethyl ether (35 mL) was slowly added with rapid stirring, and the suspension was stirred for 30 minutes. The ether was decanted, and the precipitate was washed with ether (2 × 15 mL) and dried under high vacuum to afford the crude product as a hygroscopic, powdery solid. The solid was redissolved in water/methanol (2:1, v/v, 30 mL) and concentrated in vacuo and under high vacuum. The gum was redissolved in DMF (2 mL) and diethyl ether (25 mL) was added slowly with vigorous stirring. After stirring
for 30 minutes, the ether was decanted, the solid was washed with diethyl ether (2 × 75 mL) and dried in vacuo and under high vacuum to give the product as an isomeric mixture $\text{141,142} (\text{ca. 1:1})$, as an off-white chalky solid (0.14 g, 0.59 mmol, 98%).

$R_f$ 0.13, 0.23 (5:1:1, ethyl acetate/methanol/conc. aq NH$_3$, A'); LRMS (ES') $m/z$: 280 ([M + Na]$^+$, 24), 264 ([M + Na]$^+$, 100).

3-N-[2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-β-D-ribofuranosyl]-6-[3-(trifluoroacetamido)phenyl]-2,3-$H$-furano[2,3-$d$]pyrimidine-2-one-3'-O-(2-O-cyanoethyl-N,N-diisopropyl) phosphoramidite, 145$^{206}$

To a solution of nucleoside 155 (0.49 g, 0.66 mmol) in distilled THF (4.0 mL) and distilled DIPEA (0.29 mL, 1.67 mmol, 2.5 eq), strictly under an argon atmosphere and excluding moisture, was added chloro-phosphitylating reagent 70 (0.18 mL, 0.81 mmol, 1.2 eq) dropwise, and the reaction was stirred at rt for 80 minutes. The reaction was diluted with argon-degassed ethyl acetate (40 mL), then washed, under an argon atmosphere, with degassed sat aq KCl (3 × 10 mL). The organic fraction was dried (Na$_2$SO$_4$), the inorganics were washed with degassed ethyl acetate (3 × 10 mL) and the combined fraction was transferred under argon and concentrated in vacuo to give a pale yellow residue. Following purification by column chromatography (degassed 30% ethyl acetate/CH$_2$Cl$_2$ + 0.5% Et$_3$N), on silica gel pre-equilibrated with Et$_3$N, under argon pressure, and after drying under high vacuum, the product phosphoramidite 145 was afforded, as a diastereomeric mixture (ca. 3:2), as an air-sensitive, off-white foam (0.40 g, 0.42 mmol, 64%).
reaction was quenched with argon-degassed sat aq KCl (1 ml), diluted with degassed
mL, 0.36 mmol, 0.2 eq) was added and the reaction was stirred for 45 minutes. The
1.2 eq) dropwise, and the reaction was stirred at rt for 2 hours. Further reagent
excluding moisture, was added chloro-phosphitylating reagent
1.13 (3H, d, 1J = 1.5 Hz, H15), 7.71 (1H, td, 1J = 1.8, 7.0 Hz, H15), 7.55 (1H, br s, H16),
7.53 (1H, t, 1J = 7.7 Hz, H18), 7.42 and 7.40 (2H, td, 1J = 1.6, 7.1 Hz and 1J = 1.5, 6.6 Hz, H14), 7.36-7.19 (3H, m, H15, H16), 7.28 (4H, d, 1J = 8.8 Hz, H19), 6.91 and 6.90 (2H, d, 1J = 9.2 Hz and 1J = 8.8 Hz, H11), 6.90 and 6.89 (2H, d, 1J = 9.2 Hz, H14), 6.34 and 6.32 (1H, s, H8), 6.20 and 6.16 (1H, dd, 1J = 4.2, 6.8 Hz, and 1J = 4.0, 6.6 Hz, H9), 4.73-4.57 (1H, m, H3), 4.20 and 4.15 (1H, dd, 1J = 3.7, 8.6 Hz and 1J = 3.8, 8.5 Hz, H4), 3.81-3.68 and
3.74-3.60 (2H, m, H17), 3.71, 3.71 and 3.70 (3H, 3H and 6H, s, OCH3), 3.62-3.45 (2H, m, H18), 3.46-3.35 (2H, m, H15), 3.36-3.26 (2H, m, H16), 2.77 and 2.67 (2H, t, 1J = 5.9 Hz, H19), 2.69-2.55 (1H, m, H17), 2.55-2.38 (1H, m, H20), 1.16 (3H, d, 1J = 7.0 Hz, H28), 1.13 (3H, d, 1J = 7.0 Hz, H29), 1.12 (3H, d, 1J = 7.0 Hz, H28), 1.02 (3H, d, 1J = 6.6 Hz, H29); LRMS (ES+) m/z: 980 ([M + K]+, 41), 964 ([M + Na]+, 100), 942 ([M + H]+, 52).
Analytical results consistent with reported data.206

3-N-[2’-Deoxy-5’-O-(4,4’-dimethoxytrityl)-β-d-ribofuranosyl]-6-(3-ureidophenyl)-(2,3H)‐furano[2,3-d]pyrimidine-2-one-3’-O-(2-O-cyanoethyl-N,N-diisopropyl) phosphoramidite, 146

To a solution of nucleoside 160 (1.22 g, 1.77 mmol) in distilled THF (10.0 mL) and
distilled DIPEA (0.77 mL, 4.42 mmol, 2.5 eq), strictly under an argon atmosphere and
excluding moisture, was added chloro-phosphitylating reagent 70 (0.48 mL, 2.15 mmol, 1.2 eq) dropwise, and the reaction was stirred at rt for 2 hours. Further reagent 70 (0.08 mL, 0.36 mmol, 0.2 eq) was added and the reaction was stirred for 45 minutes. The
reaction was quenched with argon-degassed sat aq KCl (1 ml), diluted with degassed
ethyl acetate (60 mL), then washed, under an argon atmosphere, with degassed sat aq KCl (2 × 20 mL). The organic fraction was dried (Na₂SO₄), the inorganics were washed with degassed ethyl acetate (2 × 10 mL) and the combined organic fraction was transferred under argon and concentrated in vacuo to give a yellow gum. Following purification by column chromatography (degassed 65% acetone/CH₂Cl₂ + 1.0% Et₃N), on silica gel pre-equilibrated with Et₃N, under argon pressure, then precipitation at rt from distilled CH₂Cl₂ (3 mL) into degassed hexane (150 mL), under an argon atmosphere, the desired product 146 was afforded as a diastereomeric mixture (ca. 2:3), as an air-sensitive, powdery white solid (1.08 g, 1.22 mmol, 69%).

**R₉** 0.33 (65:35, acetone/CH₂Cl₂ + 0.3% Et₃N, A’), 0.44 (9:1, acetone/CH₂Cl₂ + 0.3% Et₃N, A’); **³¹P NMR** (121 MHz, d₆-DMSO) δ 149.3, 149.1 (P III), 15.1 (HO(H)P³(O)OCH₂CH₂CN); **¹H NMR** (400 MHz, d₆-DMSO) δ 8.75 and 8.74 (1H, s, H⁴), 7.90 (1H, br s, NHCONH₂), 7.42 and 7.40 (2H, d, J = 8.5 Hz and J = 9.0 Hz, H¹⁴), 7.38-7.33 (1H, m, H⁵), 7.34 (2H, d, J = 7.0 Hz, H¹⁵), 7.34-7.27 (2H, m, H¹⁶, H⁸), 7.30 and 7.27 (4H, d, J = 9.0 Hz, H¹⁰), 7.24 (1H, ddd, J = 1.8, 3.3, 6.5 Hz, H⁹), 6.91, 6.91 and 6.90 (2H, 2H and 4H, d, J = 9.0 Hz, H¹⁰), 6.21 and 6.17 (1H, dd, J = 4.5, 6.5 Hz, H¹), 6.20 and 6.18 (1H, s, H⁵), 5.93 (2H, br s, CONH₂), 4.69 and 4.65 (1H, td, J = 6.4, 13.1 Hz and J = 6.1, 12.5 Hz, H³), 4.19 and 4.15 (1H, td, J = 3.5, 5.0 Hz, H⁴), 3.81-3.70 and 3.71-3.62 (2H, m, H¹⁷), 3.72, 3.72 and 3.71 (3H, 3H and 6H, s, OCH₃), 3.63-3.47 (2H, m, H¹⁹), 3.46 and 3.43 (1H, dd, J = 3.4, 10.9 Hz and 3.4, 11.4 Hz, H⁹), 3.43-3.38 and 3.39 (1H, m and dd, J = 2.8, 12.1 Hz, H⁸), 2.77 and 2.67 (2H, t, J = 6.0 Hz, H¹⁸), 2.64 and 2.60 (1H, td, J = 6.4, 12.8 Hz and J = 6.3, 12.4 Hz, H²), 2.47-2.39 (1H, m, H²), 1.15 (3H, d, J = 6.8 Hz, H¹⁸b), 1.13 (3H, d, J = 6.8 Hz, H¹⁹b), 1.12 (3H, d, J = 6.8 Hz, H¹⁸b), 1.02 (3H, d, J = 6.8 Hz, H¹⁹b); **LRMS** (ES⁺) m/z: 927 ([M + K]⁺, 9), 911 ([M + Na]⁺, 28), 889 ([M + H]⁺, 17), 618 (100).
To a solution of nucleoside 163 (1.00 g, 1.46 mmol) in distilled THF (8.0 mL) and distilled DIPEA (0.64 mL, 3.67 mmol, 2.5 eq), strictly under an argon atmosphere and excluding moisture, was added chloro-phosphitylating reagent 70 (0.39 mL, 1.75 mmol, 1.2 eq) dropwise, and the reaction was stirred at rt for 2½ hours. The reaction was quenched with argon-degassed sat aq KCl (2 ml) stirring for 5 minutes, diluted with degassed ethyl acetate (100 mL), then washed, under an argon atmosphere, with degassed sat aq KCl (2 × 20 mL). The organic fraction was dried (Na₂SO₄), the inorganics were washed with degassed ethyl acetate (2 × 15 mL) and the combined fraction was transferred under argon and concentrated in vacuo to give a pale yellow foam. Following purification by column chromatography (degassed 10-60% acetone/CH₂Cl₂ + 0.5% Et₃N), on silica gel pre-equilibrated with Et₃N, under argon pressure, then precipitation twice from distilled CH₂Cl₂ (2 mL) into degassed hexane (200/250 mL) at –78 °C/rt, under an argon atmosphere, the desired phosphoramidite 147 was afforded as a diastereomeric mixture (ca. 2:3), as an air-sensitive, pale cream foam (0.89 g) containing 18.9 mol % hydrolysed phosphitylating reagent (recalculated yield - 0.97 mmol, 66%).

Rf 0.42, 0.50 (2:3, acetone/CH₂Cl₂ + 0.3% Et₃N, A’), 0.21, 0.30 (1:9, acetone/CH₂Cl₂ + 0.3% Et₃N, A’); ³¹P NMR (121 MHz, d₆-DMSO) δ 149.3, 149.0 (PⅢ), 15.1 (HO(H)PⅤ(O)OCH₂CH₂CN); ¹H NMR (400 MHz, d₆-DMSO) δ 10.10 (1H, s, NHCOCH₃), 8.76 and 8.75 (1H, s, H⁴), 8.02 (1H, s, H²”), 7.59 (1H, br d, J = 7.5 Hz, H⁴”), 7.44-7.40 (1H, m, H⁵”), 7.42-7.37 (2H, m, H¹⁴), 7.37-7.30 (2H, m, H¹⁵, H⁶”), 7.30 and 7.27 (4H, d, J = 8.5 Hz and J = 9.0 Hz, H¹⁰), 7.30-7.25 (1H, m, H¹⁶), 6.91, 6.91 and
6.90 (2H, 2H and 4H, d, J = 8.5 Hz, J = 9.0 Hz and J = 9.0 Hz, H\textsuperscript{11}), 6.25 and 6.23 (1H, s, H\textsuperscript{5}), 6.20 and 6.10 (1H, dd, J = 4.3, 6.8 Hz and J = 4.8, 6.3 Hz, H\textsuperscript{17}), 4.67 and 4.65 (1H, td, J = 6.2, 12.3 Hz and J = 6.0, 12.1 Hz, H\textsuperscript{3}), 4.19 and 4.15 (1H, td, J = 6.2, 12.1 Hz and J = 6.0, 12.1 Hz, H\textsuperscript{3}), 3.81-3.69 and 3.73-3.61 (2H, m, H\textsuperscript{17}), 3.72, 3.71 and 3.70 (3H, 3H and 6H, s, OCH\textsubscript{3}), 3.62-3.48 (2H, m, H\textsuperscript{19}), 3.49-3.35 (2H, m, H\textsuperscript{5\prime}), 2.76 and 2.67 (2H, t, J = 6.0 Hz, H\textsuperscript{18}), 2.62 and 2.61 (1H, td, J = 6.4, 12.7 Hz and J = 6.3, 12.6 Hz, H\textsuperscript{2\prime}), 2.43 and 2.42 (1H, ddd, J = 4.0, 6.8, 13.8 Hz and J = 3.0, 7.0, 13.6 Hz, H\textsuperscript{3}), 2.08 (3H, s, COCH\textsubscript{3}), 1.15 (3H, d, J = 7.0 Hz, H\textsuperscript{3\prime}), 1.13 (3H, d, J = 7.0 Hz, H\textsuperscript{3\prime}), 1.02 (3H, d, J = 7.0 Hz, H\textsuperscript{3\prime}); LRMS (ES\textsuperscript{+}) m/z: 910 ([M + Na]\textsuperscript{+}, 100), 888 ([M + H]\textsuperscript{+}, 4); (ES\textsuperscript{–}) m/z: 949 (32), 922/924 ([M + HCl – H]\textsuperscript{–}, 3:1, 33), 886 ([M – H]\textsuperscript{–}, 100).

4-(3-Aminophenyl)-2-methyl-but-3-yn-2-ol, 151\textsuperscript{206,253}

\[\begin{align*}
\text{NH}_2 & \quad \text{C}_2\text{H}_5\text{Ni} \\
\text{mol. wt.: 219.02} & \\
\text{149} & \\
\text{CuI (1 mol.%)} & \text{PPh}_3 (3 mol.%) \quad \text{[(Ph-P)]_2PdCl}_2 (3 mol.%) \\
\text{distilled Et}_3\text{N, 50 °C - reflux} & \\
\text{150} & \text{151} \\
\text{C}_11\text{H}_{13}\text{NO} & \text{mol. wt.: 178.23}
\end{align*}\]

To a solution of 3-iodoaniline 149 (1.00 g, 4.56 mmol) in distilled Et\textsubscript{3}N (1.5 mL) under an argon atmosphere, in absence of light, was added CuI (8.7 mg, 0.05 mmol, 1.0 mol %) then 2-methyl-but-3-yn-2-ol 150 (1.10 mL, 11.4 mmol, 2.5 eq), and the mixture was stirred at rt for 5 minutes. Triphenylphosphine (36.0 mg, 0.14 mmol, 3.0 mol %) was added and the reaction was stirred for a further 5 minutes. Bis(triphenylphosphine) palladium dichloride (96.2 mg, 0.14 mmol, 3.0 mol %) was added and the reaction was stirred at 50 °C for 20 minutes then refluxed for 50 minutes. The reaction mixture was cooled and concentrated under high vacuum, co-evaporating with methanol/toluene, then the brown syrupy solid was suspended in diethyl ether (70 mL) and allowed to stand in the fridge overnight. The solution was decanted and the solid washed again with diethyl ether (9 × 30 mL), followed by ice-cold ethyl acetate/diethyl ether (1:1 v/v, 100 mL). All fractions were combined, vacuum filtered and the solid was further washed with diethyl ether (30 mL). The combined fractions were concentrated in vacuo and redissolved in hot ethyl acetate (10 mL). The hot solution was added dropwise to diethyl ether at ~70 °C with vigorous stirring. The suspension was vacuum filtered, the
solid washed with very cold diethyl ether (150 mL) and the filtrate was concentrated in vacuo. The resultant syrup was dissolved in CH₂Cl₂ (70 mL) and washed with water (3 × 100 mL). The aqueous fraction was re-extracted (CH₂Cl₂, 200 mL), and combined CH₂Cl₂ fractions were washed with sat aq KCl (100 mL), dried (Na₂SO₄) and concentrated in vacuo to give an orange/brown gum. Following purification by column chromatography (0–30% CH₂Cl₂/diethyl ether), and drying under high vacuum, the product alkyne 151 was isolated as a pale yellow solid (0.43 g, 2.43 mmol, 53%).

R_f 0.29 (1:1, ethyl acetate/hexane, A’), 0.31 (1:1, CH₂Cl₂/diethyl ether, A’), 0.04 (CH₂Cl₂, A’), 0.36 (diethyl ether, A’); ¹H NMR (300 MHz, d₆-DMSO) δ 6.97 (1H, t, J = 7.9 Hz, H₅), 6.58 (1H, dd, J = 1.3, 2.0 Hz, H²), 6.53 (1H, ddd, J = 1.1, 2.6, 8.1 Hz, H₄), 6.50 (1H, td, J = 1.3, 7.4 Hz, H₆), 5.37 (1H, s, OH), 5.14 (2H, br s, NH₂), 1.44 (6H, s, CH₃); ¹³C NMR (75 MHz, d₆-DMSO) δ 148.7 (C₃–NH₂), 129.0 (C₅), 122.8 (C¹), 118.6 (C₆), 116.3 (C²), 114.0 (C⁴), 94.6 (ArC≡C₈R), 81.1 (ArC≡CR), 63.5 (C(CH₃)₂OH), 31.7 (CH₃); LRMS (ES⁺): m/z: 277 ([M + Et₃N + H]+, 10), 217 ([M + CH₃CN + H]+, 35), 176 ([M + H]+, 37), 102 ([Et₃N + H]+, 100).

Analytical results consistent with reported data.

3-Aminophenyl acetylene (or 3-ethynylaniline), 152

Potassium hydroxide (0.21 g, 3.80 mmol, 2.3 eq) was dissolved in dry, refluxing isopropanol (5 mL) with 4Å molecular sieves. Immediately on dissolution, protected alkyne 151 (0.29 g, 1.63 mmol) was added and the mixture was refluxed for 2½ hours. Further KOH (0.10 g, 1.82 mmol, 1.1 eq) was added and the reaction was refluxed for 1 hour. The mixture was cooled and concentrated to dryness under high vacuum at rt. Dichloromethane (40 mL) was added and the mixture was vacuum filtered. The residue was washed with CH₂Cl₂ (2 × 20 mL), and the filtrate was concentrated to dryness in vacuo and under high vacuum to give a tan-coloured solid. Following purification by column chromatography (0–10% diethyl ether/CH₂Cl₂), and drying under high vacuum,
the product alkyne 152 was afforded as a pale orange/brown oil (0.08 g, 0.64 mmol, 39%).

**Rf** 0.23 (CH₂Cl₂, A’), 0.47 (1:1, diethyl ether/CH₂Cl₂, A’), 0.68 (1:9, methanol/CH₂Cl₂, A’); **¹H NMR** (300 MHz, CDCl₃) δ 7.10 (1H, t, J = 8.1 Hz, H⁵), 6.90 (1H, td, J = 1.2, 7.7 Hz, H⁶), 6.81 (1H, dd, J = 1.5, 2.2 Hz, H²), 6.67 (1H, ddd, J = 1.1, 2.6, 8.1 Hz, H⁴), 3.60 (2H, v br s, 3-NH₂), 3.02 (1H, s, C≡CH₈); **¹³C NMR** (75 MHz, CDCl₃) δ 146.4 (C₃–NH₂), 129.4 (C₅), 122.9 (C₁), 122.6 (C⁶), 118.4 (C²), 115.9 (C⁴), 84.0 (C²≡CH), 76.6 (C≡CH₈); **LRMS** (EI⁺): calcd 177.1 (M⁺), found 117.0 (M⁺).

Analytical results consistent with reported data.

