(a) STUDIES IN OLIGONUCLEOTIDE SYNTHESIS
(b) NEW PROTECTING GROUPS FOR 1,2-DIOL SYSTEMS

By

Hongbin Yan

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Supervised by

Professor Colin B. Reese, F. R. S.

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Department of Chemistry, King’s College London
To the love of my parents,
and the memory of my mother-in-law.
ABSTRACT

(a) A modified $H$-phosphonate strategy, which involves coupling at $-40^\circ$C effected by bis(2-chlorophenyl) phosphorochloridate followed by a sulphur transfer step, was applied in the assembly of an antisense oligonucleotide phosphorothioate 21-mer, ISIS 2922. Also a four-component $H$-phosphonate approach was developed. By using diphenyl phosphorochloridate as coupling agent and succinimide derivatives as sulphur-transfer agents, side reactions associated with $H$-phosphonate chemistry can be reduced, especially when the preparation is carried out on a large scale. Pivaloyl chloride was found to be an efficient coupling reagent in the $H$-phosphonate approach at $0^\circ$C. By using a combined strategy of these two approaches, ISIS 2922 was prepared on a relatively large scale (2.0 mmol). The product and the intermediates were unblocked and characterised by reverse-phase HPLC, ion-exchange LC, $^1$H NMR, $^{31}$P NMR, capillary gel electrophoresis and MALDI-TOF mass spectroscopy. Also a procedure to convert protected S-(2-cyanoethyl) phosphorothioate triesters to phosphate diesters was investigated. By alkylation of phosphorothioate diesters with bromoacetonitrile, S-(cyanomethyl) phosphorothioate triesters can be obtained, which upon 2-nitrobenzaldoximate treatment gave phosphate diesters as the only products. This approach was applied in the conversion of a series of fully-protected oligonucleotide phosphorothioate triesters to phosphate diesters, which were characterised by NMR spectroscopy and enzymatic digestion.

(b) A group of new achiral protecting groups for 1,2-diol systems was developed. $2',3'-O$-[Di-$p$-(anisyl)methylene]uridine, $2',3'-O$-(xanthen-9-ylidene)uridine and $2',3'-O$-(2,7-dimethylxanthen-9-ylidene)uridine were prepared and the rates of hydrolysis of these compounds were compared with that of $2',3'$-$O$-isopropylideneduridine in trifluoroacetic acid–water–methanol. All of these new protecting groups were found to be more labile than the commonly used isopropylidene protecting group under these conditions. The racemic form and the $\alpha$- and $\beta$- enantiomers of 1,2-$O$-(xanthen-9-ylidene)glycerol and 1,2-$O$-(2,7-dimethylxanthen-9-ylidene)glycerol were prepared. The stearoyl esters of racemic 1,2-$O$-(xanthen-9-ylidene)glycerol and 1,2-$O$-(2,7-dimethylxanthen-9-ylidene)glycerol, $\alpha$ and $\beta$1,2-$O$-(xanthen-9-ylidene)glycerol were
prepared. The xanthen-9-ylidene protecting groups were unblocked under very mild conditions (pyrrole–dichloroacetic acid).
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<table>
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<th>Description</th>
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<tr>
<td>2-NBO</td>
<td>2-nitrobenzaldoxime</td>
</tr>
<tr>
<td>A</td>
<td>adenosine</td>
</tr>
<tr>
<td>Ac</td>
<td>acetyl</td>
</tr>
<tr>
<td>AcOH</td>
<td>acetic acid</td>
</tr>
<tr>
<td>Ade</td>
<td>adenine</td>
</tr>
<tr>
<td>B</td>
<td>base residue</td>
</tr>
<tr>
<td>C</td>
<td>cytidine</td>
</tr>
<tr>
<td>CDCl₃</td>
<td>deuterated chloroform</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CPG</td>
<td>controlled pore glass</td>
</tr>
<tr>
<td>CSA</td>
<td>(±)-camphor-10-sulphonic acid</td>
</tr>
<tr>
<td>Cyt</td>
<td>cytosine</td>
</tr>
<tr>
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<td>density</td>
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<td>d</td>
<td>doublet</td>
</tr>
<tr>
<td>dd</td>
<td>doublet of doublets</td>
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<tr>
<td>DBU</td>
<td>1,8-diazabicyclo[5.4.0]undec-7-ene</td>
</tr>
<tr>
<td>DCC</td>
<td>1,3-dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DMSO-d₆</td>
<td>deuterated dimethylsulphoxide</td>
</tr>
<tr>
<td>DMTr</td>
<td>4,4'-dimethoxytrityl</td>
</tr>
<tr>
<td>DNA</td>
<td>2'-deoxyribonucleic acid</td>
</tr>
<tr>
<td>ex</td>
<td>exchangeable with D₂O</td>
</tr>
<tr>
<td>G</td>
<td>guanosine</td>
</tr>
<tr>
<td>Gua</td>
<td>guanine</td>
</tr>
<tr>
<td>h</td>
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</tr>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
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<td>IMS</td>
<td>industrial methylated spirit</td>
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<td>infrared spectrometry</td>
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<td>molar concentration</td>
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<td>---------------------------------</td>
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</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
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<td>4-methoxytrityl</td>
</tr>
<tr>
<td>mp</td>
<td>melting point</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>MSNT</td>
<td>1-(mesitylene-2-sulphonyl)-3-nitro-1,2,4-triazole</td>
</tr>
<tr>
<td>ODS</td>
<td>octadecylsilyl</td>
</tr>
<tr>
<td>Ph</td>
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</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>Rt</td>
<td>retention time</td>
</tr>
<tr>
<td>SVPD</td>
<td>snake venom phosphodiesterase</td>
</tr>
<tr>
<td>t</td>
<td>triplet</td>
</tr>
<tr>
<td>T</td>
<td>thymidine</td>
</tr>
<tr>
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<td>triethylammonium acetate</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
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<tr>
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<td>thymine</td>
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<tr>
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<td>thin layer chromatography</td>
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<tr>
<td>TMG</td>
<td>tetramethylguanidine</td>
</tr>
<tr>
<td>TMS</td>
<td>trimethylsilyl</td>
</tr>
<tr>
<td>TOF</td>
<td>time of flight</td>
</tr>
<tr>
<td>Tr</td>
<td>trityl (triphenylmethyl)</td>
</tr>
<tr>
<td>Ura</td>
<td>uracil</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet spectrometry</td>
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Introduction to Nucleic Acid Chemistry

1.1 Nucleic Acids

The chemical study of the nucleic acids dates back to 1868 when Friedrich Miescher first isolated a phosphorus-containing compound from the nuclei of pus cells which he obtained in abundant supply from surgical bandages. He named the substance 'nuclein' and carried out a precise elemental analysis giving a phosphorus content of 9.59%. In fact nuclein was nucleoprotein and it fell subsequently to Richard Altmann in 1889 to obtain the first protein-free material, to which he gave the name 'nucleic acid'. Between 1877 and 1907 the pyrimidine and purine bases were isolated and characterised by Kossel and co-workers and by Fischer. The first generally accepted direct evidence that deoxyribonucleic acid (DNA) was the genetic material was reported by Hershey and Chase in 1952, using radioactively labelled phosphorus in the DNA of bacteriophage T2. Later, the regularity in the concentration of pyrimidine and purine bases was found by Chargaff and others by analysing the composition of DNA from various organisms. In 1955, by comparison of the first chemically synthesised dinucleotide with naturally occurring DNA, Michelson and Todd confirmed the postulated primary structure of DNA.

Naturally, there exist two forms of nucleic acids, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). They are both constructed from three components: nitrogen heterocyclic bases, pentose sugars, and phosphate residues. When a heterocyclic base is joined with a pentose sugar, a nucleoside is formed. The phosphate ester of a nucleoside is referred to as a nucleotide.

The primary structures of nucleic acids, i.e. the order in which the components are linked together, are shown in Figure 1.1. DNA and RNA are poly-dialkyl phosphate
esters in which the 3'-hydroxy group of one nucleoside unit is covalently linked with
the 5'-hydroxy group of another through an internucleotide phosphodiester linkage. The nucleoside units in ribonucleic acid (RNA, 101a) and deoxyribonucleic acid
(DNA, 101b) are ß-D-ribofuranosides 102a and ß-D-deoxyribofuranosides 102b
respectively, of pyrimidine 103 and purine 106 bases. In ribonucleic acids, the four
principal base residues are uracil-1-yl 104a, cytosin-1-yl 105, adenin-9-yl 107 and
guanin-9-yl 108; in deoxyribonucleic acids, three of the four base residues, cytosin-1-
yl, adenin-9-yl and guanin-9-yl, are the same as those in ribonucleic acids, but the
other principal base residue is thymin-1-yl 104b instead of uracil-1-yl 104a.

\[
\begin{align*}
101 \text{a; } R &= \text{OH} \\
101 \text{b; } R &= \text{H} \\
102 \text{a; } R &= \text{OH} \\
102 \text{b; } R &= \text{H}
\end{align*}
\]

\[
\begin{align*}
103 \\
104 \text{a; } R' &= \text{H} \\
104 \text{b; } R' &= \text{Me} \\
105 \\
106 \\
107 \\
108
\end{align*}
\]

Figure 1.1 DNA, RNA, nucleosides and nucleobases.

In 1953, Watson and Crick\textsuperscript{5} proposed the double helical structure of DNA based on
the X-ray crystallographic data obtained by Franklin and Wilkins\textsuperscript{6,7} and further
postulated a mechanism of DNA replication. The double helix or Watson–Crick
structure is referred to as the secondary structure of DNA (Figure 1.2). The purine and
pyrimidine bases are on the inside of the helix, and the phosphate and sugar moieties are on the outside. The planes of the bases are perpendicular to the helix and the planes of the sugar molecules are nearly at right angles to those of the bases. The two chains are anti-parallel, i.e. they run in opposite directions. The two strands have different sequences, but one sequence specifies the other because of the rigid rule of complementary base pairing. Adenine always pairs with thymine (A:T) and guanine always pairs with cytosine (G:C), by hydrogen bonds (Figure 1.3).

![DNA double helix](www.accessexcellence.org/AB/GG/dna_molecule.html)

**Figure 1.2** DNA double helix (Source: [www.accessexcellence.org/AB/GG/dna_molecule.html](www.accessexcellence.org/AB/GG/dna_molecule.html)).
Chapter 1 Introduction to Nucleic Acid Chemistry

This isomorphism of purine : pyrimidine base pairing ensures that there is no major distortion of the phosphodiester backbone of a double helix. Hydrogen bonding interactions between purines and/or pyrimidines other than A:T or G:C are possible, but the distortion of the backbone introduces an energy penalty that leads to rejection of such interactions in a perfect double helix. However under certain circumstances some such base pairing can be accommodated within a double helix. Furthermore, a third oligonucleotide chain can also hybridise with a double helix to form a triplex in a Hoogsteen hydrogen-bonding way (Figure 1.4), which is the basis for the antigene therapy.

The characterisation of the semi-conservative mechanism of DNA biosynthesis followed in 1960.\(^8\) The use of novel enzymatic and chemical tools for manipulating, sequencing and synthesising oligonucleotides\(^9\) facilitated the subsequent explosion in
molecular biology. The potential use of synthetic oligonucleotides as specific inhibitors of transcription (antigene oligonucleotides) or inhibitors of translation (antisense oligonucleotides) has prompted organic chemists to undertake the synthesis of a variety of oligodeoxyribo-/oligoribo- nucleotide analogues, especially those analogues in which the sugar residues or internucleotide linkages are modified. Recently, the development of DNA chips and their analytical and diagnostic applications have invited a plethora of studies.1°11

1.2 The Chemical Synthesis of Oligonucleotides

The first chemical synthesis of a nucleoside was accomplished by Michelson and Todd4 in 1955. Methods of phosphorylation were developed and a dinucleotide [d(TpT)] 109 was synthesised which was identical to naturally occurring DNA. Over the past four decades, several methods for the synthesis of oligonucleotides, both in solution and on solid supports, have been established. However, additional studies are required for the large-scale synthesis of oligonucleotides and their analogues as the demands for oligonucleotides for therapeutic use increase.

1.2.1 Overall Strategy

Nucleic acids are highly sensitive to a wide range of chemical reactions and thus only mild reaction conditions can apply in the assembly of oligonucleotide chains. The phosphodiester backbone is vulnerable to hydrolysis, and the heterocyclic bases are prone to alkylation, oxidation and reduction. Hence, the chemical reactions in oligonucleotide synthesis are limited to (1) mild alkaline hydrolysis; (2) mild acidic
hydrolysis; (3) mild nucleophilic displacement; (4) base-catalysed elimination; and (5) certain mild redox reactions.

The key step in the synthesis of oligonucleotides is the specific formation of internucleoside 3' → 5' phosphodiester linkages which is known as a coupling (or condensing) reaction. In the formation of the linkage, functional groups, except those involved in the reaction, must be suitably protected during the reaction and subsequent purification. In the example of joining a 5'-protected-dT unit 110 to a 3'-protected-dC unit 111 (Scheme 1.1), one of the two units must be phosphorylated or phosphitylated and then condensed with the other nucleoside via a coupling reaction. Where R¹ and R² are conventional protecting groups, the synthesis is described as solution-phase; if either R¹ or R² is an insoluble or inorganic support, the process is known as solid-phase synthesis which can be carried out with an automated synthesiser.

![Scheme 1.1 Joining of a 5'-protected-dT to a 3'-protected-dC unit; R¹-R⁵ = protecting groups.](image)

1.2.2 Ways of Making an Internucleotide Linkage

The development of an efficient method for the formation of an internucleotide bond has been the most vital issue in oligonucleotide synthesis for many years. Over the
past four decades, several methods have been successfully developed, which include the phosphodiester, phosphotriester, phosphite triester, phosphoramidite and H-phosphonate approaches.

1.2.2a The Phosphodiester Approach

In a communication to *Chemistry & Industry* in 1956, Khorana and co-workers\(^\text{12}\) reported the preparation of dithymidinyl phosphate via the phosphodiester approach. With toluene-\(p\)-sulphonyl chloride 113a as activating agent, 5'-\(O\)-tritylthymidine 114 and thymidine 5'-phosphate 115 were allowed to react in anhydrous pyridine to form the desired dinucleotide 116 in 45% yield (Scheme 1.2). In their later studies, the 3'-hydroxy function of 115 was blocked with an acetyl group, and 1,3-dicyclohexylcarbodiimide\(^\text{13,14}\) (DCC, 117) and other arylsulphonyl chlorides\(^\text{15}\) were demonstrated to be effective activating agents for this application.

![Scheme 1.2 Reagents and conditions: i, 113a, C\(_{3}\)H\(_{3}\)N.](image)

\(113\ a; R^1 = H, R^2 = Me\)
\(b; R^1 = R^2 = Me\)
\(c; R^1 = R^2 = CH(CH_3)_2\)
\(d; R^1 = R^2 = H\)
Over a decade after this method was proposed, the total syntheses of the genes encoding a tyrosine suppressor tRNA from *E. coli* and an alanine tRNA from yeast were achieved.

The main drawback of this approach is that the phosphodiester functions of the growing oligonucleotide are nucleophilic and therefore vulnerable to attack in the subsequent phosphorylation steps to give a branched pyrophosphate by-product, which complicates subsequent purification and also lowers the yield.

### 1.2.2b The Phosphotriester Approach

In the mid-1950s, Michelson and Todd reported the first chemical synthesis of nucleotides via the phosphotriester approach. In their study, 5'-O-acetylthymidine-3'-O-benzyl-H-phosphonate 118 was activated by *N*-chlorosuccinimide 119 then coupled with 3'-O-acetylthymidine 121 to give a dinucleotide triester 122 (Scheme 1.3), which upon removal of the protecting groups afforded natural dithymidine monophosphate 109. Compared with the phosphodiester approach, this approach has the advantage that phosphotriester intermediates (e.g. 122) are neutral species which can be purified by chromatography and which are not susceptible to phosphorylation of the internucleotide linkages. After the desired chain has been assembled, the benzyl groups can be eliminated under basic conditions.

![Scheme 1.3 Todd’s phosphotriester approach.](image-url)
However, the benzyl protecting group was found not to be stable enough in pyridine. So the search for stable phosphate protecting groups, as well as for methods leading to their removal, was pursued intensively.

The 2-cyanoethyl group (as in 123) was first examined as an alternative to the benzyl protecting group by Letsinger and Ogilvie.\textsuperscript{18,19} Up to a tetramer triphosphate was prepared. However, the 2-cyanoethyl group is still very labile to bases, and if a comparatively long oligonucleotide is to be synthesised, loss of 2-cyanoethyl protecting groups during subsequent coupling, work-up and purification is bound to occur.