To a solution of 5’-O-(4,4’-dimethoxytrityl)-5-iodo-2'-deoxyuridine 97 (2.32 g, 3.53 mmol) in anhydrous DMF (20.0 mL), under an argon atmosphere, in absence of light, was added CuI (0.27 g, 1.41 mmol, 0.4 eq), distilled Et₃N (1.50 mL, 10.8 mmol, 3.1 eq) then 3-ethynylaniline 152 (1.19 mL, 10.6 mmol, 3.0 eq). The mixture was stirred for 10 minutes then *tetrakis*(triphenylphosphine) palladium (0) (0.41 g, 0.35 mmol, 0.1 eq) was added and the reaction was stirred at rt for 75 minutes. The reaction mixture was concentrated under high vacuum, co-evaporating with toluene to give a sticky dark brown gum. The residue was soaked in diethyl ether (2 × 50 mL), the ether was decanted, and residue was co-evaporated with toluene to yield a dark brown foamy resin. The crude material was twice-purified by column chromatography (0–15% methanol/CH₂Cl₂ + 1-5% Et₃N) to afford, and drying under high vacuum, the desired product 153 as an orange/brown glassy solid (2.09 g), containing 57 mol% Et₃N (recalculated yield - 2.67 mmol, 76%).
Analytical results consistent with reported data.

\[ \text{R}_f 0.35 \text{ (1:9, methanol/CH}_2\text{Cl}_2 + 0.3\% \text{ Et}_3\text{N, A')}; 0.35 \text{ (4:1, ethyl acetate/toluene + 0.3\% Et}_3\text{N, A').} \]

\[ \text{Mp 116-120 °C (CH}_2\text{Cl}_2); \text{ IR (solid): v 3342 (w, O-H, N-H), 3232 (w, O-H)} \]

\[ 3056, 2973, 2931, 2834 \text{ (w, C-H), 2171 (w, C=C), 1689 (br m, C=O), 1632 (s, N-H)} \]

\[ 1596 \text{ (s, C=C), 1507 (s, Aryl C=C), 1442 (C-H), 1385 (m, O-H), 1276 (s), 1246 (vs), 1174, 1087, 1030 (s, C-O), 943, 866 (m), 826 (s, C-H), 788 (s), 754, 688 (s, Aryl C-H), 583, 515 (s) cm}^{-1}; \]

\[ \text{¹H NMR (400 MHz, d}_6\text{-DMSO) } \delta 11.3 \text{ (1H, br s, NH}_3^3), 7.97 \text{ (1H, s, H}_8^6), 7.41 \text{ (2H, d, J = 7.5 Hz, H}_8^{15}), 7.32-7.26 \text{ (2H, m, H}_8^{16}), 7.30 \text{ (2H, d, J = 9.0 Hz, H}_8^{11}), 7.29 \text{ (2H, d, J = 9.0 Hz, H}_8^{11}), 7.18 \text{ (1H, br t, J = 7.3 Hz, H}_8^{17}), 6.95 \text{ (1H, t, J = 7.7 Hz, H}_8^8), 6.86 \text{ (2H, d, J = 9.0 Hz, H}_8^{12}), 6.84 \text{ (2H, d, J = 9.0 Hz, H}_8^{12}), 6.55 \text{ (1H, ddd, J = 0.6, 2.0, 8.0 Hz, H}_8^{15}), 6.52 \text{ (1H, app. t, J = 1.6 Hz, H}_8^{25}), 6.33 \text{ (1H, br d, J = 7.8 Hz, H}_8^{25}), 6.14 \text{ (1H, t, J = 6.8 Hz, H}_8^1), 5.31 \text{ (1H, br s, 3'-OH), 5.13 \text{ (2H, s, NH}_2), 4.28 \text{ (1H, td, J = 3.3, 60 Hz, H}_8^3), 3.95 \text{ (1H, td, J = 3.3, 4.8 Hz, H}_8^{14}), 3.68 \text{ (3H, s, OCH}_3), 3.67 \text{ (3H, s, OCH}_3), 3.24 \text{ (1H, dd, J = 5.3, 10.5 Hz, H}_8^5), 3.15 \text{ (1H, dd, J = 2.8, 10.5 Hz, H}_8^5), 2.30 \text{ (1H, td, J = 6.7, 13.6 Hz, H}_8^2), 2.22 \text{ (1H, ddd, J = 3.3, 6.3, 13.6 Hz, H}_8^5); \}

\[ \text{¹C NMR (100 MHz, d}_6\text{-DMSO) } \delta 161.4 \text{ (C}_6^6), 158.1, 158.0 \text{ (C}_6^{11}), 149.3 \text{ (C}_5^3), 148.5 \text{ (C}_5^8), 144.6 \text{ (C}_4^{14}), 142.4 \text{ (C}_6^6), 135.6, 135.3 \text{ (C}_8^{19}), 129.7, 129.6 \text{ (C}_1^{11}), 128.8 \text{ (C}_8^3), 127.8 \text{ (C}_6^{16}), 127.6 \text{ (C}_1^{15}), 126.6 \text{ (C}_7^7), 122.4 \text{ (C}_1^1), 118.7 \text{ (C}_6^6), 116.0 \text{ (C}_2^2), 114.4 \text{ (C}_4^4), 113.2 \text{ (C}_1^{12}-\text{OMe), 98.8 \text{ (C}_5^5), 93.0 \text{ (C}_8^8), 85.9 \text{ (C}_4^4), 85.8 \text{ (C}_8^8\text{Ar}), 85.1 \text{ (C}_1^1), 80.3 \text{ (C}_7^7), 70.5 \text{ (C}_3^3), 63.7 \text{ (C}_8^8), 54.9 \text{ (OCH}_3), 39.9 \text{ (C}_2^2); \}

\text{LRMS (ES+) m/z: 1314 ([2M + Na]^+, 22), 747 ([M + Et}_3\text{N + H}^+), 719 (41), 668 ([M + Na]^+, 44), 646 ([M + H]^+, 41); HRMS (ES+) calcd for C}_{33}\text{H}_{35}\text{N}_7\text{O}_7 \text{ (M), [M + Na]^+ = 668.2367, found 668.2375. Found also DMT}: \text{ calcd}

\[ 303.1380, \text{ found 303.1387.} \]

Analytical results consistent with reported data.206

3-N-[2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-β-D-ribofuranosyl]-6-(3-aminophenyl)-(2,3H)-furan[2,3-d]pyrimidine-2-one, 154206
To a solution of nucleoside 153 (1.94 g, 2.48 mmol) in distilled methanol/distilled Et$_3$N (7:3 v/v, 15.0 mL), under an argon atmosphere, in absence of light, was added CuI (0.57 g, 2.99 mmol, 1.0 eq). The reaction mixture was stirred at 50 °C for 17 hours then cooled to rt and concentrated in vacuo, co-evaporating with CH$_2$Cl$_2$ (2 × 50 mL). The residue was dissolved/suspended in CH$_2$Cl$_2$ (150 mL) and filtered. The filtrate was washed with 5% w/v aq Na$_2$EDTA (pH 9, 4 × 50 mL), sat aq KCl (2 × 50 mL), dried (Na$_2$SO$_4$), concentrated in vacuo and dried under high vacuum to afford the product 154 as an orange/brown glassy foam (1.53 g, 2.37 mmol, 96%) containing 3.5 mol % uncyclised material 153.

R$_f$ 0.35 (153), 0.34 (154) (1:9, methanol/CH$_2$Cl$_2$ + 0.3% Et$_3$N, A’); $^1$H NMR (300 MHz, d$_6$-DMSO) δ 8.70 (1H, s, H$_4$), 7.40 (2H, d, J = 7.5 Hz, H$_{14}$), 7.34 (2H, t, J = 7.5 Hz, H$_{15}$), 6.94-6.88 (1H, m, H$_{5''}$), 6.92 (2H, d, J = 8.8 Hz, H$_{11}$), 6.91 (2H, d, J = 9.0 Hz, H$_{11}$), 6.84 (1H, br d, J = 7.5 Hz, H$_4$), 6.62 (1H, br dd, J = 1.5, 8.0 Hz, H$_6$), 6.13 (1H, dd, J = 4.6, 6.4 Hz, H$_1'$), 5.42 (1H, d, J = 4.8 Hz, 3'-OH), 5.32 (2H, br s, NH$_2$), 4.43 (1H, td, J = 5.9, 11.1 Hz, H$_3$), 4.04-3.99 (1H, m, H$_4$), 3.72 (3H, s, OCH$_3$), 3.71 (3H, s, OCH$_3$), 3.41 (1H, dd, J = 4.0, 10.8 Hz, H$_8$), 3.32 (1H, dd, J = 2.8, 10.8 Hz, H$_8$), 2.46 (1H, td, J = 6.6, 13.1 Hz, H$_2$), 2.25 (1H, ddd, J = 4.8, 6.5, 13.4 Hz, H$_2$); $^{13}$C NMR (100 MHz, d$_6$-DMSO) δ 170.9 (C$_{7a}$), 158.2 (C$_{12}$–OCH$_3$), 154.5 (C$_2$), 149.2 (C$_{3''}$–NH$_2$), 144.4 (C$_{13}$), 137.3 (C$_4$), 135.4 (C$_8$), 135.3 (C$_6$), 135.1 (C$_8$), 129.8, 129.7 (C$_{10}$), 129.5 (C$_5$), 128.6 (C$_{11}$), 128.0 (C$_{15}$), 127.8 (C$_{14}$), 126.8 (C$_{16}$), 115.1 (C$_8$), 113.3 (C$_{11}$), 112.0 (C$_5$), 109.2 (C$_{2''}$), 106.7 (C$_{4''}$), 97.8 (C$_5$), 87.3 (C$_1'$), 86.1 (C$_8$Ar$_3$), 85.9 (C$_4$), 68.8 (C$_3$), 62.4 (C$_5$), 55.0 (OCH$_3$), 41.1 (C$_2$); LRMS (ES$^+$): m/z: 917 (20), 663 ([M + NH$_4$]$^+$, 100), 646 ([M + H]$^+$, 13); HRMS (ES$^+$): calcd for C$_{38}$H$_{35}$N$_3$O$_7$ (M), [M + Na]$^+$ = 668.2367, found 668.2359. Found also DMT$: calcd 303.1380, found 303.1382.

Analytical results consistent with reported data.
To a stirred solution of nucleoside 154 (1.37 g, 2.12 mmol) and N,N-dimethylamino pyridine (DMAP) (0.26 g, 2.13 mmol, 1.0 eq) in distilled THF (10.0 mL), under an argon atmosphere, with 4Å molecular sieves, was added distilled Et$_3$N (0.15 mL) then ethyl trifluoroacetate (0.50 mL, 4.24 mmol, 2.0 eq). The solution was stirred at rt for 2 minutes, then heated to reflux under argon pressure (80 °C). Further DMAP (1.0 eq) and ethyl trifluoroacetate (2.0 eq) were added after 6 hours and 21½ hours. The temperature was then raised to 85 °C. Further DMAP (0.5 eq) and ethyl trifluoroacetate (1.0 eq) were added at 30 hours. At 53 hours, the reaction mixture was cooled to rt, dissolved in CH$_2$Cl$_2$/methanol (1:1 v/v, 60 mL) and concentrated in vacuo, co-evaporating with CH$_2$Cl$_2$ (4 × 40 mL) to give an orange/brown solid. Following purification by column chromatography (70-90% ethyl acetate/CH$_2$Cl$_2$ + 0.5% Et$_3$N), and drying under high vacuum, the desired product 155 was afforded as an off-white foam (0.55 g, 0.74 mmol, 35%).
(C\textsuperscript{5}), 129.8, 129.7 (C\textsuperscript{10}), 128.8 (C\textsuperscript{15}), 128.0 (C\textsuperscript{14}), 126.8 (C\textsuperscript{16}), 121.9 (C\textsuperscript{4}), 121.5 (C\textsuperscript{6}), 116.7 (C\textsuperscript{2}), 113.3 (C\textsuperscript{11}), 113.2 (q, \(J_{CF} = 227.4\) Hz, COCF\textsubscript{3}), 106.4 (C\textsuperscript{9}), 99.6 (C\textsuperscript{6}), 88.1 (C\textsuperscript{4}Ar), 86.0 (C\textsuperscript{4}), 68.8 (C\textsuperscript{3}), 62.4 (C\textsuperscript{5}), 54.9 (OCH\textsubscript{3}), 41.1 (C\textsuperscript{2}); LRMS (ES\textsuperscript{+}) \textit{m/z}: 764 ([M + Na]\textsuperscript{+}, 100); (ES\textsuperscript{−}) \textit{m/z}: 740 ([M – H]\textsuperscript{−}, 100).

Analytical results consistent with reported data.\textsuperscript{206}

3-Ureidophenyl acetylene, 156\textsuperscript{206}

3-Ethynylaniline 152 (3.00 mL, 26.6 mmol) and dry phenyl carbamate 157 (7.31 g, 53.3 mmol, 2.0 eq) were heated in a sealed tube, under an argon atmosphere, excluding moisture, in dim light at 90 °C for 12 hours. The mixture was cooled to rt, transferred using warm methanol/acetone, and dried under high vacuum. Following purification by column chromatography (5-50% acetone/CH\textsubscript{2}Cl\textsubscript{2}), and drying under high vacuum, the desired urea 156 was afforded, as an off-white, papery solid (2.93 g, 18.3 mmol, 69%).

R\textsubscript{f} 0.31 (1:9, methanol/CH\textsubscript{2}Cl\textsubscript{2} + 0.3% Et\textsubscript{3}N, A’, F’), 0.04 (1:9, acetone/CH\textsubscript{2}Cl\textsubscript{2} + 0.3% Et\textsubscript{3}N, A’, F’); IR (solid): v 3467, 3329 (m, N–H), 3302 (w N–H), 3238 (m, N–H), 2100 (w, C≡C), 1649 (vs, C=O), 1586, 1575 (s, Aryl C–H), 1540 (br vs, Aryl C–H), 1473 (s), 1413 (m), 1325 (s), 1284, 1256, 1162 (m), 1120 (br w), 1029 (w), 890 (m, Aryl C–H), 801 (m), 773 (m, Aryl C–H), 683 (s), 643 (br s), 587 (s) cm\textsuperscript{−1}; \textsuperscript{1}H NMR (300 MHz, d\textsubscript{6}-DMSO) δ 8.61 (1H, s, N\textsubscript{H}), 7.64 (1H, t, \(J = 1.7\) Hz, H\textsubscript{2}), 7.32 (1H, ddd, \(J = 1.1, 2.0, 8.2\) Hz, H\textsuperscript{4}), 7.22 (1H, t, \(J = 7.9\) Hz, H\textsuperscript{5}), 7.00 (1H, td \(J = 1.3, 7.3\) Hz, H\textsuperscript{6}), 5.89 (2H, s, NH\textsubscript{2}), 4.08 (1H, s, C≡CH\textsubscript{8}); \textsuperscript{13}C NMR (100 MHz, d\textsubscript{6}-DMSO) δ 155.9 (CONH\textsubscript{2}), 140.8 (C\textsuperscript{3}), 129.0 (C\textsuperscript{5}), 124.3 (C\textsuperscript{6}), 121.8 (C\textsuperscript{1}), 120.5 (C\textsuperscript{2}), 118.4 (C\textsuperscript{4}), 83.8 (C\textsuperscript{7}=CH), 80.0 (C≡CH\textsubscript{8}); LRMS (ES\textsuperscript{+}) \textit{m/z}: 343 ([2M + Na]\textsuperscript{+}, 21), 183 ([M + Na]\textsuperscript{+}, 70), 102 ([Et\textsubscript{3}N + H]\textsuperscript{+}, 100); (ES\textsuperscript{−}) \textit{m/z}: 159 ([M – H]\textsuperscript{−}, 100); HRMS (ES\textsuperscript{+}): calcd for C\textsubscript{9}H\textsubscript{8}N\textsubscript{2}O (M), [M + Na]\textsuperscript{+} = 183.0529, found 183.0529, [2M + Na]\textsuperscript{+} = 343.1171, found 343.1170.

Analytical results consistent with reported data.\textsuperscript{206}
To a solution of 5'-O-(4,4'-dimethoxytrityl)-5-iodo-2'-deoxyuridine 97 (3.01 g, 4.58 mmol) in anhydrous DMF (15.0 mL), under an argon atmosphere, in absence of light, were added distilled Et$_3$N (3.30 mL, 23.7 mmol, 5.0 eq), Cul (0.35 g, 1.83 mmol, 0.4 eq), then alkyne 156 (2.20 g, 13.7 mmol, 3.0 eq). The mixture was stirred for 15 minutes then tetakis(triphenylphosphine) palladium (0) (0.53 g, 0.46 mmol, 0.1 eq) was added and the reaction was stirred at rt for 3 hours. The reaction mixture was concentrated under high vacuum, co-evaporating with CH$_3$CN to give a dark brown foam/gum. Following purification by column chromatography (5-8% methanol/CH$_2$Cl$_2$ + 0.5% Et$_3$N), and drying under high vacuum, the desired product 159 was afforded as a pale orange foam (2.71 g) containing 34 mol % Et$_3$N (recalculated yield - 3.66 mmol, 80%).
H); $^{13}$C NMR (100 MHz, $d_6$-DMSO) $\delta$ 161.4 (C$^4$), 158.1, 158.0 (C$^{13}$–OCH$_3$), 155.8 (CONH$_2$), 149.3 (C$^3$), 144.7 (C$^{14}$), 142.8 (C$^6$), 140.6 (C$^{3''}$–NHR), 135.6, 135.3 (C$^{10}$), 129.7, 129.6 (C$^{11}$), 128.7 (C$^{16}$), 127.8 (C$^{16'}$), 127.6 (C$^{17}$), 126.7 (C$^8$), 124.0 (C$^6'$), 122.4 (C$''$), 120.0 (C$^{2''}$), 118.0 (C$^9$), 113.2 (C$^{12}$), 98.6 (C$^5$), 92.4 (C$^4$), 86.0 (C$^8$Ar$_3$), 85.1 (C$'$), 81.2 (C$^7$), 70.5 (C$^3$), 63.7 (C$^{5'}$), 54.9 (OCH$_3$), 39.9 (C$^2'$); LRMS (ES$^+$) m/z: 711 ([M + Na]$^+$, 100), 303 ([DMT$^+$], 9); HRMS (ES$^+$): calcd for C$_{39}$H$_{36}$N$_4$O$_8$ (M), [M + Na]$^+$ = 711.2425, found 711.2409, [M + NH$_4$]$^+$ = 706.2877, found 706.2856. Found also 301.1413.

3-N-[2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-β-D-ribofuranosyl]-6-(3-ureidophenyl)-(2,3H)-furan[2,3-d]pyrimidine-2-one, 160

To a solution of nucleoside 159 (2.13 g, 3.09 mmol) in distilled/argon-degassed methanol/Et$_3$N (7:3 v/v, 15.0 mL), with 4Å molecular sieves, in absence of light and under an argon atmosphere, was added CuI (0.65 g, 3.40 mmol, 1.1 eq), and the mixture was stirred at reflux under argon pressure (80 °C) for 3.5 hours. After cooling to rt, the reaction cake was dissolved/suspended in CH$_2$Cl$_2$ (150 mL), filtered, and the solid was washed with CH$_2$Cl$_2$ (2 × 50 mL). The combined filtrate was washed with 5% w/v aq Na$_2$EDTA (pH 9, 4 × 100 mL) and sat aq KCl (100 mL), dried (Na$_2$SO$_4$), and concentrated in vacuo to give a yellow solid. The product was crystallised by addition of methanol (10 mL) and the mixture was allowed to stand in the fridge for 2 days. The solid was filtered, washed with the filtrate (×2) then cold diethyl ether (-8 °C, 3 × 10 mL), and dried under high vacuum over KOH, to afford the desired product 160 as a pale cream-coloured fine crystalline solid (1.34 g). The filtrate was concentrated in vacuo from which two further crops were obtained (0.11 g, 0.03 g) (combined yield 1.47 g, 2.14 mmol, 69%).
Rf 0.18 (9:1, acetone/CH₂Cl₂ + 0.3% Et₃N), 0.23 (acetone + 0.3% Et₃N); 0.33 (1:9, methanol/CH₂Cl₂ + 0.3% Et₃N); Mp 160-169 °C (dec.); IR (solid): ν 3472, 3305 (m, N=H, O–H), 2932, 2836 (w, C–H), 1663 (s, C=O), 1606 (s, N=H), 1584 (s, C=N), 1547 (m), 1508, 1492 (s, Aryl C=C), 1445, 1408 (m), 1382 (m, C–H), 1342 (m), 1301, 1247 (s), 1174 (vs), 1072 (br s, C–O), 1031 (vs), 972, 912, 878 (m), 828 (s, C–H), 773 (s), 701 (s, Aryl C–H), 583, 532 (s) cm⁻¹; ¹H NMR (400 MHz, d₆-DMSO) δ 7.73 (1H, br s, NHCONH₂), 8.72 (1H, d, J = 6.3 Hz, H¹), 7.90 (1H, s, H²), 7.40 (2H, d, J = 7.5 Hz, H¹⁴), 7.37-7.31 (4H, m, H₁⁵, H⁴⁺, H⁵⁺), 7.31-7.24 (1H, m, H¹⁶), 7.28 (4H, d, J = 8.3 Hz, H¹⁶), 7.28-7.22 (1H, m, H⁸⁺), 6.92 (2H, d, J = 8.8 Hz, H¹¹), 6.91 (2H, d, J = 8.8 Hz, H¹¹), 6.19 (1H, s, H⁵), 6.16 (1H, dd, J = 4.5, 6.3 Hz, H¹′), 5.92 (2H, s, CONH₂), 5.42 (1H, d, J = 4.9 Hz, 3'-OH), 4.44 (1H, td, J = 5.6, 10.8 Hz, H³), 4.02 (1H, dd, J = 3.5, 7.5 Hz, H⁴), 3.72 (3H, s, OCH₃), 3.71 (3H, s, OCH₃), 3.41 (1H, dd, J = 3.7, 10.8 Hz, H⁸), 3.34-3.27 (1H, m, H⁸), 2.47 (1H, td, J = 6.5, 13.1 Hz, H²), 2.26 (1H, ddd, J = 4.6, 6.5, 13.6 Hz, H²); ¹³C NMR (100 MHz, d₆-DMSO) δ 170.9 (C⁷⁹), 158.2 (C¹²–OCH₃), 155.9 (CONH₂), 153.7 (C¹), 144.5 (C⁵), 141.3 (C¹³), 137.8 (C⁴), 137.7 (C³=OHR), 135.3, 135.0 (C⁶), 129.8, 129.8 (C¹⁴b), 129.4 (C⁸⁺), 128.4 (C⁵⁺), 128.0 (C¹⁴b), 127.7 (C¹⁴), 126.9 (C¹⁶), 118.7 (C⁶'), 117.2 (C⁴'), 112.3 (C¹¹), 113.2 (C²'), 106.6 (C³'), 98.6 (C⁵'), 87.4 (C¹'), 86.1 (C⁸Ar), 85.9 (C⁴'), 68.8 (C⁹'), 62.4 (C⁵'), 55.0 (OCH₃), 41.1 (C²'); LRMS (ES⁺) m/z: 743 ([M + CH₃OH + Na]⁺, 100), 711 ([M + Na]⁺, 74); (ES⁻) m/z: 687 ([M – H]⁻, 100); HRMS (ES⁺): calcd for C₉₀H₅₀N₄O₆ (M), [M + Na]⁺ = 711.2425, found 711.2389, [M + H]⁺ = 689.2606, found 689.2572. Found also DMT⁺ (calcd 303.1380, found 303.1385).

3-Acetamidophenyl acetylene, 161²¹

Method 1: To a stirred solution of 3-ethynylaniline 152 (0.20 mL, 1.78 mmol) in dry, degassed diethyl ether (5.0 mL) at 0 °C, under an argon atmosphere, was added distilled Et₃N (0.04 mL, 0.29 mmol, 0.16 eq), followed by acetyl chloride (0.14 mL, 1.97 mmol, 1.1 eq) in dry, degassed diethyl ether (4.0 mL) dropwise. The reaction mixture was stirred for 2 hours, allowed to warm to rt, then stirred for a further 4.5 hours. The reaction mixture was filtered, the solid was washed with diethyl ether (3 ×
10 mL), and combined ether fractions were concentrated *in vacuo* and dried under high vacuum to give a pale yellow oil. Following trituration with water (3.0 mL), the product 161 was afforded, following drying under high vacuum over KOH, as a pale cream-coloured waxy solid (228 mg, 1.43 mmol, 81%).

**Method 2:** To a stirred solution of 3-ethynylaniline 152 (3.50 mL, 31.1 mmol) and distilled Et$_3$N (4.37 mL, 31.4 mmol, 1.0 eq) in dry, degassed diethyl ether (15.0 mL), at 0 °C, under an argon atmosphere, was added acetyl chloride (2.23 mL, 31.4 mmol, 1.0 eq) in dry, degassed diethyl ether (25.0 mL), dropwise over 45 minutes. The reaction was stirred for a further 15 minutes then allowed to warm to rt over 30 minutes. The mixture was filtered, the white precipitate was washed with diethyl ether (3 × 35 mL) and combined ether fractions were concentrated *in vacuo* to give a cream-coloured solid. The solid was triturated with water (60 mL) to which was added diethyl ether (100 mL) and the layers were separated. The ether layer was washed with water (50 mL), combined aqueous layers were re-extracted (diethyl ether, 20 mL), and combined ether fractions were concentrated *in vacuo* and under high vacuum to afford the desired product 161 a pale brown oil, which crystallised on standing to an off-white chalky/waxy solid (4.79 g, 30.1 mmol, 97%).

**Rf** 0.40 (5:95, methanol/CH$_2$Cl$_2$ + 0.3% Et$_3$N, A’); **Mp** 92-94 °C (chloroform/hexane), lit.$^{211}$ 94-96 °C (CCl$_4$); **IR** (solid): ν 3300, 3282 (m, CC–H), 1666 (m, C=O), 1605, 1583 (m, Aryl C–H), 1556 (m, C=O), 1481 (m, Aryl C–H), 1425, 1401, 1370, 1308, 1289, 1255 (m), 1015 (w), 880, 786 (m, Aryl C–H), 721, 678, 648 (m), 601 (s), 541 (m) cm$^{-1}$;

$^1$H NMR (300 MHz, $d_6$-DMSO) δ 10.00 (1H, s, NHCOCH$_3$), 7.77 (1H, app. t, $J = 1.7$ Hz, H$^5$), 7.54 (1H, ddd, $J = 1.1, 2.0, 8.1$ Hz, H$^4$), 7.30 (1H, t, $J = 7.9$ Hz, H$^5$), 7.13 (1H, td, $J = 1.3, 7.7$ Hz, H$^6$), 4.13 (1H, s, C≡CH$^8$), 2.05 (3H, s, COC$^3$H$_3$); $^{13}$C NMR (100 MHz, $d_6$-DMSO) δ 169.1 (C=OCH$_3$), 140.1 (C$^3$), 129.7 (C$^5$), 126.8 (C$^4$), 122.5 (C$^1$), 122.4 (C$^2$), 120.1 (C$^4$), 84.0 (C≡CH), 81.0 (C≡C$^8$H), 24.6 (COCH$_3$); **LRMS** (ES$^+$) $m/z$: 160 ([M + H]$^+$, 100), 182 ([M + Na]$^+$, 11), 201 ([M + CH$_2$CN + H]$^+$, 7), 223 ([M + CH$_2$CN + Na]$^+$, 17), 242 ([M + AcOH + Na]$^+$, 12); (ES$^-$) $m/z$: 158 ([M – H]$^-$, 100); **HRMS** (ES$^+$): calcd for C$_{10}$H$_3$NO (M), [2M + Na]$^+$ = 341.1260, found 341.1239, [M + Na]$^+$ = 182.0576, found 182.0565, [M + H]$^+$ = 160.0757, found 160.0755.
To a solution of 5'-O-(4,4'-dimethoxytrityl)-5-iodo-2'-deoxyuridine 97 (3.02 g, 4.61 mmol) in anhydrous DMF (15.0 mL), under an argon atmosphere, in absence of light, were added distilled Et3N (3.20 mL, 23.0 mmol, 5.0 eq), Cul (0.35 g, 1.84 mmol, 0.4 eq), then alkyne 156 (2.20 g, 13.8 mmol, 3.0 eq). The mixture was stirred for 15 minutes then tetrakis(triphenylphosphine) palladium (0) (0.53 g, 0.46 mmol, 0.1 eq) was added and the reaction was stirred at rt for 2 hours. After concentration under high vacuum and co-evaporation with methanol/toluene, the dark brown foam was purified four times by column chromatography (3-10% methanol/CH2Cl2 + 0.5% Et3N; 20-50% acetone/CH2Cl2 + 0.5% Et3N; 40-100% acetone/CH2Cl2 + 0.5% Et3N; 92:6:2-82:16:2, ethyl acetate/methanol/conc. aq NH3). The desired product 162 was afforded, after drying under high vacuum, as a pale orange foam (2.37 g, 3.44 mmol, 75%).

Rf 0.39 (1:9, methanol/CH2Cl2 + 0.3% Et3N, A'), 0.33 (82:16:2, ethyl acetate/methanol/conc. aq NH3, A'). 0.64 (1:4, methanol/ethyl acetate + 0.3% Et3N, A'); 1H NMR (400 MHz, d6-DMSO) δ 11.63 (1H, extremely br s, NH), 9.98 (1H, s, NHCOCH3), 8.04 (1H, s, H6), 7.74 (1H, t, J = 1.5 Hz, H3), 7.45 (1H, br dd, J = 1.2, 8.0 Hz, H4), 7.41 (2H, d, J = 7.5 Hz, H15), 7.30 (2H, d, J = 9.0 Hz, H11), 7.30-7.25 (1H, m, H16), 7.29 (2H, d, J = 9.0 Hz, H11), 7.22 (1H, t, J = 8.0 Hz, H16), 7.16 (1H, t, J = 7.3 Hz, H17), 6.85 (2H, d, J = 8.5 Hz, H12), 6.84 (2H, d, J = 9.0 Hz, H15), 6.74 (1H, br d, J = 7.5 Hz, H16), 6.14 (1H, t, J = 6.8 Hz, H17), 5.32 (1H, br s, 3'-OH), 4.29 (1H, br s, H3), 3.95 (1H, td, J = 3.2, 5.0 Hz, H4), 3.66 (6H, s, OCH3), 3.25 (1H, dd, J = 5.3, 10.8 Hz, H5), 3.15 (1H, dd, J = 2.5, 10.5 Hz, H8), 2.32 (1H, td, J = 6.8, 13.6 Hz, H9), 2.22 (1H, ddd, J = 3.5, 6.5, 13.6 Hz, H5); 13C NMR (100 MHz, d6-DMSO) δ 168.4 (COCH3), 161.4 (C4), 158.0, 158.0 (C13–OCH3), 149.3 (C2), 144.7 (C14), 142.9 (C6), 139.3 (C3–NHR), 135.6, 135.3 (C18), 129.7, 129.6 (C11), 128.8 (C5'), 127.8 (C16), 127.6 (C15), 126.6 (C17), 125.8 (C8'), 122.5 (C7'), 121.3 (C4'), 119.1 (C5'), 113.2 (C12), 98.4 (C6), 92.0 (C7), 86.0 (C4'), 85.8
(C9Ar3), 85.2 (C1), 81.6 (C5), 70.4 (C3), 63.7 (C6), 54.9 (OCH3), 40.0 (C2), 24.0 (COCH3); LRMS (ES+) m/z: 710 ([M + Na]+, 100), 705 ([M + NH4]+, 8); (ES-) m/z: 784 ([M + HPO4 − H]−, 100), 732 ([M + HCO2H − H]−, 45), 686 ([M − H]−, 12); HRMS (ES−): calcd for C40H37N3O8 (M), [M + Na]+ = 710.2473, found 710.2480.

3-N-[2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-β-D-ribofuranosyl]-6-(3-acetamido-phenyl)-(2,3H)-furano[2,3-d]pyrimidine-2-one, 163

To a solution of nucleoside 159 (1.94 g, 2.82 mmol) in distilled/argon-degassed methanol/Et3N (7:3 v/v, 20.0 mL), with 4Å molecular sieves, in absence of light and under an argon atmosphere, was added CuI (0.59 g, 3.10 mmol, 1.1 eq), and the mixture was stirred at reflux under argon pressure (80 °C) for 2 hours. The reaction was diluted with methanol (20 mL), filtered, and the filtrate was concentrated in vacuo. The green foam was dissolved in CH2Cl2 (100 mL) and washed with 5% w/v aq Na2EDTA (pH 10, 5 × 100 mL). The combined aqueous fraction was re-extracted (CH2Cl2, 2 × 70 mL) and the combined CH2Cl2 layer was washed with sat aq KCl (2 × 100 mL), dried (Na2SO4), and concentrated in vacuo to give a green foam. Following purification by column chromatography (20-60% acetone/ethyl acetate + 0.5-1.0% Et3N), and drying under high vacuum, the desired cyclised product 163 was afforded as a pale yellow foam (1.73 g, 2.52 mmol, 89%).

Rf 0.31 (1:2, acetone/ethyl acetate + 0.3% Et3N, A’), 0.62 (1:4, methanol/ethyl acetate + 0.3% Et3N, A’); 1H NMR (400 MHz, d6-DMSO) δ 10.10 (1H, s, NHCOCH3), 8.73 (1H, s, H9), 8.03 (1H, t, J = 1.5 Hz, H13), 7.59 (1H, td, J = 1.6, 7.8 Hz, H4), 7.44-7.40 (1H, m, H5), 7.41 (2H, d, J = 7.5 Hz, H14), 7.37 (1H, td, J = 1.5, 8.0 Hz, H6), 7.34 (2H, t, J = 8.0 Hz, H15), 7.29 (2H, d, J = 9.0 Hz, H16), 7.28 (2H, d, J = 9.0 Hz, H17), 7.27 (1H, td, J = 1.3, 7.0 Hz, H18), 6.92 (2H, d, J = 9.0 Hz, H19), 6.91 (2H, d, J = 9.0 Hz, H20), 6.23
air-sensitive pale peach-coloured foam (5.44 g, 6.19 mmol, 84%) afforded, after drying under high vacuum, as a diastereomeric mixture (ca. 1:2), as an air-sensitive pale peach-coloured foam (5.45 g, 6.19 mmol, 84%).

\[ \text{13C NMR} \ (100 \text{ MHz}, \text{d}_6-\text{DMSO}) \delta \ 170.9 \ (\text{C}^8), \ 168.6 \ (\text{COCH}_3), \ 158.2 \ (\text{C}^{12}-\text{OCH}_3), \ 153.6 \ (\text{C}^2), \ 153.4 \ (\text{C}^6), \ 144.5 \ (\text{C}^{13}), \ 140.0 \ (\text{C}^{3'}-\text{NHR}), \ 137.9 \ (\text{C}^4), \ 135.4, \ 135.0 \ (\text{C}^9), \ 129.8, \ 129.7 \ (\text{C}^{10}), \ 129.5 \ (\text{C}^8), \ 128.5 \ (\text{C}^{1'}), \ 128.0 \ (\text{C}^{15}), \ 127.7 \ (\text{C}^{14}), \ 126.8 \ (\text{C}^{16}), \ 119.8 \ (\text{C}^5), \ 119.0 \ (\text{C}^6), \ 114.5 \ (\text{C}^7), \ 113.3 \ (\text{C}^{11}), \ 106.5 \ (\text{C}^4), \ 98.9 \ (\text{C}^5), \ 87.5 \ (\text{C}^5), \ 86.1 \ (\text{C}^8\text{Ar}_3), \ 85.9 \ (\text{C}^4), \ 68.8 \ (\text{C}^3), \ 62.4 \ (\text{C}^5), \ 55.0, \ 55.0 \ (\text{OCH}_3), \ 41.1 \ (\text{C}^2), \ 24.0 \ (\text{COCH}_3); \]

LRMS (ES+) m/z: 710 ([M + Na]+, 100); (ES−) m/z: 686 ([M − H]−, 100); HRMS (ES+): calcd for C_{40}H_{37}N_{12}O_{8} (M), [M + Na]^+ = 710.2473, found 710.2462.

5′-O-(4,4′-Dimethoxytrityl)-5-(3-trifluoroacetamidopropynyl)-2′-deoxyuridine-3′-O-(2′-O-cyanoethyl-N,N-diisopropyl) phosphoramidite, 164\textsuperscript{117,170}

To a stirred solution of nucleoside 165 (5.02 g, 7.39 mmol) in distilled THF (25.0 mL), strictly under an argon atmosphere and excluding moisture, was added distilled DIPEA (3.22 mL, 18.5 mmol, 2.5 eq) followed by chloro-phosphitylating reagent 70 (1.82 mL, 8.14 mmol, 1.1 eq) dropwise, and the reaction was stirred at rt for 1¼ hours. The THF was removed in vacuo and the residue was dissolved in argon-degassed ethyl acetate (100 mL). The solution was washed with degassed sat. aq KCl (3 × 50 mL), dried (Na$_2$SO$_4$), and concentrated in vacuo to give an orange foam. Following purification by column chromatography (10% acetone/CH$_2$Cl$_2$ + 1.0% pyridine) on silica gel pre-equilibrated with Et$_3$N, under argon pressure, the desired phosphoramidite 164 was afforded, after drying under high vacuum, as a diastereomeric mixture (ca. 1:2), as an air-sensitive pale peach-coloured foam (5.45 g, 6.19 mmol, 84%).
R, 0.41 (1:9, acetone/CH₂Cl₂ + 0.5% pyridine, A’); ³¹P NMR (121 MHz, d₆-DMSO) δ 148.9, 148.5 (P⁰); ¹H NMR (400 MHz, d₆-DMSO) δ 11.66 (NH¹), 9.98 (1H, s, CONH), 7.97 and 7.96 (1H, s, H⁶), 7.42-7.38 (2H, m, H¹⁶), 7.33-7.28 (2H, m, H¹⁷), 7.29 and 7.27 (4H, d, J = 7.8 Hz and J = 8.0 Hz, H¹⁵), 7.25-7.19 (1H, m, H¹⁸), 6.89 and 6.87 (4H, d, J = 8.8 Hz and J = 9.0 Hz, H¹³), 6.10 and 6.08 (1H, t, J = 6.7 Hz and J = 6.8 Hz, H¹¹), 4.50 and 4.47 (1H, ddd, J = 3.6, 6.7, 14.2 Hz and J = 3.9, 6.6, 14.2 Hz, H⁹), 4.10 and 4.06 (2H, s, H⁶), 4.08-4.00 (1H, m, H⁴), 3.77-3.67 (1H, m, H¹⁹), 3.74 (3H, s, OCH₃), 3.73 (3H, s, OCH₃), 3.67-3.59 (1H, m, H¹⁹), 3.60-3.44 (1H, m, H²¹), 3.33-3.24 (1H, m, H⁵), 3.19 and 3.14 (1H, dd, J = 3.3, 10.5 Hz and J = 3.0, 10.3 Hz, H⁵), 2.75 and 2.64 (2H, t, J = 5.9 Hz, H²⁰), 2.44 and 2.43 (1H, dd, J = 6.8, 13.6 Hz and J = 6.9, 13.9 Hz, H²), 2.35 and 2.30 (1H, ddd, J = 3.9, 6.4, 13.7 Hz and J = 3.6, 6.4, 13.9 Hz, H²), 1.13 (3H, d, J = 6.8 Hz, H²²), 1.11 (3H, d, J = 7.0 Hz, H²²), 1.10 (3H, d, J = 6.8 Hz, H²²), 0.99 (3H, d, J = 6.8 Hz, H²²); LRMS (ES⁺) m/z: 902 ([M + Na⁺], 100), 897 ([M + NH₄⁺], 38), 880 ([M + H⁺], 32), 303 (DMT⁺, 24); (ES⁻) m/z: 914/916 ([M + HCl – H], 2:1, 21), 878 ([M – H], 97), 825 ([M – CH₂CH-CN – H], 100).

Analytical results consistent with reported data.¹¹⁷,¹⁷⁰

5'-O-(4,4'-Dimethoxytrityl)-5-(3-trifluoroacetamidopropynyl)-2'-deoxyuridine, 165¹¹⁷,¹⁷⁰,²⁵⁵

To a stirred solution of 5'-O-(4,4'-dimethoxytrityl)-5-iodo-2'-deoxyuridine 97 (10.1 g, 15.4 mmol) in anhydrous DMF (70.0 mL), under an argon atmosphere, was first added distilled Et₃N (15.0 mL, 108 mmol, 7.0 eq) followed by, in absence of light, Cul (0.59 g, 3.09 mmol, 0.2 eq) and 3-(trifluoroacetamido)prop-1-ynyl 68 (2.41 mL, 21.5 mmol, 1.4 eq). After stirring for 20 minutes, tetrakis(triphenylphosphine) palladium (0) (1.78 g, 1.54 mmol, 0.1 eq) was added and the reaction was stirred at rt for 1½ hours, when solid Na₂EDTA (1.20 g) was added. After stirring for 5 minutes, the reaction
mixture was concentrated under high vacuum to give a dark brown syrup. The syrup was dissolved in ethyl acetate (300 mL) and washed with 5% w/v aq Na$_2$EDTA (pH 9, 3 × 150 mL) and sat. aq KCl (2 × 150 mL), dried (Na$_2$SO$_4$), and concentrated in vacuo and under high vacuum to give a golden brown foam. This reaction was repeated on the same scale as described above.

Following joint purification by column chromatography (10-30% acetone/CH$_2$Cl$_2$ + 1.0% pyridine), the desired product 165 was afforded, after drying under high vacuum, as a very pale orange foam (16.6 g, 24.4 mmol, 80%). A mixture of starting material 97 and product 165 (1.84 g) was also isolated.