Subsequently, Reese and Saffhill\textsuperscript{20} proposed aryl groups (as in 124) as potential protecting groups even though their removal by alkaline hydrolysis led to significant cleavage of internucleotide linkages. The extent of this cleavage was controlled by incorporation of electron-withdrawing substituents in the phenyl group\textsuperscript{21-23} and replacement of the alkaline hydrolysis step by treatment with the conjugate base of either (E)-2-nitrobenzaldoxime 125 or (E)-pyridine-2-carboxaldoxime\textsuperscript{24} 126. Typically, reaction of dinucleotide O-(2-chlorophenyl)phosphotriester with the 1,1,3,3-tetramethylguanidinium salt of E-2-nitrobenzaldoxime resulted in the complete removal of the 2-chlorophenyl phosphate protecting group within 30 min, while the cleavage of internucleotide linkages occurred to less than 0.1%.\textsuperscript{24} As a result of these elegant studies, the 2-chlorophenyl group was established to be the phosphotriester protecting group of choice in the phosphotriester approach, and (E)-2-
nitrobenzaldoximate became the standard unblocking reagent for the 2-chlorophenyl protecting group.

It is noteworthy that in the course of these investigations, a stepwise chain elongation strategy was developed. In conjunction with the use of trityl (or MMTr, or DMTr) as 5'-hydroxy protecting group, the β-benzoylpropionyl group (as in 127) was used to mask the 3'-hydroxy function. Treatment of a 3'-O-(β-benzoylpropionyl) phosphotriester with hydrazine hydrate in pyridine–acetic acid removed the benzoylpropionyl protecting group without internucleotide linkage cleavage. On the other hand, the 5'-trityl protection can be removed by acidic treatment. Thus, chain assembly can be directed as required. This stepwise synthetic strategy was demonstrated by Letsinger and co-workers in the preparation of a tetrathymidinyl triphosphate (Scheme 1.4).
A critical step in the phosphotriester approach is the activation of the phosphodiester prior to chain extension. Arylsulphonyl chlorides (phenyl-113d, p-tolyl-113a, 2,4,6-trimethylphenyl-113b, 2,4,6-triisopropylphenyl-113c, and 8-quinolinyl-131) have been examined for this purpose. To minimize sulphonlation of the 5'-hydroxyl
function, sterically hindered arylsulphonyl chlorides are preferred. Hindered arylsulphonyl azolides, which were found to have lower reactivity towards alcoholic functions, have been used more successfully than the chlorides. While various imidazolides, triazolides, and 4-nitroimidazolides have been found to be only marginally useful as coupling agents, 2,4,6-triisopropylbenzenesulphonyl tetrazolide and mesitylenesulphonyl 3-nitrotriazolide (MSNT) were found to be very efficient. Because MSNT is considerably more stable than 2,4,6-triisopropylbenzenesulphonyl tetrazolide, it was established as the coupling agent of choice in the phosphotriester approach.

![Chemical structures](image)

**1.2.2c The Phosphite Triester Approach**

The first investigation of the phosphite triester approach was reported in the mid-1970s by Letsinger and co-workers. The basic aspects of this method are shown in Scheme 1.5. 5'-O-Protected nucleoside was allowed to react with the bifunctional phosphitylating agent (2,2,2-trichloroethyl)phosphorodichloridite to generate a 3'-O-phosphorochloridite intermediate at low temperature (−78°C); underwent rapid coupling with a second nucleoside. The phosphite triester formed was oxidised in situ by aqueous iodine treatment to generate a phosphate triester. This approach was employed in solid phase synthesis using a silica support attached through a 3'-O-succinate linkage. By adding 1H-tetrazole to the deoxynucleoside chlorophosphite, coupling rates and yields were increased significantly. A dodecanucleotide was prepared in an overall yield of 55%. Despite the rapid coupling rate, this approach suffers from several drawbacks. The preparation of the nucleoside chlorophosphite from the bifunctional phosphitylating agents such as had to be performed at low temperature in the absence of moisture; and because of the sensitivity of the reagents to oxidation and hydrolysis, they have to be prepared immediately before use. A more serious problem
with this approach is the formation of $3' \rightarrow 3'$ and $5' \rightarrow 5'$ symmetrical isomers due to the use of the bifunctional phosphorodichloridite reagents. Efforts to improve this approach eventually led to the emergence of phosphoramidite chemistry.

1.2.2d The Phosphoramidite Approach

In 1981, the preparation of a new class of reagent called nucleoside phosphoramidites was reported by Beaucage and Caruthers; this led to a new strategy called the phosphoramidite approach. As a modification to the phosphite triester approach, the reactive chlorophosphite in the phosphite triester approach was replaced with a phosphoramidite, which is stable to hydrolysis and oxidation under normal laboratory
conditions, but is readily activated to form an internucleotide linkage in virtually quantitative yield with very few side reactions.

This approach consists of reacting the protected deoxynucleoside 141 with chloro-
N,N-dimethylaminomethoxyphosphine 142 and N,N-diisopropylethylamine. The product 143, upon activation by N,N-dimethylaniline hydrochloride 144 affords the corresponding chlorophosphite 145 which couples rapidly with a second nucleoside with a free hydroxyl function 146 to give a phosphite triester 147. As in the phosphite triester approach, iodine oxidation of the phosphite triester 147 produces the corresponding phosphotriester 148 (Scheme 1.6).

Scheme 1.6 The original phosphoramidite chemistry.
Chapter 1: Introduction to Nucleic Acid Chemistry

Tertiary amine hydrochlorides however are hygroscopic, which leads to unreliable and unreproducible results. 1H-Tetrazole 140, which is a non-hygroscopic weak acid, was then chosen as a suitable activating agent in the phosphoramidite approach.\textsuperscript{38} 3'-O-(Methoxy-N,N-dimethyl)phosphoramidites 143 were however found to be unstable in acetonitrile which is the solvent most commonly used in solid phase synthesis. Therefore, more detailed investigation was directed to the exploration of stable phosphoramidite derivatives.

Several methyl N,N-dialkylaminophosphoramidites, for example methoxy-morpholinophosphine derivative 149 and (methyl N,N-diisopropylphosphoramidite) derivative\textsuperscript{39,40} 150 were later found to be stable in acetonitrile. After the chain assembly process is complete, the methyl protecting groups can be removed by treatment with thiophenol, which is rather unpleasant. To eliminate this thiolate treatment, more phosphoramidite derivatives were prepared. Among them, (2-cyanoethyl N,N-diisopropylphosphoramidite) derivative\textsuperscript{41} 151 were found to be stable in acetonitrile and the cleavage of the cyanoethyl protecting groups from the oligonucleotides could be effected under basic conditions required for the removal of the nucleobase protecting groups, i.e. aqueous ammonia treatment. This proved to work extremely well and has since been widely used in standard phosphoramidite oligonucleotide synthesis. By using these phosphoramidites, relatively large oligonucleotides (up to 150-mer) were successfully synthesised by solid phase synthesis on nonporous silica microbeads\textsuperscript{42} and rigid non-swelling polystyrene beads\textsuperscript{43} with stepwise coupling yields averaging from 98% to 99%.
1.2.2e The \(H\)-Phosphonate Approach

Nucleoside \(H\)-phosphonates were first used in the synthesis of oligonucleotides by Todd and co-workers\(^{44}\) in the late 1950s. In their studies, 2',3'-protected uridine \(5'-H\)-phosphonate 152 was activated by diphenyl phosphorochloridate 153 and coupled with 2',3'-protected adenosine 154, affording the corresponding dinucleotide \(H\)-phosphonate 155 in high yield. The \(H\)-phosphonate diester linkage was easily oxidised by \(N\)-chlorosuccinimide 119 to give phosphodiester 156 (Scheme 1.7).

![Scheme 1.7 Original studies on \(H\)-phosphonate chemistry.](image-url)
This approach was applied to polymer-supported solid phase synthesis of short oligomers (di, tri, and tetramers) in the early 1970s but only gave poor to moderate yields (46–80%). The H-phosphonate approach did not therefore receive intensive study until the mid-1980s. In 1985, Stawiński and co-workers found that 5′-O-dimethoxytrityl thymidine 3′-H-phosphonate reacted rapidly with 3′-O-benzoylthymidine in the presence of diphenyl phosphorochloridate and 1-methylimidazole to give the dithymidinyl H-phosphonate 159 in nearly quantitative yield. It was postulated that a reactive mixed-anhydride was formed from the activation of the H-phosphonate with diphenyl phosphorochloridate, which underwent rapid condensation with the nucleoside with a free hydroxyl function, 158 (Scheme 1.8).

Scheme 1.8 Activation of H-phosphonate.

It was also found that a trinucleoside metaphosphate could be formed when the activated H-phosphonate (i.e. mixed-anhydride) was allowed to stand in pyridine for 5 min. This metaphosphate could then be converted to phosphorodichloridite depending on the amount of diphenyl phosphorochloridate used.
Apart from diphenyl phosphorochloridate, other activating agents were also evaluated. Sterically hindered acyl chlorides, such as pivaloyl chloride\textsuperscript{49} 163 and adamantancarbonyl chloride\textsuperscript{50} 164 were found to be very efficient coupling agents. These acyl chlorides activate the H-phosphonates 157 by the same mechanism as diphenyl phosphorochloridate does, and a mixed-anhydride intermediate 165 is generated as the reactive species\textsuperscript{51,52} (Scheme 1.9). In the presence of excess acyl chloride, the mixed-anhydride further reacts with the activating agent to form a bis-acylphosphite 166 or a more complicated by-product 167.

\begin{center}
\begin{tikzpicture}
\node at (0,0) [draw] {163};
\node at (1.5,0) [draw] {164};
\node at (3,0) [draw] {168};
\end{tikzpicture}
\end{center}

\textbf{Scheme 1.9} Acyl chloride as activating agent.
A less reactive coupling agent, dipentafluorophenyl carbonate 168 was later demonstrated to minimise the side reactions discussed above. 53

Although the H-phosphonate approach had shown great potential for the preparation of oligonucleotides, it was not until very recently that it emerged as an approach of choice in large-scale synthesis of oligonucleotides in solution. A couple of years ago, Reese and co-workers 54,55 developed a modified H-phosphonate strategy involving the coupling of an H-phosphonate 169 with a nucleoside (or nucleotide) with a free hydroxyl function 170, followed by a sulphur-transfer step, to give a fully protected S-aryl or S-alkyl phosphorothioate triester 172. As soon as the coupling had gone to completion, the H-phosphonate diester 171 was converted in situ to a stable phosphorothioate triester 172 by reacting with a sulphur-transfer agent 173 or 174 in one pot (Scheme 1.10).

![Scheme 1.10 A modified H-phosphonate approach.](image)

According to the choice of sulphur-transfer agent (S-(2-cyanoethyl) or S-aryl) and unblocking reagent (DBU or oximate), either a phosphorothioate diester 175a or phosphate diester 175b could be obtained (Scheme 1.11). It is therefore possible to
extend this strategy to the preparation of chimeric sequences with both phosphate and phosphorothioate linkages.

Scheme 1.11 Unblocking of the fully-protected phosphorothioate triesters.

1.2.3 Solid Phase Synthesis of Oligonucleotides

The idea of carrying out an organic synthesis on a solid support was proposed by Merrifield\textsuperscript{56} in relation to peptide synthesis. This strategy was afterwards adapted to oligonucleotide synthesis.\textsuperscript{57} The attractive feature of this strategy is obvious: the separation of the growing oligomer products can be easily effected through simple filtration and further washing, which avoids time-consuming isolation and purification involved in solution synthesis. Therefore, tremendous work has since been directed towards this innovative field.

As a result of intensive studies, several solid supports emerged to be suitable for the synthesis of oligonucleotides. HPLC grade silica\textsuperscript{36,37} and controlled-pore glass (CPG)\textsuperscript{58} have been used successfully in solid phase oligonucleotide synthesis. Particularly, CPG has been used extensively in automated oligonucleotide synthesis. At the present time, the leader nucleoside is anchored covalently to the commercially available ‘long-chain alkylamine’ CPG through a succinate linkage (Figure 1.5).
Over the past four decades, all the approaches discussed above have been evaluated on solid supports for the preparation of oligonucleotides. However, solid phase synthesis of oligonucleotides using the phosphoramidite approach has so far been the most successful strategy and has been established as the standard methodology. The optimised solid phase cycle is shown in Scheme 1.12. Four basic steps are involved in this approach: detritylation, coupling, capping and oxidation. The cycle is repeated until the desired oligonucleotide has been assembled. After the desired chain has been assembled, it is then cleaved from the solid support by treatment with aqueous ammonia, which removes the exocyclic NH protecting groups on the nucleobases as well.

Phosphoramidite-based solid phase synthesis offers great versatility in assembling oligonucleotides of moderate chain length and the time required for this process is very much shorter than for solution phase synthesis. But whether it can be scaled up to a manufacturing scale is yet to be investigated.
Scheme 1.12 A phosphoramidite solid phase synthesis cycle.
Antisense Oligonucleotides and Analogues

In 1978, synthetic oligonucleotides were for the first time proposed by Zamecnik and Stephenson$^{59}$ as a class of potential therapeutics to inhibit gene expression through an antisense mechanism. In the January 1990 issue of Scientific American, an introduction to the field of antisense nucleic acids was presented by Weintraub,$^{60}$ in which he defines the field as follows:

Antisense RNA and DNA

'Molecules that bind with specific messenger RNAs can selectively turn off genes. Eventually certain diseases may be treated with them.'

After this novel therapeutic strategy was conceived, a great deal of work was directed towards the elucidation of the mechanism of antisense inhibition, the development of new antisense oligonucleotides and studies on their pharmacokinetics. The intensive investigation of this strategy led to a clinical trial of the first drug of this kind in the mid-1990s.

2.1 Mechanism of Antisense Inhibition

It was established a few decades ago that the genetic information is encoded in double-stranded DNA and transcribed into single-stranded mRNA, which is then expressed as protein, the functioning biological species. Whereas traditional drug discovery targeted the protein or later stages of metabolism, it has been assumed that a drug which operates at the DNA or RNA level could be superior to traditional drugs. It is the progress in biological techniques which opens up the possibility of introducing normal, functioning genes to replace defective, non-functioning genes, and also that of selectively preventing the expression of unwanted genes. By such
Chapter 2 Antisense Oligonucleotides and Analogues

means, it may be possible to develop highly effective antiviral agents which suppress viral genes without simultaneously affecting the expression of host cellular genes. Such selective inhibition is possible in theory due to the known complementary Watson–Crick base pairing rules. Oligonucleotides which bind with single-stranded RNA are named as antisense oligonucleotides, while those which interact with double-stranded DNA are called antigene oligonucleotides.

The mechanism of action of an antisense oligonucleotide depends on the target sequence it seeks. Although the structural requirements to be met by an effective antisense oligonucleotide have not yet been clearly defined, there are concrete theories about the mechanism of its action. To understand the principles behind the design of antisense oligonucleotides, it is necessary to recall briefly the biosynthesis of protein.

At the beginning of the process, DNA is transcribed. But in addition to the coding sequences which are called exons, the precursor mRNA also contains extra nucleotide sequences called introns. In the next step which is called splicing, introns are excised from the precursor mRNA and the exons are joined together to form the mature mRNA. The mature mRNA, which contains a 5'-cap region, an untranslated leader sequence, an AUG initiation codon which signals the ribosome to start protein synthesis, the coding regions, a terminating codon which signals the ribosome to stop protein synthesis, and a 3'-untranslated sequence with a polyadenylate tract, is then transported through the nuclear membrane to the cytoplasm, where protein synthesis takes place (Figure 2.1).
The mechanism of antisense therapy can be simply illustrated as in Figure 2.2. An antisense oligonucleotide binds specifically with a complementary area within an mRNA, according to Watson–Crick base pairing rules. Thus the biological synthesis of a protein is switched off.

To view the process in more detail, several possible antisense mechanisms can be considered.


2.1.1 Transcription Inhibition

At the stage of transcription, RNA polymerase binds to DNA to form a closed complex. The closed complex is then converted into an open complex where the double-stranded DNA is unwound. When this open complex is attacked by an antisense oligonucleotide, transcription is inhibited (Figure 2.3).

2.1.2 Inhibition of Splicing

When an antisense oligonucleotide targets at an exon/intron junction, the complementary complex formed will block the access of the spliceosome to this junction, resulting in an improperly spliced mRNA which degrades in the nucleus.
2.1.3 Non-Sequence-Specific Mechanism

Although it is generally believed that antisense oligonucleotides work on the basis of specific binding of the oligos with mRNA, there is evidence which supports non-sequence-specific mechanisms. Inhibition of Rauscher leukaemia virus by homopolynucleotides⁶¹ and inhibition of HIV by phosphorothioates⁶² based on inhibition of reverse transcriptase have been shown to be possible.

2.1.4 RNase H Mechanism

In some circumstances, RNase H selectively cleaves the RNA strand at the antisense oligonucleotide-binding site.⁶³-⁶⁵ The antisense oligonucleotide itself is not cleaved and therefore is free to bind to another RNA molecule. However, this action of RNase H entails the risk of a certain lack of specificity because even brief interaction
of only 5–6 base pairs may be recognised as a DNA : RNA substrate.\textsuperscript{66,67} Especially at the site of injection, the transient high local concentration of oligos can bring about a non-specific mRNA breakdown.\textsuperscript{68}

2.2 Requirements of Antisense Oligonucleotides

For oligonucleotides to be useful as antisense therapeutic agents, they must meet the following requirements:

1. The oligonucleotides must have good stability towards extra- and intra-cellular enzymes. Oligonucleotide phosphates, the natural nucleotides, are therefore not suitable for this purpose because they are degraded by nucleases quickly \textit{in vivo}.