$R_f$ 0.19 (1:9, acetone/CH$_2$Cl$_2$ + 0.5% pyridine, A’, B’); $^{19}$F NMR (282 MHz, d$_6$-DMSO) δ -73.9 (NHCO CF$_3$), $^1$H NMR (400 MHz, d$_6$-DMSO) δ 11.66 (N)$_3$, 9.98 (1H, s, NHCO CF$_3$), 7.40 (2H, d, $J$ = 7.5 Hz, H$_{16}$), 7.31 (2H, t, $J$ = 7.5 Hz, H$_{17}$), 7.29 (2H, d, $J$ = 9.0 Hz, H$_{12}$), 7.28 (2H, d, $J$ = 9.0 Hz, H$_{13}$), 7.22 (1H, tt, $J$ = 1.5, 7.3 Hz, H$_{18}$), 6.89 (2H, d, $J$ = 9.0 Hz, H$_{13}$), 6.89 (2H, d, $J$ = 9.0 Hz, H$_{15}$), 6.10 (1H, t, $J$ = 6.7 Hz, H$_1$), 5.32 (1H, d, $J$ = 4.5 Hz, 3’-OH), 4.27 (1H, td, $J$ = 4.0, 10.3 Hz, H$_4$), 4.06 (2H, br d, $J$ = 3.8 Hz, H$_8$), 3.92 (1H, dd, $J$ = 10.4 Hz, H$_5$), 3.09 (1H, dd, $J$ = 2.9, 10.4 Hz, H$_5$), 2.29 (1H, td, $J$ = 6.8, 13.6 Hz, H$_2$), 2.19 (1H, ddd, $J$ = 3.8, 6.3, 13.3 Hz, H$_2$); $^{13}$C NMR (100 MHz, d$_6$-DMSO) δ 161.5 (C$_4$), 158.1, 158.1 (C$_{14}$–OMe), 156.0 (q, $^{3}$J$_{CF}$ = 36.9 Hz, COCF$_3$), 149.3 (C$_2$), 144.8 (C$_{15}$), 143.7 (C$_6$), 135.6, 135.2 (C$_{11}$), 129.7, 129.6 (C$_{12}$), 127.8 (C$_{17}$), 127.5 (C$_{16}$), 126.6 (C$_{18}$), 115.7 (q, $^{4}$J$_{CF}$ = 288.7 Hz, COCF$_3$), 113.2, 113.2 (C$_{13}$), 97.8 (C$_5$), 87.4 (C$_{18}$Ar$_3$), 85.8 (C$_4$,C$_7$), 85.1 (C$_1$), 75.1 (C$_8$), 70.4 (C$_3$), 63.8 (C$_5$), 55.0 (OCH$_3$), 39.7 (C$_2$), 29.4 (C$_9$); LRMS (ES$^+$) m/z: 803 ([M + Et$_3$N + Na]$^+$, 14), 718 ([M + K]$^+$, 14), 702 ([M + Na]$^+$, 100), 697 ([M + NH$_4$]$^+$, 5), 303 (DMT$^+$, 5); (ES$^-$) m/z: 678 ([M – H]$^-$, 100). Analytical results consistent with reported data.\textsuperscript{170,255}
To a stirred solution of nucleoside 197 (5.02 g, 7.39 mmol) in distilled CH₂Cl₂ (25.0 mL), strictly under an argon atmosphere and excluding moisture, was added distilled DIPEA (3.22 mL, 18.5 mmol, 2.5 eq) followed by chloro-phosphitylating reagent 70 (1.82 mL, 8.14 mmol, 1.1 eq) dropwise, and the reaction was stirred at rt for 4¾ hours. The reaction mixture was diluted with argon-degassed CH₂Cl₂ (15 mL) and washed, under an argon atmosphere, with degassed sat. aq KCl (15 mL). The aqueous was re-extracted (degassed CH₂Cl₂, 3 × 5 mL) and combined CH₂Cl₂ fractions were dried (Na₂SO₄), and concentrated in vacuo and dried under high vacuum to give a yellow foam. Following purification by column chromatography (degassed 1% acetone/CH₂Cl₂ + 0.5% Et₃N → 5% acetone/CH₂Cl₂ + 0.5% Et₃N), on silica gel pre-equilibrated with Et₃N, under argon pressure, then precipitation at rt from degassed CH₂Cl₂ (3 mL) into degassed hexane (180 mL), the desired phosphoramidite 178 was afforded, after drying under high vacuum, as a diastereomeric mixture (ca. 2:1), as a pale yellow foam (0.18 g) containing 38.0 mol % Et₃N.HCl (recalculated yield - 0.18 mmol, 70%).

Rf 0.50, 0.57 (1:1, acetone/CH₂Cl₂ + 0.5% Et₃N, A‘), 0.29, 0.34 (1:4, acetone/CH₂Cl₂ + 0.5% Et₃N, A‘), 0.18, 0.25 (CH₂Cl₂ + 0.5% Et₃N, A‘); ³¹P NMR (121 MHz, d₆-DMSO) δ 149.1, 148.9 (PⅢ); ¹⁹F NMR (282 MHz, d₆-DMSO) δ -73.5 (NHCOCF₃); ¹H NMR (400 MHz, d₆-DMSO) δ 11.47 (NHCOCF₃), 8.71 and 8.68 (1H, s, H⁴), 7.85 (1H, br s, H⁵), 7.75 (1H, br dd, J = 1.1, 8.2 Hz, H⁴), 7.54 (1H, t, J = 8.0 Hz, H⁶), 7.42 and 7.40 (2H, d, J = 8.3 Hz and J = 8.8 Hz, H⁴), 7.16 (1H, br dd, J = 0.9, 7.9 Hz, H⁶), 7.34-7.28 (2H, m, H⁵), 7.30 and 7.27 (4H, d, J = 8.5 Hz and J = 8.8 Hz, H⁴), 7.28-7.23 (1H, m, H⁶), 6.89, 6.88 and 6.87 (2H, 2H and 4H, d, J = 8.8 Hz, J = 9.0 Hz and J = 8.5 Hz,
the combined solution was washed with degassed sat. aq NaHCO₃. The sticky yellow precipitate was washed with degassed ethyl acetate (3 × 5 mL), and the reaction was stirred at rt. After 18½ hours, further reagent was added (0.11 mL, 0.36 mmol, 1.1 eq), and the reaction was stirred at 24 hours, further DIHT 190 (27.8 mg, 0.16 mmol, 0.5 eq) was added, then at 24 hours, further reagent 189 was added (0.10 mL, 0.32 mmol, 1.0 eq). After 41 hours, THF was removed in vacuo, and the syrup was dissolved in argon-degassed ethyl acetate (5 mL). The sticky yellow precipitate was washed with degassed ethyl acetate (3 × 5 mL), and the combined solution was washed with degassed sat. aq NaHCO₃ (3 × 8 mL) and sat.

Method 1: To a stirred solution of cyclised nucleoside 188 (0.22 g, 0.32 mmol) in distilled THF (1.5 mL) and anhydrous DMF (0.5 mL), under an argon atmosphere and excluding moisture, was added DIHT 190 (0.03 g, 0.16 mmol, 0.5 eq) followed by phosphitylating reagent 189 (0.11 mL, 0.36 mmol, 1.1 eq), and the reaction was stirred at rt. After 18½ hours, further reagent 189 was added (0.05 mL, 0.16 mmol, 0.5 eq). At 22 hours, further DIHT 190 (27.8 mg, 0.16 mmol, 0.5 eq) was added, then at 24 hours, further reagent 189 was added (0.10 mL, 0.32 mmol, 1.0 eq). After 41 hours, THF was removed in vacuo, and the syrup was dissolved in argon-degassed ethyl acetate (5 mL). The sticky yellow precipitate was washed with degassed ethyl acetate (3 × 5 mL), and the combined solution was washed with degassed sat. aq NaHCO₃ (3 × 8 mL) and sat.
aq KCl (2 × 8 mL), dried (Na₂SO₄), concentrated *in vacuo* and dried under high vacuum to give a viscous yellow syrup. Following purification by column chromatography (degassed 2% methanol/acetone + 0.5% Et₃N) on silica gel pre-equilibrated with Et₃N, under argon pressure, and drying under high vacuum, the desired phosphoramidite 179 was afforded as a diastereomeric mixture (*ca.* 1:1), as an air-sensitive, pale yellow foam (0.08 g, 0.09 mmol, 29%).

**Method 2:** To a stirred solution of cyclised nucleoside 188 (0.92 g, 1.31 mmol) in distilled THF (5.0 mL) and anhydrous DMF (1.0 mL), under an argon atmosphere and excluding moisture, was added distilled DIPEA (0.58 mL, 3.30 mmol, 2.5 eq) followed by chloro-phosphitylating reagent 70 (0.33 mL, 1.46 mmol, 1.1 eq), and the reaction was stirred at rt for 2 hours. The reaction mixture was concentrated *in vacuo* and under high vacuum to give a viscous orange syrup, which was dissolved in argon-degassed CH₂Cl₂ (80 mL). The solution was washed with degassed sat. aq KCl (60 mL) and the aqueous was re-extracted (degassed CH₂Cl₂, 3 × 10 mL). The combined CH₂Cl₂ fractions were dried (Na₂SO₄) and concentrated *in vacuo* and under high vacuum to give an orange foam. Following purification by column chromatography (degassed acetone + 0.5% Et₃N → 2% methanol/acetone + 0.5% Et₃N) on silica gel pre-equilibrated with Et₃N, under argon pressure, and precipitation at rt from degassed CH₂Cl₂ (6 mL) into degassed hexane (350 mL), the desired phosphoramidite 179 was afforded, after drying under high vacuum, as a diastereomeric mixture (*ca.* 1:1), as an air-sensitive pale yellow foam (0.60 g, 0.67 mmol, 51%).

\[
R_f \ 0.30 \ (9:1, \ acetone/CH₂Cl₂ + 0.3\% \ Et₃N, \ A'), \ 0.24, \ 0.31 \ (5:95, \ methanol/acetone + 0.3\% \ Et₃N, \ A'), \ 0.17 \ (acetone + 0.3\% \ Et₃N, \ A');
\]

³¹P NMR (121 MHz, d₆-DMSO) \(\delta\) 149.2, 149.0 \(\text{P} \text{III}\); ¹H NMR (400 MHz, d₆-DMSO) \(\delta\) 8.69 (1H, s, NHCONH₂), 8.66 and 8.63 (1H, s, \(\text{H}^4\)), 7.63 (1H, br s, \(\text{H}^2\)), 7.44-7.38 (1H, m, \(\text{H}^6\)), 7.42 and 7.40 (2H, d, \(J = 8.0 \text{ Hz and } J = 8.5 \text{ Hz, } \text{H}^1\)), 7.34 (1H, t, \(J = 7.9 \text{ Hz, } \text{H}^5\)), 7.34-7.24 (3H, m, \(\text{H}^{15}, \text{H}^{16}\)), 7.30 and 7.27 (4H, d, \(J = 8.0 \text{ Hz and } J = 8.8 \text{ Hz, } \text{H}^{10}\)), 7.02 (1H, br d, \(J = 7.5 \text{ Hz, } \text{H}^5\)), 6.89 and 6.87 (2H, d, \(J = 8.3 \text{ Hz and } J = 8.6 \text{ Hz, } \text{H}^{11}\)), 6.89 and 6.87 (2H, d, \(J = 8.5 \text{ Hz, } \text{H}^{11}\)), 6.31 and 6.27 (1H, app. t, \(J = 5.8 \text{ Hz, } \text{H}^4\)), 5.92 (2H, br s, NH₂), 5.63 and 5.59 (1H, s, \(\text{H}^8\)), 4.68 and 4.64 (1H, td, \(J = 5.4, 16.1 \text{ Hz and } J = 5.9, 17.9 \text{ Hz, } \text{H}^3\)), 4.17 and 4.13 (1H, dd, \(J = 3.4, 7.7 \text{ Hz and } J = 3.8, 8.0 \text{ Hz, } \text{H}^3\)), 3.81-3.68 (1H, m, \(\text{H}^7\)), 3.71, 3.70 and 3.70 (3H, 3H and 6H, s, OCH₃), 3.69-3.61 (1H, m, \(\text{H}^7\)), 3.63-3.49 (2H, m, \(\text{H}^9\)), 3.46 (3H, s, NCH₃), 3.49-3.44 and 3.41 (1H, m and dd, \(J = 3.5, 11.5 \text{ Hz, } \text{H}^5\)), 192
3.46-3.41 and 3.37 (1H, m and dd, J = 2.9, 10.8 Hz, H5), 2.77 and 2.67 (1H, t, J = 5.9 Hz, H18), 2.59 (1H, td, J = 6.7, 13.6 Hz, H2'), 2.34 (1H, td, J = 6.0, 11.9 Hz, H2'), 2.59 (1H, td, J = 6.7, 13.6 Hz, H2'), 1.12 (3H, d, J = 6.8 Hz, H20), 1.15 (3H, d, J = 7.3 Hz, H20), 1.13 (3H, d, J = 7.3 Hz, H20), 1.02 (3H, d, J = 6.8 Hz, H20); LRMS (ES') m/z: 940 ([M + K]+, 8), 924 ([M + Na]+, 45), 902 ([M + H]+, 78), 303 (DMT+, 100); HRMS (ES'): calcd for C49H56N7O8P (M), [M + Na]+ = 924.3820, found 924.3825; [M + H]+ = 902.4001, found 902.4000. Found also DMT+ (calcd 303.1380, found 303.1381).

5'-O-(4,4'-Dimethoxytrityl)-5-[(3-ureidobenzoyl)methyl]-4N-methyl-2'-deoxycytidine, 183

A suspension of nucleoside 160 (0.20 g, 0.29 mmol) in 33 wt% MeNH2/ethanol (2.50 mL) was stirred under an argon atmosphere, at rt for 1 hour. The suspension dissolved over 25 minutes. The solvent was removed in vacuo to give a green crystalline solid. Following purification by column chromatography (5-20% methanol/ethyl acetate + 0.5% Et3N), and drying under high vacuum, the desired ring-opened nucleoside 183 was afforded as a yellow glassy solid (0.13 g, 0.18 mmol, 61%).

Rf 0.11 (9:1, acetone/ethyl acetate + 0.3% Et3N, A'), 0.25 (1:4, methanol/ethyl acetate + 0.3% Et3N, A'); LRMS (ES') m/z: 758 ([M + K]+, 10), 742 ([M + Na]+, 30), 720 ([M + H]+, 5), 303 (DMT+, 100); (ES') m/z: 816 ([M + H3PO4 – H]–, 100), 718 ([M – H]–, 41), 675 ([M – O=C=NH – H]–, 52); HRMS (ES'): calcd for C40H40N5O8 (M), [M + H]+ = 720.3028, found 720.3024.
3-N-[2’-Deoxy-5’-O-(4,4’-dimethoxytrityl)-β-D-ribofuranosyl]-6-(3-ureidophenyl) (2,3H)-N-methylpyrrolo[2,3-d]pyrimidine-2(7H)-one, 184

To a solution of 5’-O-(4,4’-dimethoxytrityl)-5-iodo-4’N-methyl-2’-deoxycytidine 185 (1.25 g, 1.87 mmol) in anhydrous DMF (6.0 mL), under an argon atmosphere and in absence of light, were added distilled Et₃N (1.30 mL, 9.33 mmol, 5.0 eq), CuI (0.14 g, 0.75 mmol, 0.4 eq), then alkynê 156 (0.90 g, 5.61 mmol, 3.0 eq). The reaction mixture was stirred for 15 minutes then tetrais(triphenylphosphine) palladium (0) (0.53 g, 0.46 mmol, 0.1 eq) was added and the reaction was stirred at rt for 2½ hours. The reaction mixture was diluted with methanol/acetone (1:1 v/v, 10 mL), filtered and the residue was washed with methanol/acetone (1:1 v/v, 3 × 10 mL). The combined filtrate was concentrated in vacuo and under high vacuum, co-evaporating with methanol/toluene to give a dark brown foam. The crude material was partially-purified by column chromatography (80-100% acetone/CH₂Cl₂ + 0.5% Et₃N → 5-20% methanol/acetone + 0.5% Et₃N), to afford, after drying under high vacuum, the product 188 plus alkynê-dimer impurity, as a pale brown foam (1.49 g).

Rₜ 0.18 (188), 0.12 (alkynê dimer) (1:9, methanol/CH₂Cl₂ + 0.3% Et₃N, A’), 0.11 (188), 0.21 (alkynê dimer) (4:1, acetone/CH₂Cl₂ + 0.3% Et₃N, A’), 0.44 (188 + alkynê dimer), (1:9, methanol/acetone + 0.3% Et₃N, A’).

To a stirred solution of partially-purified product 188 (1.48 g) in anhydrous DMF (10.0 mL) and distilled Et₃N (2.60 mL, 18.7 mmol, 10.0 eq), under an argon atmosphere, in absence of light, were added 4Å molecular sieves then CuI (0.39 g, 2.05 mmol, 1.1 eq), and the reaction was stirred at 125 °C for 40 minutes. The mixture was allowed to cool and diluted with methanol (20 mL). The mixture was filtered, the residue was washed with methanol (3 × 10 mL) and the combined filtrate was concentrated in vacuo and under high vacuum to give a green foam/resin. The compound was twice-purified by column chromatography (0-10% methanol/acetone + 0.5% Et₃N), then by precipitation
from methanol/acetone (1:1 v/v, 8 mL) into diethyl ether (230 ml), to afford, after
drying under high vacuum, the desired N-methylpyrrolo-dC product 184, as a very pale
yellow powdery solid (1.03 g, 1.47 mmol, 79% over two steps).

Rf 0.24 (1:9, methanol/acetone + 0.3% Et3N, A’); IR (solid): ν 3324 (br m, O–H, N–H), 2932, 2836 (w, C–H), 1651 (s, C=O),
1606 (s, C=C), 1556 (vs, C=N), 1507 (s, Aryl C=C), 1479 (s), 1441 (m), 1402 (s), 1338,
1303 (m), 1248 (s, O–H), 1176 (s), 1095, 1032 (s, C–O), 904 (m), 828 (s, C–H), 773,
726 (s), 701 (s, Aryl C–H), 659, 584, 534 (s) cm⁻¹; ¹H NMR (400 MHz, d₆-DMSO) δ 8.71 (1H, s, NHCONH₂), 8.63 (1H, s, H²), 7.63 (1H, t, J = 1.8 Hz, H¶), 7.41 (1H, dd,
J = 1.0, 2.0, 7.0 Hz, H⁴), 7.41 (2H, d, J = 7.0 Hz, H¹⁴), 7.34 (1H, t, J = 8.0 Hz, H⁵),
7.32 (2H, tt, J = 1.5, 7.0 Hz, H¹⁵), 7.02 (1H, td, J = 1.3, 7.5 Hz, H⁶), 6.89 (2H, d,
J = 9.0 Hz, H¹¹), 6.26 (1H, dd, J = 5.0, 6.5 Hz, H²); ¹³C NMR (100 MHz, d₆-DMSO) δ 158.5 (C⁷), 158.1 (C¹²–OCH₃), 155.9 (CONH₂), 153.5 (C²),
144.5 (C¹³), 142.6 (C⁶), 140.9 (C¹⁵–NHR), 136.2 (C⁴), 135.4, 135.1 (C⁶), 130.8 (C¹),
129.8, 129.7 (C¹⁰), 129.1 (C⁸), 127.9 (C¹⁵), 127.7 (C¹⁴), 126.8 (C¹⁶), 120.9 (C⁶), 118.1
(C⁴), 117.4 (C⁸), 113.3 (C¹¹), 107.6 (C⁸), 98.6 (C⁴), 86.7 (C⁵), 86.1 (C³Ar), 85.7
(C⁴), 69.0 (C⁵), 62.6 (C³), 55.0 (OCH₃), 41.5 (C²), 29.3 (NCH₃); LRMS (ES⁺) m/z:
1425 ([2M + Na]⁺, 8), 724 ([M + Na]⁺, 100), 702 ([M + H]⁺, 50); HRMS (ES⁺): calcd for C₄₀H₃₉N₇O₇ (M), [M + Na]⁺ = 724.2742, found 724.2733. Found also DMT⁺ (calcd
303.1380, found 303.1382); Anal. Calcd C₄₀H₃₉N₇O₇ (701.77), C 68.46%, H 5.60%, N
9.97%, found C 65.70%, H 5.82%, N 9.35%.
Method 1: To a stirred solution of acetylated nucleoside 187 (1.44 g, 2.07 mmol) and N-methylimidazole (2.10 mL, 26.3 mmol, 12.8 eq) in distilled pyridine (50.0 mL), under an argon atmosphere, at 0 °C, distilled phosphorous oxychloride (0.73 mL, 7.83 mmol, 3.8 eq) was added dropwise over 10 minutes. The yellow suspension of intermediate 186 was stirred at rt for 1 hour. A 40 wt% MeNH$_2$/water solution (10.0 mL, 116 mmol MeNH$_2$, 55.9 eq MeNH$_2$) was added whilst cooling in ice, and the cloudy solution was stirred at rt for 90 minutes. The pyridine was removed under high vacuum, and the mixture was diluted with water (200 mL). The aqueous solution was extracted with CH$_2$Cl$_2$ (4 × 100 mL), then the combined organic layer was washed with sat. aq KCl (200 mL) and dried (Na$_2$SO$_4$). Triethylamine (6.0 mL) was added to prevent detritylation and the solution was concentrated in vacuo, and dried under high vacuum. The viscous brown oil was dissolved in 33 wt% MeNH$_2$/ethanol (10.0 mL, 80.3 mmol MeNH$_2$, 38.8 eq MeNH$_2$) and stirred at rt for 40 minutes, then concentrated in vacuo and dried under high vacuum. Following purification by column chromatography (0-2% methanol/CH$_2$Cl$_2$ + 0.5% Et$_3$N), and drying under high vacuum, the 4N-methyl-dC nucleoside 185 was afforded, as a pale yellow foam (1.24 g, 1.86 mmol, 90%).