2. The oligonucleotides must be able to penetrate through the cell membrane.

3. The oligonucleotides must specifically bind to the targets to form duplexes. Non-sequence-specific interaction with other macromolecules should be avoided.

4. For the antisense oligonucleotides to be useful in clinical therapy, they must be synthesised and scalable easily. Phosphoramidite based solid-phase chemistry has greatly eased synthesis and improved availability of oligonucleotides. Large-scale oligonucleotide synthesis is still pursued intensively.

2.3 Deciding the Length of Antisense Oligonucleotides

To achieve a high degree of selectivity of an antisense oligonucleotide in the interaction with its target, it should be capable of binding only to its target mRNA and not to other mRNAs.

Statistically, a 17-mer oligonucleotide sequence occurs just once in the human genome.\textsuperscript{69} Furthermore, at a particular point in time, only 10–20\% of the genes in the cell are being transcribed into the corresponding mRNA. Also, if one takes into account the percentage of the genome which is actually expressed as mRNA (0.5\%),
an oligonucleotide with 15–20 bases will be uniquely complementary to a single nucleotide sequence within the human genome, which contains approximately $4 \times 10^9$ base pairs.\(^7\)

The affinity of the binding between an oligonucleotide and its target should also be taken into account in deciding the base-pair length of an antisense oligonucleotide. This affinity is characterised by the melting temperature ($T_m$). $T_m$, which is the temperature at which 50% of the double strand has dissociated into its two single strands, is dependent on the concentration of the oligonucleotide and on the properties of the solvent. For an unmodified oligonucleotide with a length of ca. 12–20 bases, the rule of Wallace\(^7\) can be used to estimate its $T_m$ at high salt concentration:

$$T_m = n \times (2^\circ C) + m \times (4^\circ C)$$

where $n$ is the number of dA•T base pairs and $m$ is the number of dG•dC base pairs. Under physiological conditions (37°C, low salt concentration), at least 12 base pairs are required to achieve reasonably stable hybridisation of an oligonucleotide with its target sequence. In practice, a 20-mer oligonucleotide with average base pairing may be assumed to have a $T_m$ of ca. 54°C (DNA duplex in 0.1 M NaCl).\(^7\)

### 2.4 Modified Oligonucleotides

In order to improve the properties of oligonucleotides such as nuclease stability, cellular penetration and potency, there has been an explosion of effort towards the synthesis of oligonucleotide analogues. These investigations have involved modifications of the phosphodiester backbone, the base, and the sugar ring.

The development of modified oligonucleotides started with the replacement of the phosphodiester linkage, which was earlier identified as a necessary requirement for advancing antisense oligonucleotides, since unmodified oligonucleotides are rapidly degraded by nucleases that recognise the phosphodiester linkage. Virtually any modification to the phosphodiester backbone confers stability to nucleases. Anionic
Chapter 2. Antisense Oligonucleotides and Analogues

201b–e, cationic 202 and neutral phosphate analogues 203a–g, 204 have been found to be resistant to nucleases. And these backbone modifications may also allow for enhanced affinity and increased cellular penetration of the oligos. Among these analogues, phosphorothioates 201b, morpholino derivatives 205, 206 and peptide nucleic acids (PNA)82 207 have received intensive studies.

Anionic phosphodiester analogues

<table>
<thead>
<tr>
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<th>Y</th>
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<td>O</td>
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<tr>
<td>c,</td>
<td>O</td>
<td>S</td>
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<tr>
<td>d,</td>
<td>O</td>
<td>O</td>
<td>BH3</td>
</tr>
<tr>
<td>e,</td>
<td>NH</td>
<td>O</td>
<td>O</td>
</tr>
</tbody>
</table>

Cationic analogue77

![Diagram of cationic and anionic analogues]
Chapter 2. Antisense Oligonucleotides and Analogues

Neutral backbone analogues

<table>
<thead>
<tr>
<th></th>
<th>X</th>
<th>Y</th>
<th>Z</th>
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</thead>
<tbody>
<tr>
<td>a</td>
<td>S</td>
<td>CH₂</td>
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<td>b</td>
<td>O</td>
<td>CH₂</td>
<td>O</td>
</tr>
<tr>
<td>c</td>
<td>CH₂</td>
<td>NMe</td>
<td>O</td>
</tr>
<tr>
<td>d</td>
<td>O</td>
<td>C=O</td>
<td>NH</td>
</tr>
<tr>
<td>e</td>
<td>CH₂</td>
<td>SO₂</td>
<td>CH₂</td>
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<tr>
<td>f</td>
<td>NH</td>
<td>SO₂</td>
<td>CH₂</td>
</tr>
<tr>
<td>g</td>
<td>O</td>
<td>SO₂</td>
<td>NH</td>
</tr>
</tbody>
</table>

Methylphosphonate

Morpholino-carbamate
Modification of the ribose portion has also been pursued. The ribose ring has been appended to and substituted, conformationally restricted or replaced by hexose. The introduction of substituent groups at the 2' position is the most straightforward modification. 2'-O-Methyl, 2'-fluoro-2'-deoxy, and 2'-O-(2-methoxyethyl) ribose derivatives have been investigated. The trend is that nuclease stability increases, and binding affinity decreases, with increasing substituent size. The replacement of the ribose with a hexose scaffold confers significantly enhanced affinities to RNA and improves the stability of the phosphodiester linkage towards nucleases.
Modifications of the aromatic base residues are also made to enhance affinity towards RNAs. A simple modification is to introduce a propynyl group on the 5-position of pyrimidines\(^{85}\) (209, 210) or 7-position of 7-deazapurines\(^{86,87}\) (211, 212). These substitutions significantly improve affinity.

\[
\begin{array}{c}
\text{209} & \text{210} \\
\text{211} & \text{212}
\end{array}
\]

However, some of these modifications do not allow the oligonucleotides to mediate the destruction of the target mRNA by an RNase H mediated mechanism. RNase H requires an RNA/DNA hybrid molecule as a substrate. If the oligonucleotide is modified too much (e.g. by modification at the 2' position of the sugar ring), it is no longer recognised by RNase H. To get around this problem, oligonucleotides have been designed such that some of their bases are modified to allow for increased stability and affinity, while others are left unmodified to allow hybrids of the oligonucleotides with RNA to remain viable substrates for RNase H.\(^{88-90}\) Another advantage of these chimeric oligonucleotides is that they have been found to have even fewer toxic side effects than oligonucleotide phosphorothioates. These ‘second-generation’ antisense oligonucleotides are beginning to make their way into clinical trial and may replace oligonucleotide phosphorothioates altogether in the near future. Two examples of the chimeric oligonucleotides are shown in Figure 2.4\(^1\) (213, 214).
2.5 Oligonucleotide Phosphorothioates (PS-Oligos)

In this context, our attention is drawn towards first generation oligonucleotide phosphorothioates.

There are three constitutionally isomeric forms of internucleotide phosphorothioate linkage. Replacement of either the 3'- or 5'-oxygen in a phosphodiester linkage, 215, results in the respective thiolo structures 216 or 217, whereas substitution of a non-bridging oxygen with sulphur gives a thiono (phosphorothioate) structure 218, 219.
Compared with oxygen, sulphur has a larger atomic radius and lower electronegativity, and displays less multiple bonding.\textsuperscript{92} It is generally accepted that in the P=Z bonding type, charge transfer takes place, which is greater in P=O than in P=S, and therefore the former bond is always stronger; on the other hand, the P=S bond is more polarizable than the P=O bond.\textsuperscript{93} Therefore, the phosphorothioate linkage is better represented by the thio ion \textbf{218} than by the oxy ion \textbf{219}.

Oligonucleotide phosphorothioates possess the following physicochemical and biological properties:

1. The substitution of oxygen with sulphur generates chirality at the phosphorus (\(R_P\) and \(S_P\)) centres and therefore the number of diastereoisomers is \(2^n\), where \(n\) is the number of phosphorothioate linkages.

2. In comparison to that of unmodified oligonucleotide phosphates, the enzymatic resistance of phosphorothioates is greatly improved. This improved resistance was first observed by Eckstein\textsuperscript{94} and later it was found that snake venom phosphodiesterase (SVPD) only digests the \(R_P\) configuration phosphorothioates\textsuperscript{95} and nuclease \(P_1\) only accepts the \(S_P\) configuration phosphorothioates. Studies on the digestion of oligonucleotide phosphorothioates by three nucleases (\(S_1\), \(P_1\) and SVPD) showed that\textsuperscript{96} the rates of digestion of oligonucleotide phosphorothioates with nuclease \(S_1\) and \(P_1\) were \(ca. \) 10–100 times slower than those of unmodified oligonucleotide phosphates, and oligonucleotide phosphorothioates were digested some \(10^2\)–\(10^3\) times slower than unmodified phosphate oligonucleotides with SVPD. In
in vivo studies showed that they are cleaved only slowly by metabolism, with elimination half lives of 48 hours or longer in all species studied.97

3. The oligonucleotide phosphorothioates retain water solubility and chemical stability of charged phosphate analogues.

4. The oligonucleotide phosphorothioates are capable of hybridising effectively with their complementary strands to form stable duplexes. However, in terms of $T_m$, the duplexes formed between phosphorothioate oligonucleotides and their complementary strands are less stable than normal duplexes, with lower $T_m$ values.96

5. The duplexes formed between oligonucleotide phosphorothioates and their complementary strands remain as substrates for RNase H.98

So far, oligonucleotide phosphorothioate analogues have received intensive studies and clinical trials of a few oligonucleotide phosphorothioates are under way (Table 2.1). Among these analogues, the anti-human-cytomegalovirus (CMV) agent ISIS 2922 (known as VitraveneTM commercially, 220) (Figure 2.5) has been licensed for therapeutic use.99
Table 2.1 Some oligonucleotide phosphorothioates in clinical trials.

<table>
<thead>
<tr>
<th>Molecular Target</th>
<th>Sequence (5’→3’)</th>
<th>Disease Target</th>
<th>Clinical Status</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Viral diseases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV</td>
<td>GCG TTT GCT CTT CTT GCG</td>
<td>CMV-induced retinitis</td>
<td>Approved</td>
</tr>
<tr>
<td>CMV</td>
<td>UGG GGC TTA CCT TGC GAA CA</td>
<td>CMV-induced retinitis</td>
<td>Phase II</td>
</tr>
<tr>
<td>HIV-1</td>
<td>CTC TCG CAC CCA TCT CTC TCC TTC T</td>
<td>HIV-1-AIDS</td>
<td>Withdrawn</td>
</tr>
<tr>
<td>HPV</td>
<td>TGT CTT CCA TCT TCC TGC TC</td>
<td>Genital warts</td>
<td>Withdrawn</td>
</tr>
<tr>
<td><strong>Oncological and hematological diseases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bcl-2</td>
<td>TCT CCC AGC GTG CCG CAT</td>
<td>Prostate; non-Hodgkin’s lymphoma</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>bcr-abl</td>
<td>CGC TGA AGG GCT TCT TCC TTA TTG AT</td>
<td>CML, advanced phase</td>
<td>Pilot</td>
</tr>
<tr>
<td>bcr-abl</td>
<td>CGC TGA AGG GCT TTT GAA CTG TGC TT</td>
<td>CML, blast crisis</td>
<td>Pilot</td>
</tr>
<tr>
<td>c-myb</td>
<td>TAT GCT GTG CCG GGG TCT TCG GGC</td>
<td>CML, blast crisis, refractory leukaemia</td>
<td>Phase I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CML, chronic/accelerated phase</td>
<td>Pilot</td>
</tr>
<tr>
<td>c-myc</td>
<td>GCT AAC GTT GAG GGG CAT</td>
<td>Restenosis</td>
<td>Withdrawn</td>
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<td>c-raf</td>
<td>TCC CGC CTG TGA CAT GCA TT</td>
<td>Prostate, breast, ovarian, pancreas, colon, lung</td>
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</tr>
<tr>
<td>Ha-ras</td>
<td>GGG ACT CCT CGC TAC TGC CT</td>
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<td>ICAM-I</td>
<td>GCC CAA GCT GGC ATC CGT CA</td>
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<tr>
<td>p53</td>
<td>CCC TGC TCC CCC CTG GCT CC</td>
<td>AML and myelodysplastic syndrome, refractory or relapsed</td>
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<tr>
<td>PKA-RIα</td>
<td>GCG UGC CTC CTC ACU GGC</td>
<td>AML and myelodysplastic syndrome</td>
<td>Pilot</td>
</tr>
<tr>
<td>PKC-α</td>
<td>GTT CTC GCT GGT GAG TTT CA</td>
<td>Ovarian, prostate, breast, brain, lung, colon, melanoma</td>
<td>Phase II</td>
</tr>
</tbody>
</table>

Note: a. AML, acute myeloid leukemia; CML, chronic myeloid leukemia; CMV, cytomegalovirus; HPV, human papilloma virus; ICAM, intercellular adhesion molecule; PKA, protein kinase A; PKC, protein kinase C; RIα, regulatory subunit Iα. b. All the sequences contain phosphorothioate interlinkages; plain and bold letters indicate deoxy- and 2'-O-methyl-ribonucleosides, respectively.
2.6 Fomivirsen (ISIS 2922 sodium salt)

Human cytomegalovirus (CMV), a ubiquitous herpes virus, is the most common cause of viral retinitis in immunocompromised individuals, including patients with HIV infection.\textsuperscript{100} Cytomegalovirus retinitis is a sight-threatening disease, characterised by the progressive destruction of retinal cells, and is a major cause of morbidity in patients with AIDS.\textsuperscript{101}

Fomivirsen (ISIS 2922) is an antisense oligonucleotide phosphorothioate with 21 base residues (Figure 2.5), which specifically inhibits replication of human cytomegalovirus and is the first antisense drug evaluated in the treatment of patients with AIDS-related CMV retinitis.\textsuperscript{99}

The antiviral action of fomivirsen is based on a combination of antisense and non-antisense mechanisms. Immediate-early gene expression, which is essential for viral replication, is inhibited by fomivirsen via an antisense mechanism and the adsorption of cytomegalovirus to host cells can also be inhibited through a sequence-independent mechanism.\textsuperscript{102,103} The administration of fomivirsen significantly delays progression of CMV in patients with AIDS in preliminary clinical trials.\textsuperscript{104,105} The common adverse events with this drug reported in clinical trials include increased intraocular pressure and mild to moderate intraocular inflammation, which is generally transient or reversible with topical steroid treatment.\textsuperscript{106,107} On July 22 1998, the Ophthalmic Subcommittee of the Dermatologic and Ophthalmic Drugs Advisory Committee of the FDA recommended approval of the new drug application for fomivirsen sodium (Vitravene\textsuperscript{TM}) for the treatment of patients with cytomegalovirus-induced retinitis by intravitreal injection.
Figure 2.5 Fomivirsen (ISIS 2922) 220.
Synthesis of Oligonucleotide Phosphorothioates

3.1 Chemical Synthesis of Oligonucleotide Phosphorothioates

Oligonucleotide phosphorothioates can be and have been prepared by all the approaches used for unmodified oligonucleotides. Among these strategies, the phosphotriester, phosphoramidite and $H$-phosphonate approaches have proved to be effective for this application. So far, the phosphotriester approach has been mainly used in the preparation of oligonucleotide phosphorothioates in solution, whereas the phosphoramidite and $H$-phosphonate approaches have almost exclusively been used in solid phase synthesis.

3.1.1 The Phosphodiester Approach

The chemical synthesis of oligonucleotide phosphorothioates via the phosphodiester approach was first reported by Eckstein\textsuperscript{94,108} in 1967. By using triisopropylbenzenesulphonyl chloride (TPS-Cl, 113c) as condensing agent, 3'-O-acetylthymidine 5'-phosphorothioate 301 was coupled with 5'-O-tritylthymidine 114 in pyridine. The desired dithymidine phosphorothioate 302 was obtained in 18\% yield. Loss of sulphur, which generated dithymidine phosphate 303 in 33\% yield, was also observed (Scheme 3.1).

The low yield is not surprising. Firstly, one of the starting materials 301 itself is bifunctional and the product 302 is also open to further phosphorylation; secondly, TPS-Cl may not be a suitable condensing agent to activate the thio ion. It was actually observed that when DCC 117\textsuperscript{94} was used as coupling agent, complete loss of sulphur occurred and only fully-desulphurised product 303 was formed.
Chapter 3 Synthesis of Oligonucleotide Phosphorothioates

Scheme 3.1 Preparation of phosphorothioate via phosphodiester approach.

3.1.2 The Phosphotriester Approach

Perhaps the most straightforward remedy for the phosphodiester approach is to introduce a suitable protecting group (as in 304) on the phosphorus centre of the phosphorothioate monoester. By doing this, the side-reactions resulting from the bifunctionality of the starting material 301 and the vulnerability of the coupling product to further phosphorylation can be suppressed. Also, loss of sulphur due to the lack of selectivity of activation is altogether avoided.