Method 2: Acetylated nucleoside 187 (6.85 g, 9.80 mmol) was treated with NMI and POCl$_3$ in pyridine, then 40 wt% MeNH$_2$/water and worked up, as described in Method 1. After treatment with 33 wt% MeNH$_2$/ethanol as described above, the resulting syrup was twice-purified by column chromatography (0-3% methanol/CH$_2$Cl$_2$ + 0.5% Et$_3$N, 0-15% acetone/CH$_2$Cl$_2$ + 0.5% Et$_3$N → 60% acetone/CH$_2$Cl$_2$ + 0.5% Et$_3$N) to yield a syrup containing significant NMI. Two further purifications (30-100% acetone/toluene + 0.5% Et$_3$N; 10-25% acetone/toluene + 1.0% pyridine) failed to remove NMI, however the product was successfully recrystallised from toluene (100 mL), and dried under high vacuum over KOH to afford the product 185 as a fluffy white crystalline solid (5.26 g, 7.86 mmol, 80%).
Method 3: Acetylated nucleoside 187 (8.27 g, 11.8 mmol) was treated with NMI and POCl₃ in pyridine, then 40 wt% MeNH₂/water and the reaction concentrated as described in Method 1. The resulting syrup was treated with 33 wt% MeNH₂/ethanol as described above, without an aqueous workup, then twice-purified by column chromatography (0-100% acetone/CH₂Cl₂ + 0.5% Et₃N → 5-15% methanol/acetone + 1.0% Et₃N). The orange/brown syrup was further purified by a combination of precipitation from toluene into diethyl ether, removal of NMI by soaking in diethyl ether, and recrystallisation from ethyl acetate to afford the product 185 as a white to off-white powdery/crystalline solid (3.62 g, 5.40 mmol, 46%).

Rf 0.40 (1:9, methanol/CH₂Cl₂ + 0.3% Et₃N, A’), 0.36 (4:1, acetone/CH₂Cl₂ + 0.3% Et₃N, A’), 0.57 (1:9, methanol/acetone + 0.3% Et₃N, A’); Mp 141-144 °C (ethyl acetate/diethyl ether); IR (solid): ν 3291 (br w, N–H), 3059, 2930, 2835 (w, C–H), 1634 (s, C=O), 1606 (vs, C=O, C=C), 1547 (s, C=O), 1505 (vs, C=C), 1397 (m, O–H), 1284 (s, C–O), 1246 (vs, C–N), 1173 (s), 1092, 1030 (m, C–O), 825, 779 (s, Aryl C=C), 700, 583 (s) cm⁻¹; ¹H NMR (400 MHz, d₆-DMSO) δ 7.93 (1H, s, H₆), 7.40 (2H, d, J = 7.5 Hz, H₁₃), 7.32 (2H, t, J = 7.3 Hz, H₁⁴), 7.29 (4H, d, J = 9.0 Hz, H₉), 7.22 (1H, tt, J = 2.0, 7.3 Hz, H₁⁵), 7.06 (1H, q, J = 4.5 Hz, NHCH₃), 6.89 (4H, d, J = 9.0 Hz, H₁⁰), 6.12 (1H, dd, J = 6.2, 7.4 Hz, H₁'), 5.25 (1H, d, J = 4.3 Hz, 3'-OH), 4.20 (1H, dt, J = 3.3, 6.4 Hz, H₃'), 3.91 (1H, dd, J = 3.3, 6.5 Hz, H₄'), 3.74 (6H, s, OC₃H₃), 3.20 (1H, dd, J = 3.3, 10.8 Hz, H₅'), 3.17 (1H, dd, J = 4.5, 10.8 Hz, H₆'), 2.80 (3H, d, J = 4.5 Hz, NHCH₃), 2.21 (1H, ddd, J = 3.0, 6.0, 13.3 Hz, H²), 2.09 (1H, td, J = 6.8, 13.6 Hz, H²); ¹³C NMR (100 MHz, d₆-DMSO) δ 163.0 (C₄), 159.8 (C₁₁–OMe), 155.3 (C²), 146.8 (C₆), 146.4 (C₁₂), 137.2, 137.1 (C₈), 131.4 (C₉), 129.6 (C₁⁴), 129.4 (C₁³), 128.4 (C₁⁵), 115.0 (C₉'), 87.5 (C₇Ar), 87.4 (C₄'), 87.0 (C¹'), 72.4 (C³'), 65.4 (C₈'), 59.9 (C₈–I), 56.8 (OCH₃), 42.4 (C²'), 30.4 (NHCH₃); LRMS (ES⁺) m/z: 692 ([M + Na]⁺), 670 ([M + H]⁺, 3), 303 (DMT⁺, 100); (ES⁻) m/z: 668 ([M – H]⁻, 100); HRMS (ES⁺): calcd for C₃₁H₃₂N₃O₆I (M, [M + K]⁺ = 708.0967, found 708.0962, [M + Na]⁺ = 692.1228, found 692.1219, [M + H]⁺ = 670.1409, found 670.1414. Found also DMT⁺ (calcd 303.1380, found 303.1386); Anal. Calcd C₃₁H₃₂N₃O₆I (669.51), C 55.61%, H 4.82%, N 6.27%, found C 54.80%, H 4.75%, N 6.05%.

3’-O-Acetyl-5’-O-(4,4’-dimethoxytrityl)-5-iodo-2’-deoxyuridine, 187²⁰⁹
To a stirred solution of nucleoside 97 (10.0 g, 15.2 mmol) in distilled pyridine (40.0 mL), under an argon atmosphere, at 0 °C was added acetic anhydride (7.20 mL, 76.3 mmol, 5.0 eq) dropwise over 20 minutes. The reaction was stirred at 0 °C for 15 minutes then allowed to warm to rt. After 2¾ hours, the reaction mixture was concentrated in vacuo and under high vacuum, co-evaporating with acetone (2 × 50 mL) and diethyl ether (2 × 50 mL), to give white foam/syrup. The syrup was dissolved in ethyl acetate (300 mL) and washed with water (3 × 75 mL). The aqueous was re-extracted (ethyl acetate, 40 mL) and combined organic fractions were washed with sat. aq KCl (120 mL), dried (Na₂SO₄) and concentrated in vacuo and under high vacuum to give a white foam. Following purification by column chromatography (0-2% methanol/CH₂Cl₂ + 0.5% Et₃N), and drying under high vacuum, the desired product 187 was afforded as a white foam (10.3 g, 14.7 mmol, 97%).

**Rf** 0.31 (1:9, acetone/CH₂Cl₂ + 0.3% Et₃N, A’), 0.48 (5:95, methanol/CH₂Cl₂ + 0.3% Et₃N, A’), 0.33 (7:3, ethyl acetate/hexane, A’); **IR** (solid): ν 3056 (w, C–H, N–H), 2933, 2835 (w, C–H), 1683 (br s, C=O), 1606, 1506 (m, Aryl C=C), 1442 (m, C–H), 3056 (w, C–H, N–H), 303 (DMT, 100); **LRMS** (ES⁺) m/z: 721 ([M + Na]+, 12), 303 (DMT+, 100); (ES⁻) m/z: 697 ([M – H]⁻, 100); **HRMS** (ES⁺): calcd for C₆₀H₃₅N₂O₁₂I (M), [M +
Et₃N + H]⁺ = 800.2402, found 800.2369; [M + Na]⁺ = 721.1017, found 721.1007. Found also DMT⁺ (calc 303.1380, found 303.1383).

3-N-[2’-Deoxy-5’-O-(4,4’-dimethoxytrityl)-β-D-ribofuranosyl]-6-(3-acetamidophenyl)-(2,3H)-N-methylpyrrolo[2,3-d]pyrimidine-2(7H)-one-3’-O-(2-O-cyanoethyl-N,N-diisopropyl) phosphoramidite, 191

To a stirred solution of nucleoside 193 (1.37 g, 1.95 mmol) in distilled THF (3.5 mL) and anhydrous DMF (4.5 mL), strictly under an argon atmosphere and excluding moisture, was added distilled DIPEA (0.86 mL, 4.91 mmol, 2.5 eq) followed by chloro-phosphitylating reagent 70 (0.48 mL, 2.15 mmol, 1.1 eq) dropwise, and the reaction was stirred at rt for 1½ hours. The THF was removed in vacuo and the residue was dissolved in argon-degassed ethyl acetate (60 mL). The solution was washed with degassed sat. aq KCl (3 × 40 mL), dried (Na₂SO₄), and concentrated in vacuo and dried under high vacuum to give a pale orange foam. Following purification by column chromatography (7:3, acetone/CH₂Cl₂ + 0.5% Et₃N), on silica gel pre-equilibrated with Et₃N, under argon pressure, the desired phosphoramidite 191 was afforded, after drying under high vacuum, as a diastereomeric mixture (ca. 1:1), as an air-sensitive, pale milky yellow foam (1.51 g, 1.68 mmol, 86%).

Rf 0.33, 0.46 (4:1, acetone/CH₂Cl₂ + 0.3% Et₃N, A’); ³¹P NMR (121 MHz, d₆-DMSO) δ 149.2, 149.0 (P III); ¹H NMR (400 MHz, d₆-DMSO) δ 10.08 (1H, s, NΗCOCH₃), 8.68 and 8.65 (1H, s, H₄”), 7.79 (1H, br s, H₂”), 7.62 (1H, br d, J = 8.0 Hz, H₄”), 7.42 (1H, t, J = 7.8 Hz, H₅”), 7.42 and 7.40 (2H, d, J = 8.5 Hz and J = 8.0 Hz, H₄”), 7.34-7.29 (2H, m, H₁”), 7.30 and 7.28 (4H, d, J = 9.0 Hz, H₁”), 7.30-7.23 (1H, m, H₁”), 7.16 (1H, br d, J =
7.5 Hz, H\(^6\)), 6.89, 6.88 and 6.87 (2H, 2H and 4H, d, J = 9.0 Hz, J = 8.5 Hz and J = 9.0 Hz, H\(^1\)), 6.31 and 6.27 (1H, app. t, J = 5.8 Hz, H\(^4\)), 5.66 and 5.63 (1H, s, H\(^6\)), 4.68 and 4.65 (1H, ddd, J = 5.5, 16.3 Hz and J = 6.0, 16.7 Hz, H\(^3\)), 4.18 and 4.14 (1H, dd, J = 3.3, 7.8 Hz and J = 3.8, 8.3 Hz, H\(^4\)), 3.82-3.69 (1H, m, H\(^7\)), 3.70, 3.70 and 3.69 (3H, 3H and 6H, s, OCH\(_3\)), 3.70-3.60 (1H, m, H\(^7\)), 3.62-3.48 (2H, m, H\(^9\)), 3.47 (3H, s, NCH\(_3\)), 3.47 and 3.43-3.39 (1H, dd and m, J = 4.5, 11.0 Hz, H\(^5\)), 3.44 and 3.38 (1H, dd, J = 3.5, 10.5 Hz and J = 3.0, 10.5 Hz, H\(^5\)), 2.77 and 2.67 (1H, t, J = 6.0 Hz, H\(^10\)), 2.61 and 2.59 (1H, td, J = 6.9, 13.4 Hz and J = 6.5, 13.1 Hz, H\(^2\)), 2.35 (1H, td, J = 6.3, 13.1 Hz, H\(^3\)), 2.08 (3H, s, COCH\(_3\)), 1.15 (3H, d, J = 7.0 Hz, H\(^20\)), 1.13 (3H, d, J = 7.0 Hz, H\(^20\)), 1.12 (3H, d, J = 7.0 Hz, H\(^20\)), 1.02 (3H, d, J = 7.0 Hz, H\(^20\)); LRMS (ES\(^+\)) m/z: 939 ([M + K]\(^+\), 5), 923 ([M + Na]\(^+\), 100), 901 ([M + H]\(^+\), 9); HRMS (ES\(^+\)): calcd for C\(_{50}H_{77}N_{16}O_{13}P\) (M), [M + Et\(_3\)N + H][\(^+\)] = 1002.5253, found 1002.5261; [M + Na][\(^+\)] = 923.3868, found 923.3886; [M + H][\(^+\)] = 901.4048, found 901.4033.

3-N-[2’-Deoxy-5’-O-(4,4’-dimethoxytrityl)-β-D-ribofuranosyl]-6-(3-acetamidophenyl)-(2,3H)-N-methylpyrrolo[2,3-d]pyrimidine-2(7H)-one, 193

To a solution of 5’-O-(4,4’-dimethoxytrityl)-5-ido-4N-methyl-2’-deoxycytidine 185 (1.69 g, 2.53 mmol) in anhydrous DMF (6.0 mL), under an argon atmosphere and in absence of light, were added distilled Et\(_3\)N (1.77 mL, 12.7 mmol, 5.0 eq), CuI (0.19 g, 1.01 mmol, 0.4 eq), then alkyne 161 (1.21 g, 7.60 mmol, 3.0 eq). The reaction mixture was stirred for 20 minutes then tetrakis(triphenylphosphine) palladium (0) (0.29 g, 0.25 mmol, 0.1 eq) was added and the reaction was stirred at rt for 1 hour. The reaction mixture was concentrated under high vacuum, co-evaporating with acetone to give a black/brown foam. The residue was dissolved in CH\(_2\)Cl\(_2\) (100 mL) and washed with 5% w/v aq Na\(_2\)EDTA (pH 9, 3 × 70 mL). The aqueous was re-extracted (CH\(_2\)Cl\(_2\), 2 × 50 mL) and combined organic fractions were washed with sat. aq KCl (100 mL), dried
(Na₂SO₄), and concentrated in vacuo and under high vacuum to give a golden brown foam. The crude material was partially-purified by column chromatography (10-30% acetone/CH₂Cl₂ + 1.0% pyridine → 50-70% acetone/CH₂Cl₂ + 1.0% pyridine), to afford, after drying under high vacuum, the product 192 plus alkyne-dimer impurity, as a yellow glassy foam (1.99 g).

Rf 0.16 (3:2, acetone/CH₂Cl₂ + 0.3% Et₃N, A⁺), 0.04 (1:4, acetone/CH₂Cl₂ + 0.5% pyridine, A⁺), 0.28 (4:1, acetone/CH₂Cl₂ + 0.3% Et₃N, A⁺), 0.32 (4:1, acetone/CH₂Cl₂ + 1.0% pyridine, A⁺); ^1H NMR (400 MHz, d₆-DMSO) δ 9.97 (1H, s, NHOCH₃), 8.00 (1H, s, H⁶), 7.79 (1H, s, H²⁵), 7.46 (1H, dd, J = 1.0, 2.0, 8.5 Hz, H¹⁵), 7.43 (1H, q, J = 5.0 Hz, NHCH₃), 7.40 (1H, dd, J = 1.3, 8.3 Hz, H¹⁴), 7.29 (2H, d, J = 9.0 Hz, H¹¹), 7.29-7.25 (1H, m, H⁸), 7.28 (2H, d, J = 8.5 Hz, H¹¹), 7.24 (2H, t, J = 7.8 Hz, H¹⁰), 7.15 (1H, tt, J = 1.3, 7.3 Hz, H¹⁷), 6.90 (1H, td, J = 1.3, 7.8 Hz, H⁶), 6.84 (2H, d, J = 9.0 Hz, H¹²), 6.83 (2H, d, J = 9.0 Hz, H¹³), 6.16 (1H, t, J = 6.8 Hz, H¹⁰), 5.28 (1H, d, J = 4.5 Hz, 3'-OH), 4.26 (1H, dt, J = 3.5, 6.7 Hz, H³⁵), 3.97 (1H, td, J = 3.0, 5.0 Hz, H⁶), 3.66 (6H, s, OCH₃), 3.23 (1H, dd, J = 5.0, 10.5 Hz, H⁶), 3.15 (1H, dd, J = 2.5, 10.5 Hz, H⁶), 2.86 (3H, d, J = 4.5 Hz, NHCH₃), 2.27 (1H, ddd, J = 3.5, 6.3, 13.6 Hz, H³), 2.16 (1H, td, J = 6.7, 13.6 Hz, H²), 2.06 (3H, s, COCH₃); ^13C NMR (100 MHz, d₆-DMSO) δ 168.4 (COCH₃), 161.7 (C⁴−NHCH₃), 158.0, 158.0 (C¹³−OCH₃), 153.2 (C²), 144.7 (C¹⁴), 143.0 (C⁰), 139.1 (C³⁵), 135.6, 135.3 (C³⁹), 129.7, 129.6 (C¹¹), 128.5 (C⁸), 127.8 (C¹⁶), 127.6 (C¹⁵), 126.6 (C¹⁷), 126.4 (C⁶), 122.5 (C¹⁰), 121.9 (C⁴), 119.4 (C⁵), 113.2, 113.1 (C¹²), 94.3 (C⁸), 90.3 (C⁷), 85.9 (C⁴), 85.8 (C³Ar), 85.6 (C¹⁰), 80.4 (C⁵), 70.7 (C³), 63.8 (C⁵), 54.9 (OCH₃), 40.8 (C²), 27.8 (NHCH₃), 23.9 (COCH₃); LRMS (ES⁺) m/z: 723 ([M + Na]⁺, 100), 303 (DMT⁺, 29); (ES⁻) m/z: 699 ([M − H]⁻, 100); HRMS (ES⁺): calefd for C₄₁H₉₀N₅O₇ (M), [M + H]⁺ = 701.2970, found 701.2974. Founded also DMT⁺ (calcd 303.1380, found 303.1381).

To a stirred solution of partially-purified alkynylated product 192 (1.92 g) in anhydrous DMF (8.5 mL) and distilled Et₃N (3.40 mL, 24.4 mmol, 10.0 eq), under an argon atmosphere and in absence of light, were added 4Å molecular sieves then CuI (0.51 g, 2.68 mmol, 1.1 eq), and the reaction was stirred at 125 °C for 35 minutes. The mixture was allowed to cool then filtered and the filtrate was concentrated under high vacuum to give a sticky green/brown foam. The residue was dissolved in CH₂Cl₂ (150 mL) and filtered, and the filtrate was washed with 5% w/v aq Na₂EDTA (pH 9, 3 × 100 mL), sat. aq KCl (100 mL), dried (Na₂SO₄), and concentrated in vacuo and under high vacuum to
give a green/orange foam. Following purification by column chromatography (0-10% methanol/acetone + 0.5% Et$_3$N), and drying under high vacuum, the desired cyclised product 193 was afforded, as a yellow powdery solid (1.43 g, 2.04 mmol, 84% over two steps).