The $S$-(2-cyanoethyl) group was first used by Malkievicz and Smrt\textsuperscript{109} in the preparation of dithymidine phosphorothioates. The 3'-O-acetylthymidine 5'-phosphorothioate $S$-(2-cyanoethyl) diester 304 was activated by TPS-Cl 113c and coupled with 5'-O-dimethoxytritylthymidine 305. The desired phosphorothioate...
Chapter 3 Synthesis of Oligonucleotide Phosphorothioates

triester DMTr-Tp(s)T-Ac*, 306, was obtained in 80% yield (Scheme 3.2). Unblocking of this triester was carried out in two steps. After treatment with 90% acetic acid, methanolic ammonolysis of the material gave the fully-unblocked phosphorothioate diester Tp(s)T in quantitative yield. However 3'-O-acetylthymidine 5'-phosphorothioate S-cyanoethyl diester 304 was difficult to prepare and the yield was poor.

In the following decade, several other activating agents were investigated for this approach. Reese et al.111 and van Boom et al.112 introduced 2,5-dichlorophenyl phosphorodichloridothioate 307 as a coupling agent. This is a very reactive activating agent but it is very sensitive to moisture. In addition, it is a bifunctional phosphorylating agent; therefore, some symmetrical (3'→3')-phosphotriester 308 is formed along with the activated intermediate 309. Both 309 and 307 can condense with a nucleoside with free 5'-hydroxy function to produce the desired asymmetrical (3'→5')- and the respective symmetrical (5'→5')-phosphotriester 310 and 311. The symmetrical products 308 and 311 are undesirable because they lower the yield of 310 and also create purification problems113 (Scheme 3.3).

* A number of years ago, Reese and co-workers110 introduced a system of abbreviations for protected oligonucleotides in which nucleoside residues and internucleotide linkages are italicised if they are protected in some defined way. In the present context, T, G and C represent thymidine protected on O-4 with a phenyl group, 2'-deoxyguanosine protected on N-2 and O-6 with isobutyryl and 2,5-dichlorophenyl groups, and cytidine protected on N-4 with a benzoyl group, respectively; and p(s) represents an S-(2-cyanoethyl)-protected phosphorothioate.
Van Boom\textsuperscript{112} also proposed the use of 1-hydroxybenzotriazole derivatives of 2,5-dichlorophenyl phosphorodichloridothioate, 312\textsuperscript{a,b} as condensing agents. By using 2,5-dichlorophenyl-\textit{O,O}-bis(6-nitro-1-benzotriazolyl) phosphorothioate 312\textsuperscript{b} as activating agent, hexadecamers were assembled, and the yields for 10-min synthesis cycles were greater than 90%.

A facile preparation of nucleoside 3'-phosphorothioate \textit{S}-(2-cyanoethyl) diesters was reported by Reese\textsuperscript{114} (Scheme 3.4). Suitably protected nucleoside 313 was treated with phosphorus trichloride and 1\textit{H}-1,2,4-triazole 314 to give \textit{H}-phosphonate monoester 315 in good yield (98%). Then a sulphur transfer step was incorporated. \textit{H}-Phosphonate monoester 315 was allowed to react with chlorotrimethylsilane and \textit{N}-[(2-cyanoethyl)sulphanyl]phthalimide 173\textsuperscript{a} to form 3'-phosphorothioate \textit{S}-(2-cyanoethyl) ester 316 in quantitative yield.
Chapter 3 Synthesis of Oligorucleotide Phosphorothioates

Scheme 3.4 Reagents and conditions: i, (a) PC13, 314, Et3N, THF, -35°C; (b) Et3N – water, room temp.; ii, 173a, Me3SiCl, 4-methylmorpholine, CH2Cl2, room temp.; iii, aq. Et3NH+HCO3-.

This methodology proved very effective and was later successfully applied to the preparation of an octamer Tp(s)Tp(s)Gp(s)Gp(s)Gp(s)Gp(s)Tp(s)T 317 using blockmer strategy115 (Figure 3.1).

Figure 3.1

3.1.3 The Phosphite and Phosphoramidite Approaches

In the late 1970s, Burgers and Eckstein116 described the first synthesis of diribonucleoside phosphorothioates by oxidation of phosphite triesters with sulphur (Scheme 3.5). After diribonucleoside phosphite triester 318 was formed at -78°C, the sulphurisation was effected by treating phosphite triester 318 with a 1 M solution of S8 in pyridine and the reaction was complete in 16 hours at room temperature to give the corresponding diribonucleoside phosphorothioate triester 319.
This methodology was later extended to oligodeoxynucleotide synthesis on solid supports. By using controlled pore glass (CPG) as solid support, the standard phosphoramidite method was adapted for this application (Scheme 3.6). The sulphurisation reagents used by Eckstein\textsuperscript{117} and Zon\textsuperscript{118} were a 0.2 \textit{M} solution of \( S_8 \) in \( \text{CS}_2\)-pyridine (1:1 v/v) (30 minutes at room temperature) and a 0.4 \textit{M} solution of \( S_8 \) in 2,6-lutidine (15 minutes at 60\textdegree{}C). But the poor solubility of elemental sulphur in these systems, and actually in most organic solvents, caused the reaction to be slow. In automated synthesis, this may also lead to blockage of the synthesizer. Therefore, for this strategy to work well, alternative sulphur-transfer reagents are required.

For a compound to be suitable as a sulphur transfer agent, it should be prepared readily and handled easily under laboratory conditions; it should be stable and its solubility in various solvents should be compatible with automated oligonucleotide synthesis; and finally it must convert phosphite triesters to phosphorothioate triesters fast and quantitatively without modification of the nucleosides.
Chapter 3 Synthesis of Oligonucleotide Phosphorothioates

So far, various compounds have been examined. Among them several have been shown to be suitable for this application. Beaucage reagent,\textsuperscript{119,120} 3H-1,2-benzodithiol-3-one 1,1-dioxide \textsuperscript{323}, van Boom’s reagents,\textsuperscript{121} di-(phenylacetyl) disulphide \textsuperscript{324} and dibenzoyl disulphide \textsuperscript{325}, ABI’s reagent,\textsuperscript{122} tetraethylthiuram disulphide (TETD) \textsuperscript{326}, Reese’s reagent,\textsuperscript{123} dibenzoyltetrasulphide \textsuperscript{327}, Stec’s reagent,\textsuperscript{124} bis-(O,O-diisopropoxy phosphinothioyl)disulphide \textsuperscript{328} and Cruachem’s reagent,\textsuperscript{125} benzyltriethylammonium tetrathiomolybdate \textsuperscript{329} were found to be very promising. Among these, the Beaucage reagent \textsuperscript{323} appeared most rapid and efficient, with average sulphurisation efficiency being 99.75\% in a synthesis of S-d(TTG CTT CCA TCT TCC TCG TC) on a 10 \textmu mol scale.\textsuperscript{126} However, use of this reagent on an automated synthesizer requires some precautions or special treatment of the reagent bottle.\textsuperscript{127} The reagent bottle has to be treated with dilute sulphuric acid followed by washing with water and methanol. Prior to use, the bottle has to be treated with Sigmacote or 2\% dichlorodimethylsilane solution in carbon tetrachloride followed by methanol. The metal filter also has to be replaced with a Porex filter, and the solution should be covered with aluminium foil to minimize exposure to light. The ABI reagent \textsuperscript{326} offers the advantages of being a relatively low-cost chemical commodity that is nonhazardous (used also as a pharmaceutical), and is both very soluble (0.5 \textit{M}) and indefinitely stable in acetonitrile – the prevailing solvent for solid phase synthesis. By using the ABI reagent as sulphur transfer agent, an overall sulphurisation efficiency greater than 99\% was achieved for the synthesis of S-d(TCA CAG TCT GAT CTC GAT) on a 0.1 mmol scale.\textsuperscript{128}
3.1.4 The H-Phosphonate Approach

The H-phosphonate approach was first adapted by Hata\(^\text{129}\) for the preparation of oligonucleotide phosphorothioates, by using elemental sulphur (S\(_8\)) to convert H-phosphonate diester 330 into phosphorothioate diester 331 (Scheme 3.7). This methodology was shortly afterwards employed in solid phase synthesis of oligonucleotide phosphorothioates,\(^\text{130}\) and oxidation was effected in a single-step sulphurisation at the end of chain assembly in virtually quantitative yield.
It is generally recognised that the advantages of this strategy are:\textsuperscript{131}

i. Nucleoside $H$-phosphonates are very stable solids; they can be stored under argon for more than 3 years. In a pyridine–CH$_3$CN solution, $H$-phosphonates are also very stable; when decomposition does take place, it is the slow loss of the acid labile 5'-dimethoxytrityl protecting group.\textsuperscript{132} Nucleoside $H$-phosphonates are resistant to oxidation and hydrolysis, and are only oxidized by strong oxidants, such as MnO$_4^-$\textsuperscript{133}

ii. Protection at the phosphorus centre is not required.

iii. Oxidation can be performed either as one synthetic step after the assembly of an oligonucleotide chain is complete, or stepwise.

iv. This approach offers easy access to oligonucleotide analogues by changes in the oxidation step. Therefore, combined backbone linkage oligonucleotides having PO, PS, and PN linkages can be prepared by this methodology.\textsuperscript{134}

An efficient and rather general approach to the preparation of nucleoside $H$-phosphonates was based on phosphorus trichloride–base–imidazole\textsuperscript{46} or 1,2,4-triazole\textsuperscript{49} and the yields are normally greater than 75%. The preparation is outlined in Scheme 3.8. Generally, no detectable 5'-dimethoxytrityl nucleoside \textsuperscript{141} was observed during aqueous work-up of the intermediate 332, but the PX$_3$ derivatives are very
sensitive to moisture, so the solvent must be extremely dry and this reagent must only be generated in situ prior to synthesis.

Another method for the preparation of H-phosphonate monoesters was suggested by van Boom and co-workers,\textsuperscript{135} where PX\textsubscript{3} was replaced with 2-chloro-4\(H\)-1,3,2-benzodioxaphosphorin-4-one 334. The reaction was rapid and virtually quantitative with formation of very little of the 3'→3'-symmetrical dimer. However, hydrolysis of the intermediate phosphite leads to ca. 5% of starting material and the salicyclic acid generated on aqueous work-up can be difficult to separate from the product 333.

Another phosphite-based preparation of H-phosphonate monoesters involves the use of tris-(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite\textsuperscript{136} 335. This reagent was used in the preparation of 2'-Ctmp protected ribonucleoside H-phosphonates 336.
Several other methods have also been developed for the synthesis of nucleoside $H$-phosphonates. Phosphonic acid 337, when used in conjunction with triphosgene 338, offers an alternative way to prepare $H$-phosphonate monoesters\textsuperscript{137} (Scheme 3.9). However, only moderate yields were achieved (58–75\%).

\begin{equation*}
\text{DMT} \text{DMTr} \quad \begin{array}{c}
\text{O} \\
\text{O} \\
\text{OH} \\
\text{141}
\end{array} + \begin{array}{c}
\text{HO} \\
\text{P} \text{OH} \\
\text{337}
\end{array} + \begin{array}{c}
\text{Cl}_3\text{CO} \\
\text{OCl}_3 \\
\text{338}
\end{array} \xrightarrow{\text{DMTrO}} \begin{array}{c}
\text{B} \\
\text{O} \\
\text{P} \text{O}^{-} \\
\text{333}
\end{array}
\end{equation*}

Scheme 3.9

A better method based on a P(V) starting material was described by Stawinski and co-workers.\textsuperscript{138,139} When suitably protected nucleosides were mixed with diphenyl $H$-phosphonate 339 in pyridine, phenyl $H$-phosphonate diester 340 was generated. Upon aqueous work-up, $H$-phosphonate monoester 333 was formed in good yields (>80\%) (Scheme 3.10). Compared with the methods described earlier in this context, this methodology is simple and the reaction goes to completion in only 15 min. The formation of symmetrical dimers can also be suppressed to a minimal amount (1.5\%) by changing the ratio of the nucleoside to diphenyl $H$-phosphonate.

\begin{equation*}
\text{DMTrO} \text{DMTr} \quad \begin{array}{c}
\text{O} \\
\text{O} \\
\text{OH} \\
\text{141}
\end{array} + \begin{array}{c}
\text{H} \\
\text{P} \text{O}^{-} \\
\text{333}
\end{array} \xrightarrow{\text{DMTrO}} \begin{array}{c}
\text{B} \\
\text{O} \\
\text{P} \text{O}^{-} \\
\text{333}
\end{array}
\end{equation*}

Scheme 3.10

A common feature for the above procedures is that the phosphorus-donating agents are all multifunctional, so formation of symmetrical products is bound to occur to
some extent. A convenient and effective procedure for the preparation of H-phosphonate monoesters from a monofunctional starting material was developed by Reese and co-workers.\textsuperscript{140} In this approach, ammonium aryl H-phosphonates 342a–d, which were prepared from phenols and phosphorus trichloride (Scheme 3.11), were used as starting materials.

\[ \text{ArOH} \xrightarrow{i, ii, iii} \text{ArO} + \text{P} - \text{NH}_4 \]

Scheme 3.11 Reagents and conditions: i, PCl$_3$, 160$^\circ$C, 3 h; ii, tert-butanol, 100$^\circ$C, 30 min; iii, conc. aq. NH$_3$, 0$^\circ$C.

Treatment of reagent 343a–d with suitably protected nucleoside 141 in the presence of pivaloyl chloride 163 generates aryl H-phosphonate diester 344 which breaks down upon aqueous work-up to give the desired nucleoside H-phosphonate monoester 333 (Scheme 3.12). After purification by chromatography, virtually quantitative yields were achieved on all four deoxynucleosides. This methodology, in our view, is superior to other known methods and is a method of choice for the preparation of H-phosphonate monoesters.

\[ \text{ArO} + \text{P} - \text{NH}_4 \xrightarrow{i} \text{ArO} + \text{P} - \text{NH}_3 \]

Scheme 3.12 Reagents and conditions: i, Et$_3$N, pyridine; ii, 343, Me$_2$CCOCl, $-35^\circ$C, 30 min; iii, (a) H$_2$O, pyridine, room temperature, 1 h; (b) pH 7.0 triethylammonium phosphate buffer.
In the earlier studies on the \( H \)-phosphonate approach, oxidation of \( H \)-phosphonate diesters was generally accomplished as one synthetic step after the assembly of an oligonucleotide chain was complete. The obvious disadvantage, as has been reported elsewhere,\textsuperscript{141} is that \( H \)-phosphonate diesters are not stable to long-term exposure to the coupling conditions. An interesting and indeed novel modification was proposed by Reese and Song\textsuperscript{54,55} a couple of years ago which incorporated an \textit{in situ} sulphur transfer step after the coupling was complete (Scheme 1.10, Scheme 1.11 and Scheme 3.13). This offers a flexible methodology for preparation of phosphate and phosphorothioate diester, or chimeric sequences. More advantages include virtually quantitative coupling and the possibility of scaling up.
Scheme 3.13 Reagents and conditions: i, 347, pyridine, CH$_2$Cl$_2$, -40°C, 5-10 min; ii, 173 or 174, pyridine, CH$_2$Cl$_2$, 15 min; iii, 125, TMG, MeCN, room temperature, 12 h; iv, conc. aq. NH$_3$, 55°C, 15 h; v, DBU, TMS-Cl, CH$_2$Cl$_2$, room temp., 30 min; vi, DBU, 125, MeCN, room temp., 12 h; vii, HSCH$_2$CH$_2$OH, conc. aq. NH$_3$, 55°C, 15 h.
3.2 Stereocontrolled Synthesis of Oligonucleotide Phosphorothioates

As was described in the last chapter, incorporation of sulphur into the internucleotide linkage generates chirality at the phosphorus centre, therefore there exist $2^n$ possible diastereoisomers of a given sequence having $n$ centres of such chirality. The $R$ and $S$ phosphorothioate configurations are shown in Figure 3.2.

![Figure 3.2](image)

It appears that the configuration of the phosphorothioate linkage affects the stability of the duplex formed between oligonucleotide phosphorothioates and complementary single-stranded nucleic acids. In a self-complementary oligodeoxyribonucleotide containing a single phosphorothioate linkage, the $R_p$ isomer melted approximately 2°C lower than the oligomer with normal phosphodiester linkage.\textsuperscript{142} So far, separation of diastereoisomers has been achieved by means of chromatographic techniques, but effectiveness of the separation depends on the number of phosphorothioate linkages within the oligonucleotide chain and is practically limited to $n = 1, 2$.\textsuperscript{143}

The preparation of diastereoisomerically pure (or enriched) oligonucleotide phosphorothioates in cases with only one, two or three phosphorothioate linkages has been reported.\textsuperscript{118,144}
An example was shown by Lesnikowski\textsuperscript{144,145} in the preparation of \([S_pS_p]-\) and \([R_pR_p]-\) isomers of triuridine phosphorothioate by using 5'-O-monomethoxytrityluridine-3'-O-[S-(2-nitrobenzyl)-O-(4-nitrophenyl)phosphorothioate] \textsuperscript{348} as substrate.

Despite little hope of practical application to assemble oligonucleotide chains of antisense length, a strategy for the preparation of oligonucleotide phosphorothioates of pre-determined stereochemistry was described by Stec and co-workers.\textsuperscript{146} This method involves preparation and chromatographic separation of diastereoisomerically pure 5'-O-dimethoxytrityl nucleoside 3'-O-(2-thio-1,3,2-oxathiaphospholanes) \textsuperscript{349} for stereospecific (99\%) coupling with support-bound 5'-hydroxy functions under conditions of basic catalysis.

Preparation of the diastereoisomerically pure starting material \textsuperscript{349} was achieved by treatment of protected nucleoside \textsuperscript{141} with 2-\(N,N\)-diisopropylamino-1,3,2-oxathiaphospholane \textsuperscript{350} in the presence of 1\(H\)-tetrazole \textsuperscript{140} followed by \textit{in situ} treatment with elemental sulphur (Scheme 3.14). A mixture of diastereoisomers (in 55:45 ratio) was formed and the two isomers were separated by chromatography. By using these diastereoisomerically pure starting materials, up to eight contiguous phosphorothioate linkages of predetermined stereochemistry were obtained on solid support with an average of 95\% effectiveness for a single coupling step (Scheme 65).
3.15). The purity of the material, as indicated by polyacrylamide gel electrophoresis and HPLC, was comparable with that of the sample prepared by phosphoramidite-stepwise sulphurisation or \( H \)-phosphonate-tandem sulphurisation methods.\(^{147}\)

Although further studies on this methodology are in progress, one has to realise that as high as 99% stereospecificity of a single step of synthesis does not yield a diastereoisomerically pure oligonucleotide. Using this protocol, the chemical synthesis of a 27-mer oligonucleotide phosphorothioate leads to material of at best 75% diastereoisomeric purity. It is worthwhile to mention here that by using ring-restricted diastereoisomerically pure oxazaphospholidine phosphoramidites \(^{353, 354}\), larger oligonucleotide phosphorothioates (up to 20-mers) containing stereo-enriched all \([R_p]\) or all \([S_p]\) linkages have been synthesized.\(^{148}\) Also, oligonucleotide phosphorothioates containing a central \([R_p]\) segment and \([S_p]\) segments on either side or \textit{vice versa} were prepared using the appropriate phosphoramidite.
Another phosphoramidite-chemistry-based procedure was suggested by Beaucage and co-workers\textsuperscript{149} recently. By using stereoisomerically pure cyclic $N$-acylphosphoramidites $355, 356$ as starting materials, stereo-controlled syntheses of oligothymidyl and oligocytidyl phosphorothioates were carried out.

Oligonucleotide phosphorothioates prepared via a polymerase-based process utilizing 2'-deoxynucleotide 5'-O-(1-thiotriphosphate) (dNTPoS) substrates are believed to be 100% [all $R_p$] diastereoisomerically pure.$^{150, 151}$ The pivotal limitation of enzymatic synthesis is caused, however, by the fact that oligonucleotide phosphorothioates of only all-$R_p$ configuration are easily obtainable, although it was later announced$^{152}$ that when 2'-deoxynucleotide 5'-O-(pyrophosphoryl methylphosphonate) [d(NTPoCH$_3$)] was used as substrate, all-$S_p$ oligonucleotide phosphorothioates could be obtained as well.

In a recent study on a 15-mer, LR-3280 (5'-AAC GTT GAG GGG CAT-3'), both the all-$R_p$ and all-$S_p$ versions of this sequence were obtained by using 3'-O-(2-thio-1,3,2-oxaselenaphospholanes) $357$ ($X = \text{Se}$) as building blocks.$^{153}$ The relative melting temperatures for the LR-3280 duplexes with complementary RNA were found to be
in the following order: all-\(R_p\) (\(T_m = 55.2^\circ C\)) > stereorandom (\(T_m = 50.7^\circ C\)) > all-\(S_p\) (\(T_m = 48.3^\circ C\)). The activities of these compounds in \textit{in vivo} studies show the same trend: all-\(R_p\) > stereorandom > all-\(S_p\) (Figure 3.3).

\[\text{Figure 3.3 Growth inhibition in human smooth muscle cells by } R_p \text{, stereorandom and } S_p \text{ LR-3280.}\]

Although the all-\(R_p\) phosphorothioate oligonucleotides show consistent and reproducible biological effects, the current opinion is that their use in clinical settings is unwarranted due to their high cost and much lower accessibility.\(^{154}\)

3.3 Preparation of ISIS 2922 21-Mer by the Modified \(H\)-Phosphonate Approach

The aim of the present work is to carry out synthesis of a specific oligonucleotide phosphorothioate to evaluate the modified \(H\)-phosphonate methodology as shown in Scheme 3.13. ISIS 2922 (220) with sequence GCG TTT GCT CTT CTT CTT GCG was selected as the target.
3.3.1 Protection of 5'-Hydroxy Function

The protection of 5'-hydroxy functions of nucleosides can be categorized into three systems according to the unblocking method: \(^{155}\) (i) acid conditions, (ii) alkaline or ammoniacal conditions, or (iii) selective unblocking reagents applied essentially in the absence of acid or base. Whichever method is chosen must be integrated into an orthogonal protection system for the synthesis of nucleotides. So far, a number of 5'-hydroxy protecting groups have been developed, which include: triarylmethyl (trityl) derivatives \(^{14,156}\) 358, pixyl derivatives \(^{157}\) 359, acyl protecting groups \(^{158,159}\) 360, carbonate protecting groups \(^{160,161}\) 361, silyl protecting groups and photosensitive protecting groups.

![Chemical structures]

The dimethoxytrityl 358c and pixyl 359a derivatives are so far most widely used for masking of the 5'-hydroxy function. They can both be removed in very mild acid conditions. Since 4,4'-dimethoxytrityl protected nucleosides are commercially available, this group was chosen as protecting group for the 5'-hydroxy function in our current studies. Removal of this group was effected with a solution of hydrogen chloride in dioxane at \(-50^\circ C\) (Scheme 3.16). No depurination was observed under these conditions.
3.3.2 Protection of 3'-Hydroxy Function

The levulinyl (Lev) group\textsuperscript{162} \textbf{364} was chosen as the protecting group for the 3'-hydroxy function. The introduction of the Lev group is facile. For suitably protected T, dC, and dA, treatment of the nucleoside with levulinic anhydride in the presence of \textit{N},\textit{N}-dimethylanilinopyridine (DMAP) and triethylamine gives the corresponding 3'-levulinyl derivatives. However, when protected dG was treated under these conditions, base modification occurred; thus milder conditions have to be used. It was found that acylation went to completion when suitably protected dG was treated with a solution of levulinic anhydride in dry pyridine over a period of 12 h.

The unblocking of the levulinyl group was effected under nearly neutral conditions, \textit{i.e.} treatment with hydrazine hydrate in glacial acetic acid–pyridine (1:4 \textit{v/v})\textsuperscript{163} (Scheme 3.17). No internucleotide linkage cleavage was observed.
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Scheme 3.17 Reagents and conditions: i, hydrazine hydrate, glacial acetic acid–pyridine (1:4 v/v), 0°C, 10 min; ii, 2,4-pentanedione, 15 min.

3.3.3 Protection of Base Residues

The choice of nucleobase protecting groups and unblocking protocols is of paramount importance in oligonucleotide synthesis. The protecting groups should meet several criteria:

i. they should be able to be introduced using a stable reagent that is readily obtainable;

ii. they should be achiral;

iii. they should be readily introduced on the nucleoside;

iv. they should enhance the solubility of the nucleoside in organic solvents;

v. they should be stable to the conditions used in assembling the chain;

vi. they should not cause other structural changes in the nucleoside during their introduction or removal, or during oligonucleotide assembly.

The exocyclic amino group at the 2-position of guanine residues is susceptible to electrophilic attack and is therefore protected with the isobutyryl group. 2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-2-N-isobutyrylguanosine 368 is currently commercially available from several suppliers. At the end of the chain assembly, this protecting group can be removed by ammonolysis at 55°C.
It was found\textsuperscript{167,168} that base modification may also occur at the 4-\textit{O}-position of thymine and 6-\textit{O}-position of guanosine; protecting groups are required not only to prevent possible side reactions but also to improve lipophilicity and solubility in organic solvents which facilitates column chromatography.

\begin{scheme}
\begin{center}
\includegraphics[width=\textwidth]{scheme318}
\end{center}
\end{scheme}

\textit{Scheme 3.18 Reagents and conditions:} i, MeCN, \textit{N}-methylpyrrolidine, TMS-Cl, room temp., 1 h; ii, POCl\textsubscript{3}, 0\degree\text{C}, 20 min; iii, phenol, 0\degree\text{C}, 3 h; iv, water, room temp., overnight.

The phenyl group was found to be suitable for the protection of the 4-\textit{O}-position of thymine. The introduction of this group is illustrated in Scheme 3.18. At the beginning of the synthesis, the 3'-\textit{hydroxy} function of 5'-\textit{O}-dimethoxytrityl thymidine \textbf{305} was temporarily blocked with a trimethylsilyl (TMS) group. Then \textit{N}-methylpyrrolidine and phosphoryl chloride were added to generate an activated quaternary salt \textbf{370} which was allowed to react with phenol at 0\degree\text{C}. After the silyl
protecting group was removed by aqueous work-up, the desired 5′-O-dimethoxytrityl-4-O-phenylthymidine 371 was obtained in good yield (>80%).

Similarly, the 6-O-position of 2′-deoxyguanosine is protected with phenol derivatives. 2,5-Dichlorophenyl is a good candidate in this case. The introduction of this protecting group was accomplished in a similar way to that of the thymidine protecting group described above, except that phosphoryl chloride was replaced with mesitylenesulphonyl chloride.

Unblocking of the O-aryl protection was effected by treatment with the 1,1,3,3-tetramethylguanidinium salt of 2-nitrobenzaldoxime in acetonitrile. The unblocking proceeds by a two-step mechanism (Figure 3.4). In the case of O-phenylthymidine, the O-phenyl protecting group is firstly displaced with oximate; the intermediate formed then undergoes elimination in the presence of a strong base to regenerate thymidine in virtually quantitative yield. The complete displacement of the O-aryl protecting groups on thymine and guanine residues with oximate is essential in the unblocking of a fully-protected oligonucleotide sequence. Treatment of partially O-aryl-protected compound with concentrated ammonia generates a modified base (Scheme 3.19). In the case of a 4-O-phenylthymine residue 372, a 5-methylcytosine residue 373 could be generated.
The exocyclic 4-NH$_2$ group of 2'-deoxycytidine was protected with a benzoyl group. Three strategies can be used (Scheme 3.20). Firstly, the trimethylsilyl group is used as temporary protecting groups for the 3'- and 5'- hydroxy functions of 2'-deoxycytidine 374. The resulting compound is treated with excess of benzoyl chloride in pyridine to give 375. Upon ammonia treatment at ambient temperature, the TMS groups and one of the benzoyl groups are removed to give N-benzoyl-2'-deoxycytidine 376 (Route I). Alternatively, 2'-deoxycytidine 374 can be perbenzoylated to yield 378, which is then treated with sodium hydroxide to give N-benzoyl-2'-deoxycytidine 376 (Route II). The benzoyl protecting group can also be directly incorporated on the exocyclic amino group by using benzoic anhydride or its activated esters (Route III). The 3'-hydroxy function of N-benzoyl-2'-deoxycytidine 376 is then masked with a DMTr-protecting group.

Unblocking of the exocyclic amino protecting groups is performed by treatment of the substrate with concentrated aqueous ammonia at 55°C over a period of 15 hours. No concomitant internucleotide linkage cleavage has been observed.
3.4 Synthetic Strategy for Chain Elongation

One advantage of the modified H-phosphonate approach is that an oligonucleotide chain can be assembled by adding one nucleoside unit or a blockmer per coupling cycle. If the target sequence is dissected as indicated in Figure 3.5, only four trimers (GCT, CTT, TTT, GCG) are required for assembly of the chain. The advantage of adding a blockmer each time is that the purification of the product may turn out to be easier than by adding one nucleoside unit each time, due to the bigger difference in physical properties between the by-products and the desired product. For example, if a $3 + 3 \rightarrow 6$ coupling does not go completion, it is likely to be possible to remove the truncated sequence (trimer) from the desired hexamer by means of chromatography, whereas in the case of $1 + 5 \rightarrow 6$, the chance for the separation of the desired hexamer and truncated pentamer would be very small.

The drawback of this strategy is that valuable trimer H-phosphonates have to be used. Because an excess of H-phosphonate is always required to ensure successful coupling, the cost may add up. But if a method is in place to recover the excess of H-phosphonate, this problem can then be minimized.
In practical terms, it is not recommended to use a blockmer $H$-phosphonate larger than a trimer because the elution of such big blockmer $H$-phosphonates from silica gel columns proved to be rather difficult. In our preparation of the target 21-mer, however, a $6 + 6 \rightarrow 12$-mer coupling was adopted to show the versatility of the strategy.

![Figure 3.5](image)

It is conceivable that with the increase of molecular weight, the rate of coupling drops; so it is crucial to increase the amount of $H$-phosphonate as the molecule gets larger. Starting off with 20% excess of $H$-phosphonate for a $1 + 1 \rightarrow 2$ and $1 + 2 \rightarrow 3$ coupling, an increase of 5% in the excess of $H$-phosphonate is applied, *i.e.* 25% excess for $3 + 3 \rightarrow 6$, 30% excess for $6 + 6 \rightarrow 12$ coupling and so on. It is also recommended in practice to increase the amount of the coupling agent and the amount of pyridine used in the coupling reactions with the increase of molecular weight.

3.5 Preparation of Dimer Building Blocks

The preparation of dimer building blocks is outlined in Scheme 3.21. Suitably protected 5'-O-dimethoxytrityl nucleoside 141 was allowed to react with $p$-methylphenyl $H$-phosphonate 343c at low temperature (−35°C) to give the corresponding 5'-O-dimethoxytrityl nucleoside $H$-phosphonate 333. The yields were well above 90% (Table 3.1).
The suitably protected 3'-O-levulinyl nucleosides 146 were prepared by treating the appropriate 5'-O-dimethoxytrityl nucleoside 141 with levulinic anhydride in the presence of \(N,N\)-dimethylaminopyridine and triethylamine. An exception is the preparation of the 2'-deoxyguanosine derivative, where 2'-deoxy-5'-O-dimethoxytrityl-6-O-(2,5-dichlorophenyl)-2-N-isobutyryl guanosine 380 was allowed to react with levulinic anhydride in pyridine to avoid possible side reactions. The resulting 5'-O-dimethoxytrityl 3'-O-levulinyl nucleoside was then treated with dichloroacetic acid and pyrrole in dichloromethane to give the corresponding monomer building block 146. The yields varied between individual nucleosides (Table 3.1).
Formation of the dinucleoside internucleotide linkages was carried out at low temperature (−40°C) to minimise possible side-reactions. The suitably protected nucleoside H-phosphonate 333 was coupled with a 3′-O-levulinyl-5′-hydroxyl nucleoside 146 by using bis(2-chlorophenyl) phosphorochloridate 347 as activating agent. The reaction was very fast and went to completion within 5 min with virtually no by-products as indicated by TLC. The dinucleoside H-phosphonate diester formed was then treated in situ with 4-[(2-cyanoethyl)sulphanyl]morpholine-3,5-dione 174 to give the corresponding S-(2-cyanoethyl)dinucleoside phosphorothioate triester. The 5′-O-dimethoxytrityl group was then removed by hydrogen chloride in dioxane at low temperature (−50°C). Under these conditions, no depurination was observed even for the most sensitive N-benzoyl-2′-deoxyadenosine derivative (not involved in this synthesis). The desired dinucleoside phosphorothioate triester 379 was obtained by chromatography as a colourless glass in high yield and in very good quality (Table 3.1).

Table 3.1 Yields for the preparation of monomer and dimer building blocks.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMTr-Cp(H) 381</td>
<td>95.5</td>
</tr>
<tr>
<td>DMTr-Tp(H) 382</td>
<td>95.4</td>
</tr>
<tr>
<td>DMTr-Gp(H) 383</td>
<td>92.8</td>
</tr>
<tr>
<td>HO-G-Lev 384</td>
<td>90.6</td>
</tr>
<tr>
<td>HO-T-Lev 385</td>
<td>82.1</td>
</tr>
<tr>
<td>HO-Cp(s)G-Lev 386</td>
<td>99.8</td>
</tr>
<tr>
<td>HO-Tp(s)T-Lev 387</td>
<td>93.5</td>
</tr>
<tr>
<td>HO-Cp(s)T-Lev 388</td>
<td>94.0</td>
</tr>
</tbody>
</table>
The 5'-hydroxy dinucleoside phosphorothioate triesters were characterised by HPLC, $^1$H (plus D$_2$O exchange) and $^{31}$P NMR spectroscopy. As expected, two lines were recorded in the $^{31}$P NMRs which represent the two diastereoisomers. An example is shown in Figure 3.6.

![Figure 3.6 31P NMR (DMSO-d$_6$) and HPLC profile of HO-Tp(s)T-Lev 387.](image)

### 3.6 Preparation of Trimer Building Blocks

Four trimer building blocks were used in the preparation of the ISIS 2922 21-mer, GCG, TTT, GCT and CTT. However, GCG and CTT have to be prepared in both 5'-hydroxyl and 3'-hydroxyl terminating forms (Scheme 3.22).
It was found that 20% excess of H-phosphonate was sufficient for a $1 + 2 \rightarrow 3$ coupling. For the preparation of the 5'-hydroxyl terminating trimer 389, detritylation was carried out after the coupling and sulphur-transfer steps were complete, without purification. The detritylated trimer 389 was isolated by chromatography as a colourless glass in high yield (Table 3.2).