R$_f$ 0.09 (4:1, acetone/CH$_2$Cl$_2$ + 0.3% Et$_3$N, A’), 0.14 (4:1, acetone/CH$_2$Cl$_2$ + 1.0% pyridine, A’); $^1$H NMR (400 MHz, d$_6$-DMSO) δ 10.08 (NHCOC$_3$H$_3$), 8.65 (1H, s, H$^4$), 7.79 (1H, br s, H$^5$), 7.61 (1H, br d, J = 8.0 Hz, H$^6$), 7.42 (1H, t, J = 8.0 Hz, H$^7$), 7.40 (2H, d, J = 7.0 Hz, H$^14$), 7.32 (2H, t, J = 7.0 Hz, H$^15$), 7.29 (2H, d, J = 8.5 Hz, H$^{16}$), 7.28 (2H, d, J = 9.0 Hz, H$^{18}$), 7.25 (1H, t, J = 7.0 Hz, H$^{19}$), 7.16 (1H, br d, J = 8.0 Hz, H$^{18}$), 6.90 (2H, d, J = 9.0 Hz, H$^{19}$), 6.89 (2H, d, J = 9.0 Hz, H$^{19}$), 6.26 (1H, dd, J = 5.3, 6.3 Hz, H$^{15}$), 5.62 (1H, s, H$^8$), 5.39 (1H, d, J = 5.0 Hz, 3'-OH), 4.43 (1H, td, J = 5.5, 11.0 Hz, H$^3$), 4.01 (1H, dd, J = 4.0, 8.0 Hz, H$^4$), 3.70 (3H, s, OC$_3$H$_3$), 3.59 (1H, d, J = 9.0 Hz, H$^5$), 3.30 (1H, dd, J = 3.0, 11.0 Hz, H$^3$), 2.45 (1H, td, J = 6.5, 13.6 Hz, H$^2$), 2.19 (1H, ddd, J = 5.0, 6.5, 13.6 Hz, H$^2$), 2.08 (3H, s, COCH$_3$); $^{13}$C NMR (100 MHz, d$_6$-DMSO) δ 168.5 (COCH$_3$), 158.5 (C$^7$), 158.1 (C$^{12}$), 153.4 (C$^3$), 144.5 (C$^{13}$), 142.2 (C$^6$), 139.6 (C$^3$), 136.4 (C$^4$), 135.4, 135.1 (C$^9$), 130.9 (C$^5$), 129.8, 129.7 (C$^{18}$), 129.2 (C$^{18}$), 127.9 (C$^{15}$), 127.7 (C$^{14}$), 126.8 (C$^{16}$), 122.8 (C$^6$), 119.2 (C$^4$), 118.5 (C$^2$), 113.3 (C$^{11}$), 107.5 (C$^{19}$), 98.9 (C$^5$), 86.7 (C$^7$), 86.1 (C$^8$Ar$_1$), 85.7 (C$^4$), 69.0 (C$^3$), 62.6 (C$^5$), 55.0 (OCH$_3$), 41.5 (C$^2$), 29.3 (NCH$_3$), 24.0 (COCH$_3$); LRMS (ES') m/z: 803 ([M + Et$_3$N + H]$^+$, 14), 723 ([M + Na]$^+$, 100); (ES') m/z: 699 ([M – H]$^-$, 100); HRMS (ES'): calcd for C$_{41}$H$_{38}$N$_6$O$_7$ (M), [M + H]$^+$ = 701.2970, found 701.2965. Found also DMT$^+$ (calcd 303.1380, found 303.1379).

5'-O-(4,4'-Dimethoxytrityl)-5'-(3-aminophenyl)ethynyl]-4N-methyl-2'-deoxycytidine, 194
To a solution of 5'-O-(4,4'-dimethoxytrityl)-5-iodo-4N-methyl-2'-deoxycytidine (0.97 g, 1.45 mmol) in anhydrous DMF (4.0 mL), under an argon atmosphere and in absence of light, were added distilled Et₃N (1.0 mL, 7.25 mmol, 5.0 eq) then CuI (0.11 g, 0.58 mmol, 0.4 eq) and alkyne (0.49 mL, 4.35 mmol, 3.0 eq). The reaction mixture was stirred for 20 minutes then tetrakis(triphenylphosphine) palladium (0) (0.17 g, 0.15 mmol, 0.1 eq) was added and the reaction was stirred at rt for 1½ hours. The reaction mixture was concentrated under high vacuum, co-evaporating with methanol/toluene/acetone to give an orange/brown foam. The residue was dissolved in methanol/acetone (1:1 v/v, 40 mL) and filtered. The solid was washed with methanol/acetone (1:1 v/v, 2 × 20 mL), and the combined filtrate was concentrated in vacuo and dried under high vacuum to give an orange/brown foam. Following purification by column chromatography (0-3% methanol/CH₂Cl₂ + 0.5% Et₃N), and drying under high vacuum, the product was afforded, as a pale orange foam (0.91 g, 1.39 mmol, 95%).

R₉ 0.31 (1:9, methanol/CH₂Cl₂ + 0.3% Et₃N); 0.31 (4:1, acetone/CH₂Cl₂ + 0.3% Et₃N, A’); 0.03 (1:4, acetone/CH₂Cl₂ + 0.3% Et₃N, A’); IR (solid): ν 3337 (br m, O–H, N–H), 2934, 2835 (w, C–H), 1640 (s, C=O, C=C), 1596 (s, Aryl C–C), 1556 (s), 1505 (vs, C=N), 1444 (m, C–H), 1403 (m), 1352 (m, C–H), 1285 (m, O–H), 1246, 1174 (s), 1086 (br s, C–O), 1029 (br s), 942 (m), 866 (m, C–H), 826 (s, Aryl C–H), 780 (s), 726 (m), 686 (Aryl-C–H), 583, 537, 518 (s) cm⁻¹; ¹H NMR (400 MHz, d₆-DMSO) δ 7.94 (1H, s, H₆), 7.40 (1H, dd, J = 1.1, 8.4 Hz, H¹⁴), 7.35 (1H, q, J = 4.8 Hz, NHCH₃), 7.30-7.25 (2H, m, H¹⁶), 7.29 (2H, d, J = 8.0 Hz, H¹¹), 7.29 (2H, d, J = 9.0 Hz, H¹⁴), 7.17 (1H, tt, J = 1.0, 7.3 Hz, H¹⁷), 6.96 (1H, t, J = 7.8 Hz, H⁸), 6.86 (2H, d, J = 8.5 Hz, H¹²), 6.83 (2H, d, J = 8.8 Hz, H¹³), 6.61 (1H, t, J = 1.6 Hz, H²'), 6.57 (1H, ddd, J = 1.1, 2.4, 8.0 Hz, H⁴'), 6.46 (1H, ddd, J = 1.1, 2.5, 7.5 Hz, H⁶'), 6.15 (1H, app. t, J = 6.7 Hz, H¹'), 5.30 (1H, d, J = 4.3 Hz, 3’-OH), 5.11 (1H, br s, NH₂), 4.25 (1H, td, J = 3.1, 9.0 Hz, H⁵'), 3.97 (1H, td, J = 2.4, 4.8 Hz, H⁴'), 3.68 (3H, s, OCH₃), 3.67 (3H, s, OCH₃), 3.23 (1H, dd, J = 5.0, 10.5 Hz, H⁸'), 3.14 (1H, dd, J = 2.5, 10.3 Hz, H⁵'), 2.86 (3H, d, J = 4.8 Hz, NHCH₃), 2.27 (1H, ddd, J = 3.0, 5.9, 13.4 Hz, H²'), 2.13 (1H, td, J = 6.8, 13.6 Hz, H³); ¹³C NMR (100 MHz, d₆-DMSO) δ 161.7 (C⁴–NHCH₃), 158.0, 158.0 (C¹³–OCH₃), 153.2 (C¹), 148.3 (C¹⁶), 144.6 (C³⁵), 142.5 (C⁸), 135.6, 135.2 (C¹⁰), 129.7, 129.6 (C¹¹), 128.7 (C⁸'), 127.8 (C¹⁶), 127.6 (C¹⁴), 126.6 (C¹⁷), 122.4 (C¹'), 119.2 (C⁶'), 116.4 (C²'), 114.5 (C⁴'), 113.2, 113.2 (C¹²), 95.3 (C⁸), 90.6 (C³'), 85.9 (C⁴'), 85.8 (C⁸Ar), 85.6 (C⁴'), 79.0 (C⁵), 70.8 (C³'), 63.7 (C⁸'), 54.9 (OCH₃), 40.8 (C²'), 27.9 (NHCH₃); LRMS (ES⁺) m/z:
681 ([M + Na]+, 100); HRMS (ES+): calcd for C_{41}H_{40}N_{4}O_{7} (M), [M + Na]+ = 681.2684, found 681.2671; [M + H]+ = 659.2864, found 659.2847. Found also DMT+ (calcd 303.1380, found 303.1381).

3-N-[2’-Deoxy-5’-O-(4,4’-dimethoxytrityl)-β-D-ribofuranosyl]-6-(3-aminophenyl)-(2,3H)-N-methylpyrrolo[2,3-d]pyrimidine-2(7H)-one, 195

Method 1: To a stirred solution of alkylnylated product 194 (2.21 g, 3.36 mmol) in anhydrous DMF (9.0 mL) and distilled Et3N (4.70 mL, 33.7 mmol, 10.0 eq), under an argon atmosphere, in absence of light, were added 4Å molecular sieves then CuI (0.70 g, 3.70 mmol, 1.1 eq), and the reaction was stirred at 125 °C for 50 minutes. The mixture was allowed to cool, filtered and the filtrate was concentrated under high vacuum, co-evaporating with methanol/toluene/acetone to give a dark brown/gold foam. The foam was dissolved in DMF (80 mL), filtered, the solid was washed with DMF (3 × 60 mL) and the combined filtrate was concentrated under high vacuum at 35 °C to give a sticky, dark brown foam/gum. The mixture was finally suspended/dissolved in CH2Cl2 (100 mL), soaked overnight and filtered. The solid residue was washed (CH2Cl2, 3 × 50 mL), and the combined filtrate was concentrated in vacuo to give a sticky orange/brown foam. Following purification by column chromatography (50-100% acetone/CH2Cl2 + 0.5% Et3N → 5% methanol/acetone + 0.5% Et3N), and drying under high vacuum, the desired product 195 and bis-tritylated nucleoside 196 were isolated, as pale orange foams (195, 0.71 g, 1.07 mmol, 32%; 196, 0.38 g, 0.39 mmol, 12%).
**Method 2:** Cyclised nucleoside 195 was prepared using nucleoside 194 (0.88 g, 1.34 mmol), by the same procedure as in Method 1, with a reaction time of 30 minutes. The mixture was allowed to cool, filtered, and the filtrate was concentrated under high vacuum to give a viscous brown syrup. The residue was suspended/dissolved in CH$_2$Cl$_2$ (50 mL) and filtered. The solid residue was washed (CH$_2$Cl$_2$, 2 × 25 mL) and the combined filtrate was concentrated *in vacuo* and under high vacuum to give a brown foam. Following purification by column chromatography (50-100% acetone/CH$_2$Cl$_2$ + 0.5% Et$_3$N), and drying under high vacuum, the desired cyclised product 195 was afforded, as a pale brown foam (0.71 g, 1.07 mmol, 80%).

**Product 195:**

R$_f$ 0.08 (5:95, methanol/CH$_2$Cl$_2$ + 0.3% Et$_3$N, A’), 0.14 (4:1, acetone/CH$_2$Cl$_2$ + 0.3% Et$_3$N, A’); IR (solid): ν 3336 (w, O–H, N–H), 3064, 2930, 2835 (w, C–H), 1699 (w), 1651 (s, C=O), 1605 (s, C=C), 1553 (s, C=N), 1507 (s, Aryl C–C), 1476 (s, N–H), 1445, 1398 (s), 1338 (m), 1301 (s), 1246 (vs), 1174 (s), 1095 (s, C–O), 1030 (vs), 969, 914 (m), 827 (s, C–H), 772, 726 (s), 701 (s, Aryl C–H), 659, 584, 530 (s) cm$^{-1}$; ¹H NMR (400 MHz, d$_6$-DMSO) δ 8.63 (1H, s, H$_4$), 7.41 (2H, d, J = 7.3 Hz, H$_{14}$), 7.32 (2H, t, J = 7.0 Hz, H$_{15}$), 7.29 (2H, d, J = 8.8 Hz, H$_{10}$), 7.25 (1H, tt, J = 1.8, 7.3 Hz, H$_{16}$), 7.13 (1H, t, J = 7.8 Hz, H$_{5"}$), 6.90 (2H, d, J = 8.8 Hz, H$_{11}$), 6.89 (2H, d, J = 9.0 Hz, H$_{11}$), 6.67 (1H, t, J = 1.8 Hz, H$_{2"}$), 6.64 (1H, ddd, J = 0.8, 2.2, 8.0 Hz, H$_{8}$), 6.60 (1H, td, J = 1.3, 8.0 Hz, H$_{6}$), 6.26 (1H, dd, J = 5.0, 6.3 Hz, H$_{5}$), 5.51 (1H, s, H$_5$), 5.42 (1H, d, J = 4.8 Hz, 3'-OH), 5.26 (2H, br s, NH$_2$), 4.44 (1H, td, J = 5.3, 10.5 Hz, H$_3$), 4.00 (1H, dd, J = 3.9, 7.9 Hz, H$_4$), 3.71 (3H, s, OCH$_3$), 3.70 (3H, s, OCH$_3$), 3.44 (3H, s, NCH$_3$), 3.39 (1H, dd, J = 3.9, 10.7 Hz, H$_8$), 3.30 (1H, d, J = 2.6, 10.9 Hz, H$_8$), 2.44 (1H, td, J = 6.5, 13.1 Hz, H$_2$), 2.19 (1H, ddd, J = 5.1, 6.4, 13.4 Hz, H$_2$); $^{13}$C NMR (100 MHz, d$_6$-DMSO) δ 158.5 (C$_{7a}$), 158.2 (C$_{12}$–OCH$_3$), 153.5 (C$_2$), 149.0 (C$_{16}$–NHR), 144.5 (C$_{15}$), 143.4 (C$_6$), 135.9 (C$_4$), 135.5, 135.2 (C$_9$), 131.1 (C$_{15}$), 129.8, 129.7 (C$_8$), 129.3 (C$_5$), 128.0 (C$_{15}$), 127.8 (C$_{14}$), 126.9 (C$_{16}$), 115.6 (C$_6$), 114.3 (C$_4$), 113.3 (C$_{14}$,C$_2$), 107.7 (C$_{4a}$), 98.0 (C$_5$), 86.6 (C$_4$), 86.1 (C$_8$Ar), 85.6 (C$_4$), 69.0 (C$_3$), 62.6 (C$_5$), 55.0 (OCH$_3$), 41.5 (C$_2$), 29.4 (NCH$_3$); LRMS (ES$^+$) m/z: 681 ([M + Na]$^+$, 100); HRMS (ES$^+$): calcd for C$_{39}$H$_{38}$N$_4$O$_6$ (M), [M + Na]$^+$ = 681.2684, found 681.2677. Found also DMT$^+$ (calcd 303.1380, found 303.1381); Anal. Calcd C$_{39}$H$_{38}$N$_4$O$_6$ (658.75), C 71.11%, H 5.81%, N 8.50%, found C 69.90%, H 6.11%, N 8.41%.

Analytical results are consistent with the reported data.
3-N-[2′-Deoxy-5′-O-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl]-6-[3-(4,4′-dimethoxytrityl)aminophenyl]-(2,3H)-N-methylpyrrolo[2,3-d]pyrimidine-2(7H)-one, 196:

Rf 0.33 (4:1, acetone/CH2Cl2 + 0.3% Et3N, A’); 1H NMR (400 MHz, d6-DMSO) δ 8.57 (1H, s, H4), 7.40 (2H, d, J = 7.3 Hz, H14), 7.33-7.26 (4H, m, H15, H23), 7.28 (4H, d, J = 8.8 Hz, H10), 7.26-7.19 (3H, m, H16, H24), 7.22 (4H, d, J = 8.8 Hz, H19), 7.16 (1H, tt, J = 1.6, 7.0 Hz, H25), 6.97 (1H, t, J = 7.9 Hz, H3′), 6.89 (2H, d, J = 9.0 Hz, H11), 6.88 (2H, d, J = 9.5 Hz, H12), 6.85 (4H, d, J = 9.0 Hz, H20), 6.64 (1H, br dd, J = 1.4, 8.2 Hz, H8), 6.54 (1H, d, J = 7.8 Hz, H9), 6.51 (1H, t, J = 1.8 Hz, H10′), 6.22 (1H, dd, J = 5.3, 6.3 Hz, H3′), 5.39 (1H, d, J = 4.5 Hz, 3′-OH), 5.38 (1H, s, NHDMT), 4.40 (1H, qn, J = 5.5 Hz, H5′), 3.98 (1H, dd, J = 3.8, 8.0 Hz, H4′), 3.69 (6H, s, C21-OCH3), 3.69 (3H, s, C12-OCH3), 3.68 (3H, s, C12′-OCH3), 3.36 (1H, dd, J = 3.8, 10.4 Hz, H2′), 3.28 (1H, d, J = 2.8, 10.5 Hz, H5′), 3.12 (3H, s, NCH3), 2.41 (1H, td, J = 6.4, 12.8 Hz, H3), 2.15 (1H, ddd, J = 5.3, 6.3, 13.1 Hz, H1); 13C NMR (100 MHz, d6-DMSO) δ 158.3 (C2′), 158.2, 158.1 (C12-OCH3), 157.6 (C21-OCH3), 153.4 (C2), 147.2 (C3′-NHR), 145.7 (C22), 144.4 (C13), 143.2 (C8′), 137.2 (C18′), 135.9 (C4′), 135.6, 135.2 (C9′), 129.9 (C19, C11′), 129.8, 129.6 (C10′), 128.7 (C23), 128.2 (C5′), 127.9 (C14, C24), 127.8 (C16′), 126.4 (C25), 116.8 (C6′), 116.4 (C4′), 115.3 (C2′), 113.3, 113.1 (C11, C28), 107.5 (C4′′), 98.0 (C5′), 86.6 (C1′), 86.0 (C5′Ar), 85.6 (C4′), 69.5 (C17Ar), 69.0 (C3′), 62.6 (C5′), 55.0 (OCH3), 41.5 (C2′), 29.1 (NCH3); LRMS (ES+) m/z: 1069 ([M + Et3N + H]+, 16), 983 ([M + Na]+, 59), 961 ([M + H]+, 27), 303 (DMT+, 100).

3-N-[2′-Deoxy-5′-O-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl]-6-(3-trifluoroacetamidophenyl)-(2,3H)-N-methylpyrrolo[2,3-d]pyrimidine-2(7H)-one, 197

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**Method 1:** To a stirred solution of nucleoside 195 (0.52 g, 0.79 mmol) in distilled pyridine (4.0 mL), under an argon atmosphere and excluding moisture, at rt, was added TMSCl (0.15 mL, 1.5 eq) slowly. After stirring for 2 hours, further TMSCl (0.20 mL, 2.0 eq) was added, and the reaction was stirred for a further 30 minutes. The reaction mixture was cooled to 0 °C, then trifluoroacetic anhydride (0.22 mL, 2.0 eq) was added dropwise. The reaction was stirred at 0 °C for 10 minutes, then allowed to warm to rt for 25 minutes. Water (1.0 mL) was added and the pale yellow solution was stirred for 25 minutes. The reaction mixture was reduced to approximately one-third volume, then was diluted with CH₂Cl₂ (150 mL), adding Et₃N (1.5 mL) to prevent detritylation. The solution was washed with water (60 mL), the aqueous was re-extracted (CH₂Cl₂, 2 × 50 mL), and combined CH₂Cl₂ fractions were washed with sat. aq KCl (140 mL), dried (Na₂SO₄) and concentrated in vacuo and dried under high vacuum to give a yellow gum. The compound was twice-purified by column chromatography (50-80% acetone/CH₂Cl₂ + 0.5% Et₃N; 10-60% acetone/CH₂Cl₂ + 0.5% Et₃N → 5% methanol/acetone + 0.5% Et₃N), from which the product 197 was isolated, after drying under high vacuum, as a pale orange foam (0.19 g, 0.25 mmol, 32%). A mixture containing product 197 and bis-trifluoroacetylated compound 198 (ca. 11:2) was also isolated (0.31 g).

**Method 2:** Product 197 was prepared using nucleoside 195 (0.51 g, 0.78 mmol) as described in Method 1, using 5 eq of TMSCl, with a total reaction time of 3½ hours. After addition of trifluoroacetic anhydride, white fumes were observed, hence Et₃N (2.0 mL) was added, before water, to prevent detritylation. Water (2.0 mL) was then added and the orange/brown solution was stirred for 2 hours at rt. The reaction mixture was diluted with CH₂Cl₂ (300 mL) then washed with water (300 mL). The aqueous was re-extracted (CH₂Cl₂, 2 × 50 mL), and the combined CH₂Cl₂ fraction was washed with 15% aq NH₃ (100 mL) and sat. aq KCl (140 mL), dried (Na₂SO₄) and concentrated in vacuo to give a brown gum. Following purification by column chromatography (5-80% acetone/CH₂Cl₂ + 0.5% Et₃N), and drying under high vacuum, the desired product 197 was afforded, as a pale orange foam (0.31 g, 0.41 mmol, 53%).