Fully protected trimers (e.g. 390) were prepared under the same coupling conditions and were isolated as colourless glasses by chromatography, in high yields. The products obtained were then treated with hydrazine hydrate in a mixture of acetic acid and pyridine to remove the 3'-O-levulinyl protecting group. The reaction went to completion in 10 min. Purification was effected by precipitation from diethyl ether after workup and evaporation to give 5'-O-dimethoxytrityl-3'-hydroxy trimers (e.g. 391) in virtually quantitative yields (Table 3.2).

5'-O-Dimethoxytrityl-3'-hydroxy trimers (e.g. 391) were then converted to the corresponding 3'-H-phosphonates (e.g. 392) by a similar procedure to that used for
the preparation of monomer H-phosphonates. Because of their higher molecular weight, the products could only be eluted off the column by eluents with high percentages of methanol (15–20%). As a result, it was found that upon evaporation of the appropriate fractions, the triethylammonium moiety was lost (as recorded by $^1$H NMR). An attempt to use this material directly as starting material for the next coupling reaction lead to a failed condensation. Our initial effort to resolve this problem was to replace triethylammonium with a more stable cation. DBU cation is more stable and easier to prepare, but modifications had to be made to our existing strategy. We found later that by washing with triethylammonium phosphate buffer (0.5 $M$, pH 7.0), the H-phosphonates 393, in which triethylammonium moiety was lost, were converted to the desired H-phosphonate triethylammonium salts 394 (Scheme 3.23).

![Scheme 3.23](image)

The trimer building blocks were characterised by HPLC, $^1$H (plus D$_2$O exchange) and $^{31}$P NMR spectroscopy. Up to eight lines could be recorded in the $^{31}$P NMR. For the trimer H-phosphonates, extra lines were expected at ca. 0 ppm, which integrate for one phosphorus centre. Figure 3.7 shows an example of the $^{31}$P NMR spectra and HPLC profiles of the trimer H-phosphonates. The inset NMR shows the undecoupled phosphorus NMR spectrum, where a P-H coupling constant of 593.85 Hz was observed.
Figure 3.7 $^3$P NMR (DMSO-$d_6$) and HPLC profile of DMTr-$Cp(s)Tp(s)Tp(H)$ 398.

Table 3.2 Yields for the preparation of the trimer blocks.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-$Gp(s)Cp(s)G$-Lev 389</td>
<td>91.0</td>
</tr>
<tr>
<td>HO-$Cp(s)Tp(s)T$-Lev 395</td>
<td>92.3</td>
</tr>
<tr>
<td>DMTr-$Cp(s)Tp(s)T$-Lev 396</td>
<td>95.0</td>
</tr>
<tr>
<td>DMTr-$Cp(s)Tp(s)T$-OH 397</td>
<td>95.7</td>
</tr>
<tr>
<td>DMTr-$Cp(s)Tp(s)Tp(H)$ 398</td>
<td>92.3</td>
</tr>
<tr>
<td>DMTr-$Gp(s)Cp(s)T$-Lev 399</td>
<td>95.7</td>
</tr>
<tr>
<td>DMTr-$Gp(s)Cp(s)T$-OH 3100</td>
<td>91.6</td>
</tr>
<tr>
<td>DMTr-$Gp(s)Cp(s)Tp(H)$ 3101</td>
<td>89.2</td>
</tr>
<tr>
<td>DMTr-$Tp(s)Tp(s)T$-Lev 3102</td>
<td>91.6</td>
</tr>
<tr>
<td>DMTr-$Tp(s)Tp(s)T$-OH 3103</td>
<td>95.1</td>
</tr>
<tr>
<td>DMTr-$Tp(s)Tp(s)Tp(H)$ 3104</td>
<td>90.7</td>
</tr>
<tr>
<td>DMTr-$Gp(s)Cp(s)G$-Lev 390</td>
<td>94.3</td>
</tr>
<tr>
<td>DMTr-$Gp(s)Cp(s)G$-OH 391</td>
<td>95.0</td>
</tr>
<tr>
<td>DMTr-$Gp(s)Cp(s)G$-Hp 392</td>
<td>89.6</td>
</tr>
</tbody>
</table>
3.7 Preparation of Hexamer Building Blocks

Two hexamer building blocks were required in the preparation of ISIS 2922 under the current synthetic strategy, i.e. CTT CTT and CTT GCG. The preparation is outlined in Scheme 3.24.

\[
\text{DMTr-Cp(s)Tp(s)Tp(H) + HO-Cp(s)Tp(s)T-Lev} \rightarrow \text{DMTr-Cp(s)Tp(s)Tp(s)Cp(s)Tp(s)T-Lev} \quad 398 \quad 395 \quad 3105
\]

\[
\text{DMTr-Cp(s)Tp(s)Tp(s)Cp(s)Tp(s)Tp(H)} \rightarrow \text{DMTr-Cp(s)Tp(s)Tp(s)Cp(s)Tp(s)T-OH} \quad 3107 \quad 3106
\]

\[
\text{DMTr-Cp(s)Tp(s)Tp(H) + HO-Gp(s)Cp(s)G-Lev} \rightarrow \text{HO-Cp(s)Tp(s)Tp(s)Gp(s)Cp(s)G-Lev} \quad 398 \quad 389 \quad 3108
\]

Scheme 3.24

The fully-protected hexamer \textbf{3105} was prepared by coupling of DMTr-Cp(s)Tp(s)Tp(H) \textbf{398} with HO-Cp(s)Tp(s)T-Lev \textbf{395} under the similar conditions used for the trimer blocks. After treatment with hydrazine hydrate in pyridine-acetic acid, the corresponding 5'-O-dimethoxytrityl 3'-hydroxyl hexamer \textbf{3106} was obtained in high yield. It was then converted to \textit{H}-phosphonate \textbf{3107} in the same way as described for the preparation of trimer \textit{H}-phosphonates. A triethylammonium phosphate buffer washing was also essential at the end of column chromatography to recover triethylammonium salt, which allows the next successful coupling.

The other hexamer, the 5'-hydroxyl hexamer \textbf{3108}, was prepared by assembling \textit{H}-phosphonate \textbf{398} with \textbf{389} using the same coupling and detritylation procedures described above. The product was obtained by short column chromatography in good yield (Table 3.3).
Table 3.3 Yields for the preparation of hexamers.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMT-Tr-Cp(s)Tp(s)Tp(s)Cp(s)Tp(s)T-Lev 3105</td>
<td>90.8</td>
</tr>
<tr>
<td>DMT-Tr-Cp(s)Tp(s)Tp(s)Cp(s)Tp(s)T-OH 3106</td>
<td>92.0</td>
</tr>
<tr>
<td>DMT-Tr-Cp(s)Tp(s)Tp(s)Cp(s)Tp(s)Tp(H) 3107</td>
<td>90.4</td>
</tr>
<tr>
<td>HO-Cp(s)Tp(s)Tp(s)Gp(s)Cp(s)G-Lev 3108</td>
<td>89.0</td>
</tr>
</tbody>
</table>

The hexamers were characterised by $^1$H (plus D$_2$O exchange) and $^{31}$P NMR spectroscopy. For the hexamer $H$-phosphonate 3107, a bunch of lines at around 0 ppm was also recorded which corresponds to the phosphorus centre on the $H$-phosphonate moiety. The undecoupled $^{31}$P revealed a P-H coupling constant of 575.87 Hz (Figure 3.8).

Figure 3.8 $^{31}$P NMRs of DMT-Tr-Cp(s)Tp(s)Tp(s)Cp(s)Tp(s)Tp(H) 3107 in DMSO-d$_6$. 
3.8 Preparation of Dodecamer, Pentadecamer and Octadecamer Building Blocks

The preparation of the dodecamer 3109 was conducted by joining two hexamer building blocks 3107 and 3108 and removing the 5'-terminal dimethoxytrityl group. The pentadecamer 3110 and octadecamer 3111 were prepared by adding a trimer each coupling cycle (Scheme 3.25). The yield fell below 90% (83.6, 88.2, 86.1%) because the high molecular weight made the recovery from column chromatography more difficult.

\[
\text{DMTr-Cp(s)Tp(s)Cp(s)Tp(s)Tp(s)Tp(H) \quad \text{HO-Cp(s)Tp(s)Gp(s)Cp(s)G-Lev}}
\]

\[
\text{HO-Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Gp(s)Cp(s)G-Lev}
\]

\[
\text{DMTr-Gp(s)Cp(s)Tp(H)} \quad \text{3101}
\]

\[
\text{HO-Gp(s)Cp(s)Tp(s)[Cp(s)Tp(s)Cp(s)Tp(s)]_3Gp(s)Cp(s)G-Lev}
\]

\[
\text{DMTr-Tp(s)Tp(s)Tp(H)} \quad \text{3104}
\]

\[
\text{HO-Tp(s)Tp(s)Gp(s)Cp(s)Tp(s)[Cp(s)Tp(s)Cp(s)Tp(s)]_3Gp(s)Cp(s)G-Lev}
\]

\[
\text{3111}
\]

**Scheme 3.25**

3.9 Preparation of the 21-Mer

The final step in the assembly of the target 21-mer was to join a GCG trimer building block 392 with the octadecamer 3111. After detritylation, the 5'-hydroxy 21-mer 3112 was obtained in 85.2% yield by column chromatography as a colourless glass.
3.10 Application of $^1$H NMR in the Characterisation of the Oligonucleotide Phosphorothioate Triesters

Nucleosides have relatively simple $^1$H NMR spectra. The aromatic protons of pyrimidines and purines resonate at low field ($\delta$ 7.6 to $\delta$ 8.3 with C5-H close to $\delta$ 5.9). The anomeric proton is a doublet for ribonucleosides and a double-doublet for 2'-deoxynucleosides at $\delta$ 5.8–6.4. The pentoses provide a multi-spin system which generally moves from low to high field in the series: H-3', H-4', H-5' and H-5'' (2'-deoxynucleosides). Lastly, 2'-deoxynucleosides have H-2' and H-2'' as an ABMX system near $\delta$ 2.5. An example, the proton NMR spectrum for cytidine, is shown in Figure 3.9.

![Proton NMR spectrum for cytidine (D2O at 400 MHz)](image)

However, for a large oligonucleotide, especially when it is fully protected on the base residues, 3'-hydroxy, 5'-hydroxy and internucleotide linkages, the proton NMR becomes very complex. In the case of phosphorothioate triesters, the existence of diastereoisomers makes the complete analysis of the spectra very difficult. However, in our studies, $^1$H NMR spectroscopy proved to be a useful tool in indicating the nucleoside ratios and the purities of oligonucleotides.
The first useful feature of the $^1$H NMRs of phosphorothioate triesters is the integration of the aromatic protons. Although accurate integrals are difficult to achieve, it is possible to show the purity of the oligonucleotide qualitatively.

The nucleoside ratios $(T + C)/T$ can be determined by measuring the integrals of the anomeric protons, of which T and C normally superimpose together. It is usually feasible to determine the percentage of thymidine in the oligonucleotide by measuring the integral of the 5-methyl group. The methyl groups of the isobutyryl protecting group of 2'-deoxyguanosine also provide a means for the calculation of the G-content.

Table 3.4 presents the analysis of the $^1$H NMRs of some of the building blocks in our studies.

<table>
<thead>
<tr>
<th>Compound Entries</th>
<th>12-mer 3109</th>
<th>15-mer 3110</th>
<th>18-mer 3111</th>
<th>21-mer 3112</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aromatic protons</td>
<td>Calculated</td>
<td>72</td>
<td>89</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>Observed</td>
<td>74</td>
<td>89</td>
<td>106</td>
</tr>
<tr>
<td>$(C + T)/G$</td>
<td>Calculated</td>
<td>5.00</td>
<td>4.00</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td>Observed</td>
<td>5.40</td>
<td>3.98</td>
<td>5.38</td>
</tr>
<tr>
<td>Number of T indicated by 5-Me group</td>
<td>Calculated</td>
<td>6.00</td>
<td>7.00</td>
<td>10.00</td>
</tr>
<tr>
<td></td>
<td>Observed</td>
<td>6.60</td>
<td>6.67</td>
<td>10.03</td>
</tr>
<tr>
<td>Number of G indicated by the methyl groups on 'Bu</td>
<td>Calculated</td>
<td>2.00</td>
<td>3.00</td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td>Observed</td>
<td>2.10</td>
<td>3.10</td>
<td>3.05</td>
</tr>
</tbody>
</table>

3.11 Removal of $S$-(2-Cyanoethyl) Group

The 2-cyanoethyl group has been used to protect the phosphate triester linkage for a long time and its removal is normally effected under basic conditions. The alkaline unblocking takes place via $\beta$-elimination (Figure 3.10). Various procedures have
been investigated so far. Among amines, it was found that primary amines are the most effective in achieving rapid cleavage. The following times for complete cleavage of the cyanoethyl group from phosphate were obtained: triethylamine, 180 min; diisopropylethylamine, 60 min; diethylamine, 30 min; sec-butylamine, 20 min; tert-butylamine, 10 min; n-propylamine, 2 min. Further study showed that tert-butylamine was the most suitable reagent because it did not react with protected nucleobases. Methylamine and ammonia were also fast (5 min) and effective reagents for deprotection.

![Figure 3.10 Unblocking of cyanoethyl group under basic conditions.](image)

Other bases, including aqueous ammonia in dioxane and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, \textbf{3113}) were also investigated. Fluoride ion (Bu\textsubscript{4}N\textsuperscript{+}F\textsuperscript{-}) was also found to show selective cleavage of the 2-cyanoethyl group.

![Image of DBU](image)

Although primary amines offer fast unblocking of the protecting group, there is a potential danger of primary amines attacking the 6-O-position of guanine base residues. DBU has proved superior to these primary amines because it offers fast removal of the protecting group and no danger of modification to the base residues has been observed.

Treatment of fully protected phosphorothioate triesters \textbf{172a} with DBU in dichloromethane converts the triesters to the corresponding phosphorothioate diesters \textbf{346} within 30 min. However, this reaction is very sensitive to moisture. The presence of very small amounts of moisture could lead to loss of sulphur, which produces
small amounts of phosphate 345 (Scheme 3.26). A simple solution is to add a few percent of chlorotrimethylsilane (TMS-Cl) which uses up moisture in the first instance.\textsuperscript{55} The other advantage of including TMS-Cl in the reaction is that the attack on base residues by acrylonitrile produced in the course of removal of 2-cyanoethyl group can be suppressed.

\begin{center}
\includegraphics[width=\textwidth]{Scheme_3.26}
\end{center}

\textit{Scheme 3.26 Reagents and conditions: i, DBU, TMS-Cl, CH\textsubscript{2}Cl\textsubscript{2}, room temp., 30 min.}

3.12 Unblocking of the Fully-Protected 21-Mer

The protocols for the unblocking of the 5'-hydroxy 21-mer 3112 are indicated in outline in Scheme 3.27. The material was first acetylated (step i), and the resulting fully-protected 21-mer was then unblocked by a three-step procedure. First, the internucleotide linkages were unblocked. By treatment with DBU in extremely dry dichloromethane, the S-(2-cyanoethyl) protecting groups were removed (step ii). Secondly, the 6-O-(2,5-dichlorophenyl) and 4-O-phenyl protecting groups were removed from the guanine and thymine residues respectively by oximate treatment (step iii). Finally, all the acyl protecting groups were removed by treatment with a mixture of 2-mercaptoethanol and concentrated aqueous ammonia at 55°C (step iv). The presence of 2-mercaptoethanol\textsuperscript{174} suppressed the loss of sulphur during ammonia treatment.\textsuperscript{175,176} The material obtained was then passed through an ion exchange column to replace the counterions with sodium ion (step v). The product was characterised by \textsuperscript{1}H, \textsuperscript{31}P NMR and reverse phase HPLC. No loss of sulphur was observed as indicated by the \textsuperscript{31}P NMR spectrum (Figure 3.11).
Chapter 3  Synthesis of Oligonucleotide Phosphorothioates

\[
\text{HO-Gp(s)Cp(s)Gp(s)Tp(s)Tp(s)Tp(s)Gp(s)Cp(s)Tp(s)[Cp(s)Tp(s)Tp(s)]_3Gp(s)Cp(s)G-Lev} \\
\downarrow \text{i} \\
\text{AcO-Gp(s)Cp(s)Gp(s)Tp(s)Tp(s)Gp(s)Cp(s)Tp(s)[Cp(s)Tp(s)Tp(s)]_3Gp(s)Cp(s)G-Lev} \\
\downarrow \text{ii, iii, iv, v} \\
\text{d[Gp(s)Cp(s)Gp(s)Tp(s)Tp(s)Gp(s)Cp(s)Tp(s)[Cp(s)Tp(s)Tp(s)]_3Gp(s)Cp(s)G]}
\]

Scheme 3.27 Reagents and conditions: i, acetic anhydride, pyridine, room temp., overnight; ii, DBU, TMS-Cl, CH₂Cl₂, room temp., 30 min; iii, DBU, E-2-nitrobenzaldoxime, CH₃CN, room temp., 15 h; iv, 2-mercaptoethanol, conc. aq. ammonia, 55°C, 15 h; v, Amberlite (IR120, Na⁺ form) ion exchange column.