R_f 0.31 (197), 0.41 (198) (7:3, acetone/CH₂Cl₂ + 0.5% Et₃N, A’), 0.08 (197) (1:4, acetone/CH₂Cl₂ + 0.5% Et₃N, A’); ¹⁹F NMR (282 MHz, d₆-DMSO) δ -73.6 (NHCOCF₃); ¹H NMR (400 MHz, d₆-DMSO) δ 11.36 (NH, s, H₄), 7.83 (1H, br s, H²), 7.75 (1H, td, J = 0.9, 8.3 Hz, H⁴), 7.55 (1H, t, J = 8.0 Hz, H⁵), 7.40 (2H, d, J = 8.0 Hz, H¹⁴), 7.37 (1H, d, J = 8.2 Hz, H⁶), 7.32 (2H, t, J = 7.3 Hz, H¹⁵),
7.29 (4H, d, J = 8.3 Hz, H^10), 7.28 (4H, d, J = 9.0 Hz, H^10), 7.24 (1H, t, J = 7.5 Hz, H^16), 6.89 (2H, d, J = 8.5 Hz, H^11), 6.88 (2H, d, J = 9.0 Hz, H^11), 6.25 (1H, app. t, J = 5.7 Hz, H^1), 5.65 (1H, s, H^8), 5.42 (1H, d, J = 4.3 Hz, 3'-OH), 4.42 (1H, qn, J = 5.0 Hz, H^3), 4.00 (1H, dd, J = 3.9, 7.4 Hz, H^6), 3.69 (3H, s, OCH_3), 3.69 (3H, s, OCH_3), 3.49 (3H, s, NCH_3), 3.39 (1H, d, J = 7.3 Hz, H^5), 3.35 (1H, d, J = 7.3 Hz, H^8), 2.45 (1H, td, J = 6.5, 13.1 Hz, H^2), 2.19 (1H, ddd, J = 5.5, 6.1, 13.2 Hz, H^2); ^13C NMR (100 MHz, d_6-DMSO) δ 158.6 (C^7a), 158.2 (C^{12}-OCH_3), 154.7 (q, ^2J_CF = 36.9 Hz, COCF_3), 153.4 (C^2), 144.5 (C^13), 141.6 (C^6), 136.7 (C^4,C^9), 135.5, 135.2 (C^9), 131.3 (C^1'), 129.8, 129.7 (C^10), 129.6 (C^5'), 128.0 (C^{14}), 126.8 (C^{14}), 125.4 (C^5'), 121.3 (C^4'), 120.6 (C^2'), 115.7 (q, ^1J_CF = 286.7 Hz, COCF_3), 113.3 (C^11), 107.5 (C^4a), 99.3 (C^3), 86.7 (C^1'), 86.1 (C^8Ar), 85.7 (C^4), 68.9 (C^3'), 62.6 (OCH_3), 41.5 (C^2'), 29.4 (NCH_3); LRMS (ES') m/z: 804 (16), 793 ([M + K]^+), 777 ([M + Na]^+), 99, 303 (DMT^+), 100; HRMS (ES'): calcd for C_{43}H_{57}N_{12}O_{4}F_3 (M), [M + Na]^+ = 777.2507, found 777.2509. Found also DMT^+ (calcd 303.1380, found 303.1384).

Analytical results are consistent with the reported data. 206

\[ N,N'-\text{Bis-[} (2\text{cyanoethoxy)carbonyl]-S-methyl-isothiourea, 200}^{1,2,25,226} \]

\[ \text{C}_4\text{H}_7\text{N}_2\text{O}_4\text{F}_3\text{S} \quad \text{Mol. Wt.: 398.19} \]

A mixture of S-methylisothiourea hemisulfate 205 (6.03 g, 43.3 mmol) and NaHCO_3 (10.9 g, 130 mmol, 3.0 eq) in argon-degassed, distilled water (50.0 mL) was stirred, under an argon atmosphere, at rt, for 30 minutes. To this was added degassed CH_2Cl_2 (150.0 mL) followed by (2-cyanoethyl)-N-succinimidy carbonate (CEOC-succinimide) 201 (18.9 g, 86.6 mmol, 2.0 eq) and the mixture was stirred vigorously, under an argon atmosphere, at rt for 8 hours. The mixture was diluted with CH_2Cl_2 (100 mL) and water (100 mL), agitated and separated. The aqueous layer was re-extracted (CH_2Cl_2, 3 × 100 mL) and combined CH_2Cl_2 fractions were dried (Na_2SO_4) and concentrated in vacuo and under high vacuum to give a viscous, cloudy yellow syrup.
The syrup was redissolved in distilled CH₂Cl₂ (65.0 mL), to which CEOC-succinimide 201 (9.47 g, 43.3 mmol, 1.0 eq) was added and the mixture was stirred under an argon atmosphere, at rt for 14½ hours. The yellow solution was diluted with CH₂Cl₂ (135 mL) then washed with sat. aq KCl (80 mL). The aqueous fraction was re-extracted (CH₂Cl₂, 2 × 30 mL) and the combined organic fractions were dried (Na₂SO₄) and concentrated in vacuo to give a cloudy, yellow syrup. Following purification by column chromatography (5:95, ethyl acetate/CH₂Cl₂), and drying under high vacuum, the desired product 200 was afforded as a hard, powdery white solid (11.0 g, 38.7 mmol, 89%). A mixture of reagent 201 and product 200 (ca. 7:3) (0.90 g) was also isolated.

R_f 0.46 (1:3, ethyl acetate/CH₂Cl₂, B’, F’), 0.28 (1:9, ethyl acetate/CH₂Cl₂, B'); ¹H NMR (400 MHz, CDCl₃) δ 11.77 (1H, s, NH), 4.40 (2H, t, J = 6.3 Hz, H₃), 4.36 (2H, t, J = 6.0 Hz, H₃'), 2.78 (4H, t, J = 6.5 Hz, CH₂CN), 2.44 (3H, s, SCH₃); ¹³C NMR (100 MHz, CDCl₃) δ 173.7 (NHCO), 160.4 (NC₂=O), 150.8 (NHC=O), 116.8 (C₅N), 116.3 (C₅N), 61.0 (C₃), 60.6 (C₃), 18.1 (CH₂CN), 14.8 (SCH₃); LRMS (ES⁺) m/z: 307 ([M + Na]+, 100), 285 ([M + H]+, 12), 277 (11), 236 (17).

Analytical results are consistent with the reported data.²²⁵

(2-Cyanoethyl)-N-succinimidyl carbonate, 201²²⁶

A suspension of di-succinimidyl carbonate (DSC) 202 (69.3 g, 271 mmol, 1.3 eq) in anhydrous acetonitrile (300 mL), with 4Å molecular sieves, was stirred vigorously at rt, under an argon atmosphere for 35 minutes. Distilled pyridine (23.6 mL, 291 mmol, 1.4 eq) was added and the mixture was stirred for 15 minutes. 3-Hydroxypropionitrile (3-HPN) 203 (14.2 mL, 208 mmol) was added dropwise over 35 minutes then the mixture was stirred, at rt, for 1½ hours. The pale yellow mixture was filtered, the residue was washed with CH₂CN (2 × 30 mL) and the combined filtrate was concentrated in vacuo and under high vacuum to give a viscous yellow syrup. The syrup was dissolved in argon-degassed CH₂Cl₂ (500 mL) and washed with degassed sat. aq NaHCO₃ (2 × 250 mL). The combined aqueous fraction was re-extracted (degassed CH₂Cl₂, 3 × 50 mL)
and combined CH₂Cl₂ fractions were washed with degassed sat. aq KCl (2 × 200 mL), dried (Na₂SO₄) and concentrated in vacuo and under high vacuum to give a white waxy solid. The solid was co-evaporated with anhydrous CH₃CN (2 × 50 mL) and dried under high vacuum, then triturated with diethyl ether (2 × 150 mL) and dried under high vacuum, to afford the desired product 201 as a slightly waxy, white solid (42.5 g) containing 5.1 mol % N-hydroxysuccinimide (recalculated yield - 195 mmol, 94%).

Rₛ 0.33 (1:3, ethyl acetate/CH₂Cl₂, B’, F’), 0.17 (1:9, ethyl acetate/CH₂Cl₂, B’, F”); IR (solid): ν 3001, 2968, 2941 (w, C–H), 2262 (w, C≡N), 1810, 1781 (s, C=O), 1727 (br vs, carbonate C=O), 1474, 1430 (w), 1364 (m), 1263, 1231 (s, C–O), 1076 (s, C–O), 992, 968 (s), 861, 813 (m), 763, 718 (s), 642 (s, N–O), 575, 460 (m) cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 4.52 (2H, t, J = 6.5 Hz, C₃H₂O), 2.85 (4H, s, succinimide-C₃H₂), 2.84 (2H, t, J = 6.5 Hz, C₃H₂CN); ¹³C NMR (100 MHz, CDCl₃) δ 168.4 (succinimide-NCO), 151.3 (OCO₂), 115.6 (CN), 64.8 (CH₂O), 25.6 (succinimide-C₃H₂), 18.0 (CH₂CN); LRMS (ES⁺) m/z: 276 ([M + CH₃CN + Na]⁺, 30), 235 ([M + Na]⁺, 100).

Analytical results are consistent with the reported data.⁴²


To a stirred solution of nucleoside 195 (0.39 g, 0.60 mmol) and guanidinylating reagent 200 (0.19 g, 0.66 mmol, 1.1 eq) in distilled CH₂Cl₂ (4 mL), under an argon atmosphere, was added distilled pyridine (24.1 µL, 0.30 mmol, 0.5 eq) and the solution was stirred at 35 °C. Further distilled pyridine (0.05 mL, 0.62 mmol, 1.0 eq) was added at 16½ hours
and 19 hours, when distilled CH₂Cl₂ (1.0 mL) was added and the temperature was raised to 50 °C. Molecular sieves (4Å) and distilled pyridine (0.05 mL, 0.62 mmol, 1.0 eq) were added at 20½ hours, distilled CH₂Cl₂ (2.0 mL) at 22½ hours, and further distilled pyridine (0.08 mL, 0.93 mmol, 1.6 eq) and guanidinylating reagent 200 (0.24 g, 0.84 mmol, 1.4 eq) at 23½ hours. After a further 17 hours, the reaction mixture was diluted with CH₂Cl₂ (100 mL), filtered and washed with water (50 mL). The aqueous layer was re-extracted (CH₂Cl₂, 2 × 30 mL) and combined organic fractions were washed with sat. aq KCl (75 mL), dried (Na₂SO₄), and concentrated in vacuo and under high vacuum to give a golden brown foam. Following purification by column chromatography (50-100% acetone/CH₂Cl₂), on silica gel pre-equilibrated with pyridine, the desired guanidinylated product 207 was afforded, as a yellow foam (0.23 g, 0.25 mmol, 42%). A mixture of product 207 and starting material 195 (ca. 1:2) (0.03 g) was also isolated.

Rf 0.37 (4:1, acetone/CH₂Cl₂, A’, E’); IR (solid): ν 3271 (br w, O–H, N–H), 2933, 2837 (w, C–H), 2255 (w, C=≡N), 1732 (s, C=O), 1545 (s, C=N), 1450, 1422, 1393, 1355 (s, C=O), 1327 (s), 1290, 1260, 1239, 1193 (s), 1166, 1129, 1093 (s), 1057, 1029 (s), 907 (m), 827 (s, Ar C–H), 791 (s), 770 (s, Ar C–H), 727 (s, CH₂), 700 (s, Ar C–H), 658, 583, 531 (s) cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 11.57 (1H, s, CONH), 9.91 (1H, s, NHAr), 8.52 (1H, s, H⁴), 7.48 (1H, t, J = 1.8 Hz, H²⁺), 7.23 (1H, ddd, J = 1.0, 2.0, 8.0 Hz, H⁴⁺), 7.18-7.15 (1H, m, H⁴⁺), 7.15 (2H, d, J = 7.0 Hz, H⁵⁺), 7.04 (4H, d, J = 9.0 Hz, H⁹), 7.04-6.99 (1H, m, H¹⁰), 6.98 (2H, t, J = 9.0 Hz, H¹⁵), 6.95 (1H, br d, J = 8.5 Hz, H¹⁶), 6.54 (2H, d, J = 9.0 Hz, H¹¹), 6.53 (2H, d, J = 9.0 Hz, H¹⁴), 6.19 (1H, t, J = 5.5 Hz, H¹³), 5.29 (1H, s, H⁵), 4.39 (1H, q, J = 5.5 Hz, H³), 4.19 (2H, br t, J = 5.8 Hz, H²⁴), 4.00 (2H, br t, J = 5.8 Hz, H²⁰), 3.90 (1H, td, J = 3.0, 5.0 Hz, H⁴), 3.46 (3H, s, OCH₃), 3.45 (3H, s, OCH₃), 3.34 (3H, s, NCH₃), 3.26 (1H, dd, J = 3.0, 11.0 Hz, H³⁵), 3.21 (1H, dd, J = 3.3, 10.8 Hz, H³⁶), 2.59-2.52 (1H, m, H³⁵), 2.53 (2H, br t, J = 6.0 Hz, H²⁵), 2.41 (2H, br t, J = 5.8 Hz, H¹⁹), 2.11 (1H, ddd, J = 5.5, 6.5, 13.6 Hz, H¹⁷); ¹³C NMR (100 MHz, CDCl₃) δ 163.5 (C¹⁷=N), 159.3 (C⁷′), 159.1, 158.0 (C¹³), 155.3 (NC¹⁸=O), 154.0 (C¹'), 153.6 (NH¹²=O), 144.8 (C¹³), 142.7 (C⁸), 136.8 (C⁴), 136.5 (C¹⁰=NHR), 136.1, 135.9 (C⁶), 132.3 (C²'), 130.6, 130.5 (C¹⁰), 129.9 (C⁸), 128.6 (C¹⁵), 122.5 (C¹⁴), 127.5 (C¹⁶), 126.1 (C⁶'), 123.3 (C²'), 123.2 (C⁴'), 117.2 (C¹²=N), 116.4 (C¹⁸=N), 113.8 (C¹¹), 109.2 (C¹⁴), 100.4 (C³), 88.2 (C¹'), 87.4 (C⁸Ar), 86.6 (C⁴'), 70.9 (C⁵'), 63.0 (C⁶'), 61.5 (C¹⁰), 60.3 (C¹⁵), 55.6 (OCH₃), 43.0 (C⁷'), 30.4 (NHCH₃), 18.4 (C²⁰,C²¹); LRMS (ES⁺) m/z: 918 ([M + Na]+, 100), 895 ([M + H]+, 10); HRMS
(ES\(^+\)): calcd for C\(_{48}\)H\(_{46}\)N\(_8\)O\(_{10}\) (M), [M + Na]\(^+\) = 917.3229, found 917.3234. Found also DMT\(^+\): calcd 303.1380, found 303.1386.

3-Thioureidophenyl acetylene, 209

![Diagram of 3-Thioureidophenyl acetylene reaction]

**Method 1:** Ammonium thiocyanate (1.12 g, 14.7 mmol, 1.1 eq) was dissolved, with care, in 15% aq HCl (1.5 mL). This was added to a stirred emulsion of 3-ethynylaniline 152 (1.50 mL, 13.3 mmol) in 15% aq HCl (1.3 mL), at rt. The mixture was refluxed, in absence of light, for 9¼ hours. The brown reaction mixture was allowed to cool, then diluted with ethyl acetate (180 mL) and washed with water (2 × 40 mL) and sat. aq KCl (40 mL). The organic layer ( suspension) was removed and concentrated *in vacuo* to give a golden-brown foam/gum. The gum was dissolved/suspended in acetone (100 mL), filtered, the residue was washed with acetone (2 × 20 mL) and the combined filtrate was concentrated *in vacuo*. The resulting gum was suspended in diethyl ether (50 mL) and allowed to soak overnight. The mixture was filtered, washed with diethyl ether (4 × 25 mL) and combined ether fractions were concentrated *in vacuo* and under high vacuum to give a yellow syrup. Following purification by column chromatography (0-8% methanol/CH\(_2\)Cl\(_2\) + 0.5% Et\(_3\)N), and drying under high vacuum, the desired product 209 was afforded as an orange solid (0.14 g, 0.79 mmol, 6%). Starting material 152 was also recovered (0.41 g, 3.53 mmol, 27%).

**Method 2:** Benzoylthiourea 211 (2.54 g, 9.06 mmol) was added, in one portion, to stirred 5% aq NaOH (45.0 mL) under argon, at 90 °C. After stirring for 15 minutes, the suspension was filtered hot to remove the benzamide byproduct 212, then cooled and acidified to pH 5.0 using 15% aq HCl. The mixture was then basified to pH 8.0 using...
conc. aq NH₃ and filtered. The solid was washed with water (3 × 10 mL) then dried under high vacuum over P₂O₅ for 2 days, to afford the desired deprotected thiourea 209 as a pale, cream-coloured powdery solid (1.26 g, 7.15 mmol, 79%).

Rᶠ 0.17 (5:95, acetone/CH₂Cl₂, A’); Mp 145-148 °C (CH₂Cl₂); IR (solid): ν 3418, 3394 (m, N–H), 3208 (m), 3160 (br s, water), 3015, 2981 (m, Aryl C–H), 2680 (w), 1618 (s, N–H), 1596 (m), 1580 (m, Aryl C–C), 1526, 1514, 1480, 1468 (s), 1423, 1411 (m), 1297, 1248 (s, N–CS), 1155 (m), 1065 (s, C=S), 999, 888, 831 (m), 786, 711, 688, 667, 632, 622, 579 (s), 518 (br vs) cm⁻¹; ¹H NMR (400 MHz, d₆-DMSO) δ 9.75 (1H, s, NH), 7.62 (1H, t, J = 1.8 Hz, H²), 7.54 (2H, extremely br s, NH₂), 7.42 (1H, ddd, J = 1.0, 2.0, 8.0 Hz, H⁴), 7.32 (1H, t, J = 8.0 Hz, H⁵), 7.07 (1H, td, J = 1.3, 7.7 Hz, H⁶), 4.17 (1H, s, C≡CH); ¹³C NMR (100 MHz, d₆-DMSO) δ 181.2 (C=S), 139.5 (C³), 129.0 (C⁵), 127.4 (C⁶), 125.6 (C²), 123.5 (C⁴), 121.8 (C¹), 83.2 (C≡CH), 80.8 (C≡C⁸H); LRMS (ES⁺) m/z: 353 ([2M + H]⁺, 100), 199 ([M + Na]⁺, 78), 177 ([M + Na]⁺, 78); HRMS (ES⁺): calcd for C₉H₈N₂S (M), [3M + Na]⁺ = 551.1117, found 551.1121; [2M + Na]⁺ = 375.0709, found 375.0710; [M + Na]⁺ = 199.0300, found 199.0301.

N-Benzoyl-N'-(3-ethynylphenyl) thiourea, 211

To a stirred solution of 3-ethynylaniline 152 (1.50 mL, 13.3 mmol) in dry acetone (30.0 mL), under an argon atmosphere, at rt, was added a solution of benzoyl isothiocyanate 210 (1.82 mL, 13.5 mmol, 1.0 eq) in dry acetone (20.0 mL) dropwise over 12 minutes. The solution was stirred at rt for 1 hour, then concentrated in vacuo to give a pale yellow papery solid. Cold dry acetone (15 mL) was added and the mixture was cooled at 10 °C for 2 hours. The mixture was filtered cold, then washed with cold, dry acetone (2 × 5 mL) and diethyl ether (2 × 15 mL), then dried under high vacuum over P₂O₅ to afford the desired product 211 as a pale yellow papery solid. Four further crops were obtained from the filtrate. (Combined yield - 2.81 g, 10.0 mmol, 75%).
R, 0.69 (5:95, acetone/CH₂Cl₂, A’); Mp 138-140 °C (acetone); IR (solid): v 3346 (br w, N–H), 3259 (s, alkyn C–H), 2991 (br m, Aryl C–H), 1663 (m, C=O), 1600 (s, Aryl C–C), 1557, 1517 (s, C=O), 1486, 1449 (s), 1338 (s, N–CS), 1299 (s), 1255 (N–CS), 1188, 1165 (m), 1140 (s, C=S), 1098, 1078 (s), 1024, 1000, 975, 929 (m), 878 (s, Aryl C–H), 783 (vs, Aryl C–H), 740 (m), 702 (vs, Aryl C–H), 677, 655 (vs), 591, 533, 515 (s) cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ 12.61 (1H, s, CON=O), 9.09 (1H, s, CSNHAr), 7.90 (2H, dd, J = 1.3, 6.0 Hz, H¹⁰), 7.86 (1H, t, J = 1.5 Hz, H¹²), 7.76 (1H, td, J = 2.2, 7.1 Hz, H⁴), 7.67 (1H, tt, J = 1.5, 7.3 Hz, H¹²), 7.55 (2H, tt, J = 1.7, 7.5 Hz, H¹¹), 7.41 (1H, td, J = 1.8, 7.7 Hz, H⁶), 7.37 (1H, t, J = 7.7 Hz, H⁵), 3.12 (1H, s, C≡CH); ¹³C NMR (100 MHz, CDCl₃) δ 178.6 (C=S), 167.1 (C=O), 137.8 (C³), 134.0 (C¹³), 131.7 (C⁹), 130.6 (C⁵), 129.4 (C¹¹), 129.0 (C⁶), 127.6 (C¹⁰), 127.6 (C²), 124.7 (C⁴), 123.1 (C¹²), 82.9 (C²=CH), 78.2 (C≡C=S); LRMS (ES⁺) m/z: 344 ([M + CH₂CN + Na]⁺, 50), 330 (49), 303 ([M + Na]⁺, 100), 281 ([M + H]⁺, 33); HRMS (ES⁺): calcd for C₁₀H₁₂N₂OS (M), [3M + Cu⁺]⁺ = 903.1302, found 903.1038, [2M + Cu⁺]⁺ = 623.0631, found 623.0492; [2M + H]⁺ = 561.1413, found 561.1358; [M + H]⁺ = 281.0743, found 281.0742.