Figure 3.11 ³¹P spectrum of the fully unblocked 21-mer 220 in D₂O.

To conclude this chapter, we have evaluated the modified H-phosphonate approach in the preparation of oligonucleotide phosphorothioates. By carrying out the coupling
reaction at \(-40^\circ\text{C}\) with bis(2-chlorophenyl) phosphorochloridate \(347\) as activating agent, followed by \textit{in situ} sulphur transfer, ISIS 2922 21-mer was prepared in good yield. We believe that this strategy is very effective in the preparation of both oligonucleotide phosphorothioates and phosphates of short to moderate chain length. However, it would be more attractive if this approach could be adapted for use at higher temperature, say not below \(0^\circ\text{C}\).
Large-scale Synthesis of Oligonucleotide Phosphorothioates in Solution

The solution phase synthesis of oligonucleotide phosphorothioates via the −40°C H-phosphonate approach described in the previous chapter was primarily developed to meet the anticipated demand for multikilogram quantities of oligonucleotides and their analogues for therapeutic purposes. The question of whether or not it is feasible for the process to be scaled up is a matter of great importance. It seemed likely that the chance of developing an efficient large-scale synthesis would be much greater if it were possible for both the coupling and the sulphur-transfer reactions to be carried out at room temperature or, in any case, not below 0°C. As H-phosphonate diesters 401 are sensitive intermediates, it seemed reasonable to assume that the best way to avoid undesirable side-reactions at higher temperatures would be to ensure that the sulphur-transfer reaction took place as soon as possible after coupling had occurred (Scheme 4.1). The resulting phosphorothioate triesters 402 would be expected to be stable under the coupling reaction conditions.

Scheme 4.1

4.1 Possible Side-Reactions in the H-Phosphonate Approach

Although H-phosphonates 403 and their esters 405 have been widely believed to exist in equilibrium between phosphite (404 and 406) and phosphonate (403 and 405)
forms\textsuperscript{177} (Figure 4.1), the amount of phosphite is believed to be below a level observable by any spectroscopic methods used. Therefore, these compounds are represented by their phosphonate forms in this context.

\[ RO-P-OH \quad \text{403} \quad \text{HO-P-OH} \quad \text{404} \]

\[ RO-P-OR' \quad \text{405} \quad \text{HO-P-OR'} \quad \text{406} \]

Figure 4.1

4.1.1 Activation of \textit{H}-Phosphonate Monoesters

Condensation of \textit{H}-phosphonate monoesters with a hydroxylic component in the presence of an activating agent is a multi-step reaction which involves formation of activated \textit{H}-phosphonate or phosphite intermediates, followed by their reactions with a hydroxylic component. By using \textsuperscript{31}P NMR spectroscopy, it was found that the first reaction (the activating step) is rate-determining for all studied activating agents\textsuperscript{52,178,179}

When a limited amount of a condensing agent is added to a solution of an \textit{H}-phosphonate monoester \textsuperscript{407} in pyridine, various intermediates can be recorded by \textsuperscript{31}P NMR.\textsuperscript{52} In the case of pivaloyl chloride, three intermediates are generated: the mixed acyl phosphonic anhydride \textsuperscript{408}, the corresponding \textit{H}-pyrophosphonate \textsuperscript{409}, and the bisacyl phosphite \textsuperscript{410}. When these intermediates are allowed to react with an alcohol, the corresponding \textit{H}-phosphonate diester \textsuperscript{411}, a mixture of \textit{H}-phosphonate diester and monoester, and a phosphite triester \textsuperscript{412} are formed respectively. The formation of \textsuperscript{408} and \textsuperscript{409} proceeds at a much faster rate than that of \textsuperscript{410}. In the presence of excess of acyl chloride, however, only bisacyl phosphite \textsuperscript{410} is formed initially. Upon prolonged reaction, formation of the acylphosphonate \textsuperscript{413} is observed.
The activating pathways of other coupling agents are similar to that of acyl chloride. Those of diaryl phosphorochloridates, arylsulphonyl chlorides and carbonate derivatives have been investigated in detail.

Exposure of H-phosphonate monoesters to excess of activating agents over a long period of time can lead to another side-reaction. We observed that by mixing H-phosphonate with various coupling agents (pivaloyl chloride, adamantancarbonyl chloride, diphenyl phosphorochloridate), symmetrical products were recorded by NMR spectroscopy. Thus, on mixing 5'-O-dimethoxytritylthymidine 3'-H-phosphonate with 5 molar equivalents of pivaloyl chloride in pyridine overnight, followed by addition of sulphur transfer reagent (Scheme 4.3), a 3' → 3' symmetrical dithymidine phosphorothioate triester was detected by NMR spectroscopy, which was confirmed by independent preparation. The same is true for other activating agents, and the order of rates of this reaction effected by various activating agents was observed to be: diphenyl phosphorochloridate > pivaloyl chloride > adamantancarbonyl chloride.
4.1.2 Stability of H-Phosphonate Diesters

Dialkyl H-phosphonate diesters are generally resistant to air oxidation, but they undergo slow hydrolysis in the presence of moisture. When stronger oxidants are present, e.g. aqueous iodine and sulphur, they are readily oxidized. Under the coupling conditions, they can react with excess of activating agents and form various by-products.

Non sterically hindered acyl chlorides can react with H-phosphonate diesters to give acylated phosphonates. Thus, H-phosphonate diester 411 reacts with excess of pivaloyl chloride to form pivaloylphosphonate 417 (Scheme 4.4).

\[
\text{RO-P-OR'} \quad \xrightarrow{\text{pivaloyl chloride}} \quad \text{RO-P-OR'}
\]

Scheme 4.4
This kind of reaction does not occur readily when diaryl phosphorochloridates are used as coupling agent.\textsuperscript{181}

4.2 The Aim of This Part of the Work

As one can see from the previous material, the modified H-phosphonate approach, i.e. coupling, which is effected by bis(2-chlorophenyl) phosphorochloridate at -40°C, followed by sulphur transfer reaction, has shown several advantages over other H-phosphonate approaches. However, when this approach is carried out at higher temperature, which is favoured for large-scale synthesis, various side-reactions occur. Thus, it would be advantageous if this strategy could be adapted such that fast and quantitative coupling could be achieved at higher temperature or, in any case, not below 0°C.

4.3 The Four-Component Solution Phase Synthesis

It occurred to us that the best way of ensuring that the sulphur-transfer reaction would follow as soon as possible after coupling would be by carrying out a four-component reaction. This would involve adding a mixture of the coupling and sulphur transfer agents to a solution of a mixture of the H-phosphonate monoester and the component with a free hydroxy function.

\begin{scheme}
\textbf{Scheme 4.5 Reagents and conditions:} i, 174, C\textsubscript{i}D\textsubscript{3}N, room temp.; ii, 174, 347, pyridine – CH\textsubscript{2}Cl\textsubscript{2} (98:2 v/v), 0°C.
\end{scheme}
In such a reaction mode, the coupling agent and sulphur transfer agent, which are both electrophilic, would not be expected to react with each other. Our observation is that no change can be detected if these two agents are mixed in anhydrous pyridine over a period of 72 hours. However, there is a potential problem (Scheme 4.5) in that the H-phosphonate monoester building block 157 can react with the sulphur transfer agent to give the corresponding phosphorothioate diester 418 before the coupling process is complete. This could clearly result in a diminished yield of the desired product.

The reaction between triethylammonium 5'-O-dimethoxytritylthymidine 3'-H-phosphonate 157 and 4-[(2-cyanoethyl)sulphanyl]morpholine-3,5-dione 174 to give the corresponding 3'-(2-cyanoethyl)phosphorothioate diester 418 is extremely slow. Thus when a 0.1 M solution of the H-phosphonate monoester 157 in d5-pyridine solution was allowed to react with a 1.5 fold excess of the sulphur transfer agent 174 at room temperature (Scheme 4.5, step i), the estimated percentage conversion to phosphorothioate diester 418 after 47.5 hours was only 13.3. On the other hand, when the H-phosphonate 157 was allowed to react with a 2.5 fold excess each of bis(2-chlorophenyl) phosphorochloridate 347 and the morpholine-3,5-dione reagent 174 in pyridine – dichloromethane (98:2 v/v) solution at 0°C (Scheme 4.5, step ii), the sulphur transfer reaction was very much faster and the estimated percentage conversion to phosphorothioate diester 418 after 1 hour was ca. 85. This vast difference is obviously due to the fact that the sulphur transfer reaction of the activated mixed anhydride 419 is very much faster.

Nevertheless, as the coupling reaction effected by bis(2-chlorophenyl) phosphorochloridate appears to go to completion within 10 min at -40°C, it is likely
to be fast enough at room temperature for it to go to completion well before the excess (usually 20%) of H-phosphonate has been consumed by the sulphur transfer agent. It therefore seemed likely that four-component oligonucleotide synthesis in solution would prove to be a feasible proposition. The issue here is then to find the best match between coupling and sulphur transfer agents.

To ensure the excess H-phosphonate is not consumed by the sulphur transfer agent in either way described in Scheme 4.5 before the coupling is complete, a very reactive coupling agent is required. It is believed that among the commonly used activating agents, the reactivity follows the order: diaryl phosphorochloridate > pivaloyl chloride > adamantanecarbonyl chloride. Bis(2-chlorophenyl) phosphorochloridate 347 and diphenyl phosphorochloridate 153 show similar activating properties, but the latter is commercially available and economically cheaper, therefore it is the preferred coupling agent among diaryl phosphorochloridates. Arylsulphonyl chlorides are not suitable for this application because of their more complex activating pathways and therefore more possible side-reactions.

As far as the sulphur transfer agent is concerned, it has to be reasonably soluble in the reaction medium, and more importantly the rate of sulphur transfer must match the rate of coupling. N-[(2-Cyanoethyl)sulphanyl]morpholine-3,5-dione 174 is only moderately soluble in pyridine, which is the solvent for both coupling and sulphur transfer reactions, and it reacts fairly rapidly with H-phosphonate monoesters in the presence of coupling agent. This agent is therefore not suitable for the four component reactions because its use leads to the consumption of the excess of H-phosphonates before the coupling goes to completion. Therefore, an alternative sulphur transfer agent which is both more soluble in pyridine and less reactive was sought. N-[(2-Cyanoethyl)sulphanyl]succinimide 420a proved to be more soluble in pyridine than N-[(2-cyanoethyl)sulphanyl]morpholine-3,5-dione 174, and is less reactive. As a comparison, when this agent was allowed to react with a 0.1 M solution of 5'-O-dimethoxytritylthymidine 3'-H-phosphonate 157 in d5-pyridine at room temperature, the estimated percentage conversion to phosphorothioate diester 418 after 47.5 hours was ca. 1.1 compared with 13.3 for N-[(2-cyanoethyl)sulphanyl]morpholine-3,5-dione 174. Thus the succinimide reagent 420a appears to react with the H-phosphonate monomer 157 more than an order of
magnitude more slowly than the morpholine-3,5-dione 174. For this reason, the succinimide derivatives 420 (a, N-[(2-cyanoethyl)sulphanyl]succinimide; b, N-(phenylsulphanyl)succinimide) were selected as the sulphur transfer agents in the present four-component synthetic studies.

The four-component reactions were carried out according to Scheme 4.6. To a solution of H-phosphonate monoester 333 and a component with free 5'-hydroxy function 146, a solution of coupling agent, i.e. diphenyl phosphorochloridate 153, and sulphur transfer agent 420a (or b) in pyridine was added at room temperature. After 10 to 30 min, the reaction was quenched by addition of water. A 2.5-fold excess of both the coupling and sulphur transfer agents was found to be sufficient for successful chain assembly.

Scheme 4.6 Reagents and conditions: i, a solution of 420 and 153 in pyridine, room temp., 10–30 min.

In the preparation of dimers, a 1.2-fold excess of H-phosphonate monomer was found to be sufficient. However, when a 1 + 2 → 3 coupling was examined, incomplete coupling was observed, which we believe was due to a relatively slower coupling reaction. Thus, a 1.4-fold excess of H-phosphonate monomer was used in the preparation of trimers. For this reason, it is conceivable that if a block larger than a
trimer were to be prepared in this way, more H-phosphonate monoester would be required. So far, our studies on four-component reactions have been limited to dimers and trimers.

In the four-component reactions, two by-products were recorded by $^{31}$P NMR spectroscopy. On the basis of reverse phase HPLC and $^{31}$P NMR spectroscopy data, they were identified as symmetrical $3' \rightarrow 3'$ 422 and $5' \rightarrow 5'$ 423 dimers. This has been confirmed by independent preparation (Scheme 4.7). It seems likely that the formation of these symmetrical products was due to the H-phosphonate monomers 333 being contaminated with very small quantities of their unphosphonylated precursors 141.

Two possible reasons may account for the occurrence of the unphosphonylated precursor 141. Firstly, after the H-phosphonate monoester was eluted off the silica gel column, dephosphonylation might have occurred during the concentration procedure, to give unphosphonylated precursors 141 and phosphonic acid 337. Secondly, dephosphonylation could happen during the course of activating in the

Scheme 4.7 Reagents and conditions: i, 347, $-40^\circ$C, 10 min; ii, 174, 10 min; iii, pyridine, room temp., 1.5 h; iv, 174, 10 min.
presence of activating agent, which would result in the formation of the unphosphonylated precursors 141 and phosphonic acid 337 as well (Scheme 4.8). It seems though that the latter is more likely to be the case because no signal of phosphonic acid or its salt can be identified in the $^3$P NMR spectrum of the H-phosphonate monoesters obtained after concentration of eluents from chromatography. Further evidence is that when the reaction mixture was left for a longer period of time, greater quantities of the symmetrical products were formed according to $^3$P NMR spectroscopy.

The formation of these symmetrical products can be compensated for by stepwise chromatography. Indeed, if purification is carried out after 'detritylation', the 'doubly de-tritylated' contaminants from 422 can be removed much more easily, at least at dimer or trimer stage. On the other hand, when the detritylated dimer 424 which is contaminated with the $5' \rightarrow 5'$ contaminants 423 couples with a second H-phosphonate monoester 425 to give a trimer block 426 (Scheme 4.9, step i), followed by 'delevulination' (Scheme 4.9, step ii) and conversion to trimer H-phosphonate 428 (Scheme 4.9, step iii), the $5' \rightarrow 5'$ symmetrical dimer 423 undergoes the corresponding reactions (Scheme 4.9, steps iv, v) and ends up as a bis H-phosphonate 430 whose polarity is very much different from that of the desired trimer H-phosphonate 428 and therefore can be removed by chromatography quite easily.
If the four-component approach is applied to solid phase oligonucleotide synthesis, only the 3’→3’ symmetrical by-products could be formed, which could be removed completely by acetonitrile washing after each chain assembly cycle.

### 4.4 Sequential H-Phosphonate Coupling Reaction

As has been argued in section 4.3, if the four-component reaction is to be used in the preparation of building blocks beyond trimers, a much larger excess of H-phosphonate would be required. To avoid usage of a large excess of H-phosphonate, an alternative approach was examined.

The −40°C sequential H-phosphonate approach has been demonstrated to be effective in our preparation of the ISIS 21-mer. It would be reasonable to argue that if a suitable coupling agent is selected, it may be possible to carry out coupling and sulphur transfer reactions in a sequential way at higher temperature. So, in this section we set out to investigate the efficacy of various activating agents.

For a compound to be suitable as an activating agent, it must (i), be sterically hindered to avoid reaction with the 5’-hydroxyl component; (ii), be very active in
activating the $H$-phosphonate monoester, and do so selectively; and (iii), be readily accessible and economically feasible.

Four categories of compounds were investigated in detail in the early studies of the $H$-phosphonate approach. They are acid chlorides 431, diaryl phosphorochloridates 414, arylsulphonyl chlorides 113 and carbonates 415.

In our studies, DMTr-Tp(s)T-Lev 433 was selected as the model molecule because good separation of this compound from the uncoupled 5'-hydroxyl component, i.e. HO-T-Lev 432 (Scheme 4.10) can be easily achieved by HPTLC, so the progress of the reaction can be monitored easily.

Various activating agents were evaluated (Figure 4.2). The amount of by-products was recorded by $^{31}$P NMR spectroscopy of the crude reaction mixture. Among the acid chlorides, 2,4,6-trichloro- 438, 2,4,6-tribromo- 439, 2,4,6-triiodo- 440, 2-
methoxy-441, 2-ethoxy-442, and 2-acetoxy-443 benzoyl chlorides behaved similarly and gave approximately 2% of 3' → 3’ and 0.5% of 5' → 5’ symmetrical products. Pivaloyl chloride 163, adamantanecarbonyl chloride 164, 2,2-dimethylbutyryl chloride 434, and 2-acetoxy-2,2-dimethylacetyl chloride 435 proved to be better activating agents, and smaller quantities of symmetrical products were formed. Adamantanecarbonyl chloride is particularly good, and the amount of by-products was negligible when the coupling was carried out at 0°C.

The dialkyl and diaryl phosphorochloridate series are generally too reactive to give clean couplings at temperatures above 0°C and considerable amounts of by-products could be detected by TLC and 31P NMR spectroscopy. Bis(2,4,6-tribromophenyl) phosphorochloridate 447a, however, gave improved results which was comparable to adamantanecarbonyl chloride. However, it proved difficult to prepare this reagent and its solubility in pyridine is poor.