5'-O-(4,4'-Dimethoxytrityl)-5-[(3-thioureidophenyl)ethynyl]-4-N-methyl-2'-deoxycytidine, 213

![Chemical structure](image)

To a solution of 5'-O-(4,4'-dimethoxytrityl)-5-ido-4-N-methyl-2'-deoxycytidine 185 (0.31 g, 0.46 mmol) in anhydrous DMF (2.0 mL), under an argon atmosphere and in absence of light, were added distilled Et₃N (0.33 mL, 2.33 mmol, 5.0 eq), CuI (0.10 g, 0.51 mmol, 1.1 eq), then alkyne 209 (0.20 g, 1.16 mmol, 2.5 eq). The mixture was stirred for 1 hour then tetrakis(triphenylphosphine) palladium (0) (0.38 g, 0.33 mmol, 0.7 eq) was added and the reaction was stirred at rt for 1½ hours. The reaction mixture was concentrated under high vacuum, co-evaporating with methanol/toluene, to give an
orange foam/glass. The residue was dissolved/suspended in methanol/acetone (1:1 v/v, 40 mL), and filtered. The solid was washed with methanol/acetone (1:1 v/v, 2 × 30 mL) and the combined filtrate was concentrated in vacuo and under high vacuum to give a golden orange foam/resin. Following purification by column chromatography (80-100% acetone/CH₂Cl₂ + 0.5% Et₃N), and drying under high vacuum, the desired product 213 was afforded as a yellow foam/resin (0.42 g) containing 21.1 mol % Et₃N.HI, 22.3 mol % triethylamine, and 4.0 mol% starting material 185, with some Pd-based impurities (recalculated yield - 0.47 mmol, quant.).

R_f 0.23 (5:95, methanol/ethyl acetate + 0.5% Et₃N, A’), 0.17 (4:1, acetone/CH₂Cl₂ + 0.5% Et₃N); ¹H NMR (400 MHz, d₆-DMSO) δ 9.68 (1H, s, NH), 8.01 (1H, s, H⁴), 7.49 (1H, br s, H²), 7.44-7.39 (1H, m, NHCH₃), 7.42 (2H, d, J = 7.5 Hz, H¹⁵), 7.37 (1H, br d, J = 8.3 Hz, H⁶), 7.32-7.27 (1H, m, H⁸), 7.30 (2H, d, J = 9.0 Hz, H¹⁴), 7.30 (2H, d, J = 9.0 Hz, H¹¹), 7.26 (2H, t, J = 8.0 Hz, H¹⁰), 7.17 (1H, t, J = 7.3 Hz, H¹⁷), 6.96 (1H, br d, J = 7.5 Hz, H⁶), 6.85 (2H, d, J = 9.0 Hz, H¹²), 6.84 (2H, d, J = 9.0 Hz, H¹³), 6.16 (1H, t, J = 6.5 Hz, H¹⁰), 5.30 (1H, d, J = 4.5 Hz, 3'-OH), 4.28 (1H, td, J = 3.5, 9.5 Hz, H¹²), 3.98 (1H, td, J = 3.3, 4.5 Hz, H⁴), 3.67 (3H, s, OCH₃), 3.66 (3H, s, OCH₃), 3.22 (1H, dd, J = 5.0, 10.5 Hz, H⁵), 3.16 (1H, dd, J = 2.8, 10.3 Hz, H⁶), 2.87 (3H, d, J = 5.0 Hz, NHCH₃), 2.28 (1H, ddd, J = 3.3, 6.0, 13.3 Hz, H⁵'), 2.15 (1H, td, J = 6.7, 13.6 Hz, H²'), 2.08 (2H, s, NH₂); ¹³C NMR (100 MHz, d₆-DMSO) δ 181.2 (C SNH₂), 161.6 (C⁴), 158.0, 158.0 (C¹₃), 153.2 (C²), 144.7 (C³'), 143.0 (C⁶), 139.0 (C¹₄), 135.6, 135.3 (C¹⁰), 129.7, 129.6 (C¹¹), 128.5 (C⁸'), 127.9 (C¹⁵), 127.6 (C¹⁶,C⁸'), 126.7 (C¹'), 126.1 (C²'), 123.9 (C⁴'), 122.5 (C⁵'), 113.2, 113.2 (C¹²), 94.1 (C⁸), 90.3 (C⁷), 85.9 (C⁴), 85.8 (C⁹Ar), 85.7 (C¹), 80.8 (C⁵), 70.6 (C³), 63.7 (C⁵'), 55.0, 54.9 (OCH₃), 40.9 (C²'), 27.9 (NHCH₃); LRMS (ES⁺) m/z: 740 ([M + Na]⁺, 100), 177 ([M + H]⁺, 73); HRMS (ES⁺): calcd for C₄₀H₃₈N₂O₆S (M), [M + K]⁺ = 756.2253, found 756.2260; [M + Na]⁺ = 740.2513, found 740.2550; [M + H]⁺ = 718.2694, found 718.2696.
9.2 Biophysical Studies

9.2.1 Synthesis of Oligonucleotides

Oligonucleotides were synthesised by ATDBio Ltd. and Dr. Bouamaied on an Applied Biosystems 394 automated DNA/RNA synthesiser or an ÄKTA™ oligopilot plus DNA/RNA synthesiser using a standard solid-phase 0.2 or 1.0 μmole phosphoramidite synthesis cycle. The cycle comprises acid-catalysed (TCA) detritylation, coupling, capping (Ac₂O) and oxidation (iodine/pyridine/THF). Stepwise coupling efficiencies and overall yields were determined by an automated trityl cation conductivity monitoring function and in all cases were >96%.

All synthesised phosphoramidite monomers were dissolved in anhydrous DNA grade acetonitrile or freshly distilled CH₂Cl₂ and filtered through a Millipore Millex®-FH syringe filter (0.45μm, 13/25 mm). After removing the solvent in vacuo, the monomer was dissolved in freshly distilled CH₂Cl₂ and aliquots corresponding to 100-120 mg each were transferred in to dry, sealed, argon-flushed ABI reagent bottles. After drying to constant weight, under high vacuum over KOH for 2-3 days, the monomers were stored under positive argon pressure at −20 °C until required.

All phosphoramidite monomers were dissolved in anhydrous DNA grade acetonitrile (or freshly distilled CH₂Cl₂ if poorly soluble) to a concentration of 0.1 M immediately before use.

9.2.2 Deprotection of Oligonucleotides

Oligonucleotides were deprotected/cleaved from solid-support by various conditions. Capping was carried out using acetic anhydride. Washing with 20% diethylamine/acetonitrile was introduced in the latter half of the project.

**OL1, 2, 6-9, 17:** conc. aq ammonia, 55 °C, 5 hours (Capping only).

**OL3-5:** 30% aq methylamine, rt, 6-18 hours (Capping only), followed by treatment with DOWEX-H⁺ resin for 15-17 hours.
OL10-13: 30% aq methylamine, rt, 18 hours (Capping only), followed by treatment with DOWEX-H⁺ resin for 15-17 hours.

OL14-16: 30% aq methylamine, rt, 6 hours (Capping only), followed by treatment with DOWEX-H⁺ resin for 15-17 hours.

OL18-25, 32, 33, 35-37, 40, 41, 43-59: conc. aq ammonia, rt, 24 hours, plus 1 hour at 50 °C (DEA Wash only).

OL26-31: conc. aq ammonia, 55 °C, 5 hours (DEA Wash plus Capping).

OL34, 38, 39, 42: conc. aq ammonia, rt, 12 hours, plus 2 hours at 50 °C (DEA Wash only).

OL60-63: conc. aq ammonia, rt, 12 hours (DEA Wash only).

The deprotection conditions used for oligonucleotides (OL64-70) in the Deprotection Study are discussed in Chapter 7.

9.2.3 Purification of Oligonucleotides

Oligonucleotides were purified by reversed-phase HPLC on a Gilson HPLC system using an Brownlee Aquapore column (C8, 8 × 250 mm, 300Å pore size). The system was controlled by Gilson 7.12 software, and oligonucleotide elution was monitored by UV absorption at 297 nm for non-modified and 310 nm for modified oligonucleotides.

The following purification protocol was used: Gradient of acetonitrile in ammonium acetate buffer from 0-50% buffer B over 30 minutes (flow rate: 4 mL/min; buffer A: 0.1 M ammonium acetate, pH 7.0; buffer B: 0.1 M ammonium acetate with 50% acetonitrile, pH 7.0).

9.2.4 Analysis of Oligonucleotides

Mass spectra of oligonucleotides were recorded in cooperation with Dr. Edrees-Abdou, using electrospray ionisation (ES) on a Fisons VG platform instrument in HPLC grade water, with tripropylamine to aid ionisation; or by Dr. Hong Li, using matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) analysis on a ThermoBioAnalysis Dynamo MALDI-TOF mass spectrometer in positive ion mode.
using internal $T_r$ standards. ES MS data was deconvoluted/reprocessed using the Maximum Entropy facility in MassLynx software version 2.22.

CE analysis was conducted by Louise Hagon (ATDBio Ltd.), using a Beckman Coulter P/ACE™ MDQ Capillary Electrophoresis System, using the 32 Karat Software MDQ UV application, at a concentration of ~4 OD/mL. An ssDNA 100-R gel with Tris-Borate-7 M Urea system was used.

Analytical HPLC and ion-exchange analysis were conducted by ATDBio Ltd.

9.2.5 UV Melting Studies

UV Melting experiments were performed, partly in cooperation with Dr. Edrees-Abdou, on a Varian Cary 400 Scan UV-Visible Spectrophotometer in Hellma® SUPRASIL synthetic quartz cuvettes (10 mm pathlength, 1.5 mL sample volume), monitoring at 260 nm, using Cary WinUV Thermal application software. Buffers were prepared according to the literature procedures, allowed to warm to rt and the pH checked before use.

9.2.5.1 Set-Up Procedure

Aqueous solutions of the TFO and duplex strands were mixed in a 5:1 ratio in an eppendorf tube and lyophilised. The residue was redissolved in 1.5 mL of the correct buffer, pH 6.1, 6.2, 6.4, 6.6, 7.0 or 7.0 + 2 mM spermine (10 mM sodium phosphate, 200 mM NaCl, 1 mM Na$_2$EDTA) to afford a 5 µM:1 µM concentration of TFO:duplex. The samples were degassed for 1 minute using a sonic-bath, then filtered into the cuvettes though Kinesis regenerated cellulose syringe filters (0.45 µm, 15 mm). The samples were subjected to the desired melt programme alongside a matched cell reference blank.
### 9.2.5.2 UV Melt Programmes

#### Programme 1

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<th>Hold time (min)</th>
<th>Data Interval (°C)</th>
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#### Programme 2

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#### Programme 3

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Following a fast melt/anneal for equilibration of denaturation of the triplex, heat/cool cycles up to 80 °C were recorded, followed by cycles up to 50 °C, at a slower rate and smaller data interval, for more accurate determination of the triplex $T_m$ and reduction of hysteresis.

### 9.2.5.3 Data Analysis

$T_m$ values were derived from the derivatives of melting curves for each experiment using Cary WinUV Thermal application software or OriginPro 7.5.
9.2.6 Fluorescence Melting Studies

Fluorescence melting experiments were conducted, partly in cooperation with Dr. Edrees-Abdou, on a Roche LightCycler® 1.5 instrument in LightCycler glass capillaries (20 µL volume) using Roche LightCycler Software Version 3.5. The LightCycler has one excitation source (488 nm) and change in fluorescence was monitored at 520 nm.

9.2.6.1 Set-Up Procedure

Master solutions (5 µM) of each oligonucleotide involved in each LightCycler run were made. For each experiment within that run, 4.5 µL and 45 µL of the appropriate duplex and TFO respectively were lyophilised in 100 µL eppendorf tubes then redissolved in 90 µL of the correct buffer, pH 6.0 (20 mM NaOAc, 200 mM NaCl), pH 6.2 (10 mM sodium phosphate, 200 mM NaCl) or, pH 6.2, 6.6, 7.0 or 7.0 + 2 mM spermine (10 mM sodium phosphate, 200 mM NaCl, 1 mM Na₂EDTA), to afford a 10:1 ratio of TFO:hairpin duplex (5:0.5 µM). Samples containing only the duplex were used as references within each run. The samples were mixed by vortex then centrifugation, and were degassed in a sonic bath for 1 minute. Aliquots of 20 µL were pipetted into the top of two or three LightCycler tubes per sample, which left one or two spare aliquots. The tubes were capped and centrifuged gently to avoid breakage, then loaded into the carousel and subjected to the desired LC melt programme.

9.2.6.2 LC Melt Programmes

### Programme 1

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Programme 2

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Programme 3

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<td>–</td>
<td>600</td>
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Programme 4

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<th>Hold time (s)</th>
<th>Rate (°C/min)</th>
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<tr>
<td>Cool</td>
<td>95→30</td>
<td>20.0</td>
<td>300</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Following fast heating from 30 to 95 °C, and holding at 95 °C for 10 minutes to fully denaturate the DNA, a slow stepwise cool/annealing process was recorded (1.0 °C step, 0.2-0.25 °C/min). After incubation at low temperature (27-30 °C) for 1 hour to equilibrate triplex formation, a slow stepwise heat/melting process was recorded (1.0 °C step, 0.2 °C/min). The samples were held at 95 °C for 10 minutes to equilibrate before rapid cooling back to 30 °C. Approximately ¼ of sample volume (~5 µL) is lost by evaporation during the course of the LC programme.
9.2.6.3 Data Analysis

\(T_m\) values were derived from the negative derivatives of melting curves for each experiment using the “Manual \(T_m\)” feature in Roche LightCycler Software Version 3.5.

9.2.7 Determining UV/Fluorescence Properties of Fluorescent Nucleosides

9.2.7.1 Deprotection of Nucleosides

DMT-protected nucleosides (143,182,225,226) were treated up to three times with 3% TCA/DCM followed by removal of DCM \textit{in vacuo} and trituration with diethyl ether (\(\times 3\)). The aniline 143, acetamide 225 and urea 182 deprotected nucleosides were afforded in this way as pale yellow to orange powders. The guanidine nucleoside (non-protonated) was further treated with 20% Et₂NH/CH₃CN for 24 hours, concentrated \textit{in vacuo}, then treated with a mixture of 40% NH₃/H₂O and 7N NH₃/CH₃OH (1:2 v/v) for 60 hours, before concentration \textit{in vacuo} and recrystallisation (CH₃OH/Et₂O). The fully deprotected nucleoside 226 was afforded as a yellow-orange powder. Treatment with 20% diethylamine solution cleaved only one 2-cyanoethoxycarbonyl (CEOC) protecting group from the guanidine moiety. Subsequent treatment with ammonia was necessary to cleave the second. Treatment with ammonia only would have resulted in cyclisation to form a stable triazine analogue of the guanidine moiety as reported in work by Prakash \textit{et al.}²²⁵ (Section 7.3.2.7).

9.2.7.2 UV Absorption Measurements

Solutions of nucleosides were made in either HPLC grade methanol (\(^{4}\text{PP}_{pp}\) 143, \(^{6}\text{PP}_{pp}\) 225) or anhydrous DMF (\(^{1}\text{PP}_{pp}\) 182, \(^{6}\text{PP}_{pp}\) 226) up to 0.174 µM (\(^{4}\text{PP}_{pp}\)), 0.109 µM (\(^{6}\text{PP}_{pp}\)), 0.151 µM (\(^{1}\text{PP}_{pp}\)) and 0.154 µM (\(^{6}\text{PP}_{pp}\)) concentrations. The solution being tested was filtered through a Millipore Millex\textsuperscript{®}-FH syringe filter (0.45µm, 13 mm) into a Hellma\textsuperscript{®} SUPRASIL synthetic quartz cuvette (10 mm pathlength, 1.5 mL sample volume). UV absorption/excitation spectra were recorded at this and 2-3 lower concentrations by successive dilution by factor of 2, on a Varian Cary 400 Scan UV-Visible Spectrophotometer using Cary WinUV Scan application software. Spectra for
compounds run in DMF were dominated by noise below 260 nm, due to loss of solvent transparency to UV light below ~270 nm.

Extinction coefficients (\( \varepsilon / L \text{ mol}^{-1} \text{ cm}^{-1} \)) were calculated using the Beer-Lambert Law (Equation 9.1)

\[
A = \varepsilon . c . l
\]

**Equation 9.1.** Beer-Lambert Law; \( A = \) absorbance (dimensionless), \( \varepsilon = \) molar extinction coefficient (L mol\(^{-1}\) cm\(^{-1}\)), \( c = \) concentration (M), \( l = \) pathlength (cm).

For each absorption/excitation maximum, the extinction coefficient was calculated by a three-step process:

1. At each concentration measured, the value was calculated using Equation 9.1, then the average of those values was taken (ignoring outliers).
2. The value was given as the gradient of a plot of Absorbance vs Molar Concentration, ignoring significant outliers.
3. The average of the two values was taken to give an extinction coefficient for each absorption/excitation maximum.

### 9.2.7.3 Fluorescence Measurements

Solutions of nucleosides were made in either HPLC grade methanol (\(^{\lambda}\text{PP}_p 143, \ ^{\lambda_e}\text{PP}_p 225\)) or anhydrous DMF (\(^{\lambda}\text{PP}_p 182, \ ^{6}\text{PP}_p 226\)) up to concentrations of 1-3 mg/mL. The solution being tested was filtered through a Millipore Millex\textsuperscript{®}-FH syringe filter (0.45\(\mu\)m, 13 mm) into a Hellma\textsuperscript{®} SUPRASIL synthetic quartz cuvette for fluorimetry (10 mm pathlength, 1.5 mL sample volume). An emission spectrum was run for each sample, by irradiation at the principal absorption maximum (\(\lambda \sim 360 \text{ nm}\)) for that sample, on a Varian Cary Eclipse Fluorescence Spectrophotometer using Cary Eclipse Scan application software. Each spectrum was an average of 10 consecutive scans from 380-600 nm.
References


Appendices
## A: List of Synthesised Compounds

<table>
<thead>
<tr>
<th>Cmpd No.</th>
<th>Structure</th>
<th>Name</th>
</tr>
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<tbody>
<tr>
<td>65</td>
<td><img src="image1" alt="Structure" /></td>
<td>1,3,5-tri-O-acetyl-2-O-(2-phthalimidoethyl)-D-ribofuranose</td>
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<tr>
<td>68</td>
<td><img src="image2" alt="Structure" /></td>
<td>N-trifluoroacetyl propargylamine</td>
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<tr>
<td>71</td>
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<td>benzyltriethylammonium dichloroiodate</td>
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<td><img src="image9" alt="Structure" /></td>
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<td>5'-O-(4,4'-dimethoxytrityl)-5-[(3-aminophenyl)ethynyl]-4N-methyl-2'-deoxycytidine</td>
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<td>(2-cyanoethyl)-N-succinimidyl carbonate</td>
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### B: Oligonucleotide List

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**Table.** ES MS (1 dp), MALDI-TOF MS (2 dp), Q = DABCYL (quencher), F = FAM (fluorophore), H = HEG. Key nucleotides marked in bold. * No MS analysis necessary.
C: Conferences Attended, Posters, Presentations and Publication

July 2007    Year 3 Postgraduate Talks, School of Chemistry, University of Southampton, UK. – Presentation

June 2007    3rd Nucleic Acids Summer School, University of Southern Denmark, Odense, Denmark. – Presentation


November 2006 Year 2 Postgraduate Poster Day, School of Chemistry, University of Southampton, UK. – Poster

September 2006 XVII International Roundtable Conference on Nucleosides, Nucleotides and Nucleic Acids, Bern, Switzerland. – Poster


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