Carbonate derivatives have been used in H-phosphonate chemistry. The use of dipentafluorophenyl carbonate 168 was demonstrated by Efimov53 in solid phase synthesis of oligonucleotides. However, when this reagent was applied to our solution phase reactions, an undesirable by-product, which was unstable and was not identified, was observed. The percentage of by-product amounted to ca. 30%. Although this by-product may not pose a problem in the solid phase reaction as it may well be washed away, it is a serious problem in solution phase synthesis. The chloroformate derivatives 448, 449, which are chemically similar to carbonates, were found to behave similarly to this reagent and therefore are not suitable for the current application.

We believe that pivaloyl chloride, adamantanecarbonyl chloride, 2,2-dimethylbutyryl chloride and 2-acetoxy-2,2-dimethylacetyl chloride were suitable for the high temperature (not below 0°C) H-phosphonate coupling as fast and virtually quantitative coupling reactions were produced. In economic terms, however, pivaloyl chloride is superior because it is commercially available, and cheaper. Therefore in this part of the work, pivaloyl chloride was selected for the large-scale preparation of the target compound.
In conclusion to this section, we believe that we have developed a four-component oligonucleotide synthesis in solution. This approach is very efficient for the preparation of small oligonucleotides (dimers and trimers). In conjunction with the use of a 0°C H-phosphonate coupling reaction effected by pivaloyl chloride or adamantanecarbonyl chloride followed by a sulphur transfer reaction, we believe that this protocol is suitable for the preparation of oligonucleotides of moderate chain length on a large scale. Table 4.1 and Table 4.2 show results for the preparation of some dimer and trimer building blocks using these two approaches.
Table 4.1 Preparation of some dimer and trimer building blocks with the four-component approach.

<table>
<thead>
<tr>
<th>H-Phosphonate Monomer (Mol. equiv)</th>
<th>5'-OH Component</th>
<th>Coupling Agent</th>
<th>Sulphur-transfer Agent</th>
<th>Total Reaction Time (min)</th>
<th>Product</th>
<th>Isolated Yield (%)</th>
<th>Symmetrical Dimer (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMTt-Tp(H) (1.2) 157</td>
<td>HO-T-Lev 432</td>
<td>153</td>
<td>420a</td>
<td>10</td>
<td>DMTt-Tp(s)T-Lev 433</td>
<td>99.3</td>
<td>0.7</td>
</tr>
<tr>
<td>DMTt-Tp(H) (1.2) 157</td>
<td>HO-T-Lev 432</td>
<td>153</td>
<td>420b</td>
<td>10</td>
<td>DMTt-Tp(s')T-Lev 450</td>
<td>96.7</td>
<td>0.6</td>
</tr>
<tr>
<td>DMTt-Tp(H) (1.2) 382</td>
<td>HO-T-Lev 385</td>
<td>153</td>
<td>420a</td>
<td>10</td>
<td>DMTt-Tp(s)T-Lev 451</td>
<td>97.6</td>
<td>0.8</td>
</tr>
<tr>
<td>DMTt-Ap(H) (1.2) 452</td>
<td>HO-C-Lev 453</td>
<td>153</td>
<td>420b</td>
<td>10</td>
<td>DMTt-Ap(s')C-Lev 454</td>
<td>96.7</td>
<td>-</td>
</tr>
<tr>
<td>DMTt-Cp(H) (1.2) 381</td>
<td>HO-G-Lev 384</td>
<td>153</td>
<td>420a</td>
<td>10</td>
<td>DMTt-Cp(s)G-Lev 455</td>
<td>97.0</td>
<td>1.1</td>
</tr>
<tr>
<td>DMTt-Cp(H) (1.4) 381</td>
<td>HO-Tp(s)T-Lev 387</td>
<td>153</td>
<td>420a</td>
<td>30</td>
<td>DMTt-Cp(s)Tp(s)T-Lev 396</td>
<td>97.4</td>
<td>-</td>
</tr>
<tr>
<td>DMTt-Gp(H) (1.4) 383</td>
<td>HO-Cp(s)G-Lev 386</td>
<td>153</td>
<td>420a</td>
<td>15</td>
<td>DMTt-Gp(s)Cp(s)G-Lev 390</td>
<td>97.0</td>
<td>-</td>
</tr>
<tr>
<td>DMTt-Gp(H) (1.4) 383</td>
<td>HO-Cp(s)T-Lev 388</td>
<td>153</td>
<td>420a</td>
<td>30</td>
<td>DMTt-Gp(s)Cp(s)T-Lev 399</td>
<td>94.2</td>
<td>-</td>
</tr>
<tr>
<td>DMTt-Tp(H) (1.4) 382</td>
<td>HO-Tp(s)T-Lev 387</td>
<td>153</td>
<td>420a</td>
<td>30</td>
<td>DMTt-Tp(s)Tp(s)T-Lev 3102</td>
<td>95.1</td>
<td>-</td>
</tr>
</tbody>
</table>

\[
\begin{align*}
\text{Cl} & \quad \text{O} \quad \text{Ph} \\
\text{O} & \quad \text{P} \quad \text{O} \\
153 & \quad \text{420a; } R = \text{NCCH}_2\text{CH}_2 \\
& \quad \text{b; } R = \text{Ph}
\end{align*}
\]
Table 4.2 Preparation of some dimer and trimer building blocks with the 0°C sequential coupling H-phosphonate approach.

<table>
<thead>
<tr>
<th>H-Phosphonate Monomer</th>
<th>5'-OH Component</th>
<th>Coupling Agent</th>
<th>Reaction Time (min)</th>
<th>Sulphur-transfer Agent</th>
<th>Reaction Time (min)</th>
<th>Isolated Yield (%)</th>
<th>Symmetrical Dimer (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMTri-Tp(H) 157</td>
<td>HO-T-Lev 432</td>
<td>164</td>
<td>5 (60)</td>
<td>420a</td>
<td>15</td>
<td>99.4</td>
<td>2.1</td>
</tr>
<tr>
<td>DMTri-Tp(H) 382</td>
<td>HO-T-Lev 385</td>
<td>163</td>
<td>0.5 (10.5)</td>
<td>420a</td>
<td>30</td>
<td>97.2</td>
<td>1.0</td>
</tr>
<tr>
<td>DMTri-Tp(H) 382</td>
<td>HO-T-Lev 385</td>
<td>163</td>
<td>0.5 (10.5)</td>
<td>420a</td>
<td>30</td>
<td>95.5</td>
<td>2.8</td>
</tr>
<tr>
<td>DMTri-Tp(H) 382</td>
<td>HO-T-Lev 385</td>
<td>163</td>
<td>0.5 (60)</td>
<td>420a</td>
<td>15</td>
<td>94.1</td>
<td>0.9</td>
</tr>
<tr>
<td>DMTri-Ap(H) 452</td>
<td>HO-C-Lev 453</td>
<td>164</td>
<td>5 (10)</td>
<td>420b</td>
<td>30</td>
<td>98.9</td>
<td>0.5</td>
</tr>
<tr>
<td>DMTri-Gp(H) 383</td>
<td>HO-C-Lev 453</td>
<td>434</td>
<td>0.5 (10.5)</td>
<td>420a</td>
<td>30</td>
<td>98.8</td>
<td>-</td>
</tr>
<tr>
<td>DMTri-Gp(H) 381</td>
<td>HO-Tp(s)T-Lev 387</td>
<td>163</td>
<td>0.5 (10.5)</td>
<td>420a</td>
<td>30</td>
<td>97.2</td>
<td>-</td>
</tr>
<tr>
<td>DMTri-Gp(H) 383</td>
<td>HO-Cp(s)G-Lev 386</td>
<td>163</td>
<td>0.5 (10.5)</td>
<td>420a</td>
<td>30</td>
<td>96.2</td>
<td>-</td>
</tr>
<tr>
<td>DMTri-Gp(H) 383</td>
<td>HO-Cp(s)T-Lev 388</td>
<td>163</td>
<td>0.5 (10.5)</td>
<td>420a</td>
<td>30</td>
<td>99.0</td>
<td>-</td>
</tr>
<tr>
<td>DMTri-Tp(H) 382</td>
<td>HO-Tp(s)T-Lev 387</td>
<td>163</td>
<td>0.5 (10.5)</td>
<td>420a</td>
<td>30</td>
<td>98.1</td>
<td>-</td>
</tr>
</tbody>
</table>

![Chemical structures](image)

163 164 434 420 a; R = NCCH₂CH₂
a; R = Ph

* The first number represents the time taken to add the coupling agent and the number in parentheses represents the total reaction time.

b This is the time allowed for reaction with the sulphur-transfer agent.
4.5 Detritylation

In the preparation of the ISIS 21-mer by the −40°C approach described in Chapter 3, HCl in dioxane was used to remove the 5'-O-dimethoxytrityl protecting group at very low temperature (−50°C). In conjunction with our current high temperature preparation of oligonucleotides, a method of detritylation at high temperature is required.

A couple of decades ago, Reese and co-workers\textsuperscript{182} introduced pyrrole as scavenger in the removal of the pixyl group. It was believed that when 9-phenylxanthen-9-yl protected oligonucleotide 456 was treated with acid in the presence of pyrrole, the 9-phenylxanthen-9-yl residue was quantitatively and irreversibly transferred to pyrrole to form 2-(9-phenylxanthen-9-yl)pyrrole 457 (Scheme 4.11).

![Scheme 4.11](image.png)

This procedure has since been successfully employed in the synthesis of oligodeoxy- and oligo-ribonucleotides.\textsuperscript{183–188}

Prompted by these results, we used very mild conditions, \textit{i.e.} dichloroacetic acid and pyrrole in dichloromethane, to remove the 5'-O-dimethoxytrityl protecting group in the current studies.
4.6 The Application of the Four-Component and Sequential Coupling Reactions in the Assembly of ISIS 2922 21-Mer

In order to evaluate the four-component and the sequential high temperature coupling approaches, the ISIS 2922 21-mer was chosen as the target sequence. In our current protocol, the four-component approach was used in the preparation of dimer and trimer blocks, and the pivaloyl chloride effected 0°C coupling was used in the assembly of the larger oligomers using the trimer blocks. The synthetic strategy is outlined in Figure 4.3.

\[ \text{GCGTTTGCTCTTCTTCTTGCG} \]

Figure 4.3

4.7 Preparation of the Dimer and Trimer Building Blocks

Preparation of the dimers was performed in the four-component mode by using a 20% excess of H-phosphonate monoester. Detritylation was carried out after the dimer assembly was complete without purification of the fully-protected dimers (Scheme 4.12). After removal of the 5'-O-dimethoxytrityl group, the material was purified by chromatography. The amount of impurities was below 1%, and it proved easy to remove the ‘doubly-detritylated’ symmetrical dimers by chromatography.

Trimer blocks were prepared in the same way, except for the use of a 40% excess of H-phosphonate due to relatively slower coupling reactions. Apart from the 3’-terminal trimer GCG, purification was carried out after each coupling to give fully protected trimers.
Scheme 4.12 Reagents and conditions: i, a solution of 153 and 420a in pyridine, room temp., 10-30 min; ii, dichloroacetic acid, pyrrole, CH₂Cl₂, 0°C, 15 min.

Table 4.3 Preparation of the dimers and trimers.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-Cp(s)G-Lev 386</td>
<td>94.4</td>
</tr>
<tr>
<td>HO-Tp(s)T-Lev 387</td>
<td>92.5</td>
</tr>
<tr>
<td>HO-Cp(s)T-Lev 388</td>
<td>97.0</td>
</tr>
<tr>
<td>HO-Gp(s)Cp(s)G-Lev 389</td>
<td>95.2</td>
</tr>
<tr>
<td>DMTr-Cp(s)Tp(s)T-Lev 396</td>
<td>92.5</td>
</tr>
<tr>
<td>DMTr-Gp(s)Cp(s)T-Lev 399</td>
<td>93.2</td>
</tr>
<tr>
<td>DMTr-Tp(s)Tp(s)T-Lev 3102</td>
<td>98.9</td>
</tr>
<tr>
<td>DMTr-Gp(s)Cp(s)G-Lev 390</td>
<td>93.2</td>
</tr>
</tbody>
</table>

The unblocking of the 3'-levulinyl protecting group and the conversion of the 5'-O-dimethoxytrityl-3'-hydroxy trimer building blocks to their corresponding H-phosphonates were performed in the same way as described in the previous chapter. The results are listed in Table 4.4.
Table 4.4 Removal of levulinyl protecting group and conversion of 3'-hydroxy components to corresponding H-phosphonates.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Yields (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMTr-Cp(s)Tp(s)T-OH 397</td>
<td>96.4</td>
</tr>
<tr>
<td>DMTr-Cp(s)Tp(s)Tp(H) 398</td>
<td>91.9</td>
</tr>
<tr>
<td>DMTr-Gp(s)Cp(s)T-OH 3100</td>
<td>95.1</td>
</tr>
<tr>
<td>DMTr-Gp(s)Cp(s)Tp(H) 3101</td>
<td>92.8</td>
</tr>
<tr>
<td>DMTr-Tp(s)Tp(s)T-OH 3103</td>
<td>95.1</td>
</tr>
<tr>
<td>DMTr-Tp(s)Tp(s)Tp(H) 3104</td>
<td>93.2</td>
</tr>
<tr>
<td>DMTr-Gp(s)Cp(s)G-OH 391</td>
<td>96.7</td>
</tr>
<tr>
<td>DMTr-Gp(s)Cp(s)Gp(H) 392</td>
<td>93.5</td>
</tr>
</tbody>
</table>

4.8 Coupling of Trimer Building Blocks with Oligonucleotides

Trimers prepared in the four-component mode were used to assemble larger oligomers by the pivaloyl chloride effected approach at 0°C (Scheme 4.13). In order to ensure complete coupling as the chain length grew, a larger excess of trimer H-phosphonate was used. It is also necessary to increase the amount of pyridine to keep reactants in solution, and to increase the amount of pivaloyl chloride as the chain length gets longer. Table 4.5 indicated the results of the blockmer coupling reactions.
### Table 4.5 Blockmer synthesis.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Amount of trimer H-phosphonate&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Pyridine</th>
<th>Amount of pivaloyl chloride&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 + 3</td>
<td>1.20</td>
<td>10 ml / mmol</td>
<td>2.52</td>
<td>96.4</td>
</tr>
<tr>
<td>3 + 6</td>
<td>1.25</td>
<td>11 ml / mmol</td>
<td>2.52</td>
<td>95.6</td>
</tr>
<tr>
<td>3 + 9</td>
<td>1.30</td>
<td>14.5 ml / mmol</td>
<td>2.81</td>
<td>91.0</td>
</tr>
<tr>
<td>3 + 12</td>
<td>1.35</td>
<td>20 ml / mmol</td>
<td>3.03</td>
<td>90.2</td>
</tr>
<tr>
<td>3 + 15</td>
<td>1.35</td>
<td>25 ml / mmol</td>
<td>3.12</td>
<td>90.0</td>
</tr>
<tr>
<td>3 + 18</td>
<td>1.45</td>
<td>30 ml / mmol</td>
<td>3.41</td>
<td>90.1</td>
</tr>
</tbody>
</table>

Note: <sup>a</sup>, equivalents relative to 5'-OH component.

### 4.9 Unblocking of the Partially Protected Blockmers (9-, 12-, 15-, 18-, and 21-mers)

The unblocking protocol as indicated in Scheme 3.27 was applied here to the unblocking of the blockmers. The 5'-hydroxy oligonucleotides were first acylated and then unblocked by the three-step procedure (DBU treatment, oximate treatment, ammonolysis). Caution was exercised in generating the extremely dry reaction mixture during the DBU treatment to avoid loss of sulphur. 2-Mercaptoethanol was also used in the ammonolysis to prevent loss of sulphur. The fully unblocked oligonucleotide phosphorothioates were characterised by polyacrylamide gel electrophoresis (PAGE), <sup>1</sup>H and <sup>31</sup>P NMR spectroscopy, reverse phase HPLC, ion exchange chromatography, MALDI-TOF mass spectroscopy and capillary gel electrophoresis.
Figure 4.4 Polyacrylamide gel electrophoretogram of the fully unblocked 9-mer 459, 12-mer 460, 15-mer 461, 18-mer 462 and 21-mer 220 phosphorothioates.

The polyacrylamide gel electrophoretogram (PAGE) of the fully unblocked 9-mer, 12-mer, 15-mer, 18-mer and 21-mer phosphorothioates (Figure 4.4) showed significant difference in mobility as expected.

Table 4.6 Analysis of the 'H NMR spectra of the fully unblocked oligonucleotides.

<table>
<thead>
<tr>
<th>Entries</th>
<th>Compound</th>
<th>9-mer</th>
<th>12-mer</th>
<th>15-mer</th>
<th>18-mer</th>
<th>21-mer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>459</td>
<td>460</td>
<td>461</td>
<td>462</td>
<td>220</td>
</tr>
<tr>
<td>Aromatic protons</td>
<td>Calculated</td>
<td>9.0</td>
<td>12.0</td>
<td>15.0</td>
<td>18.0</td>
<td>21.0</td>
</tr>
<tr>
<td></td>
<td>Observed</td>
<td>9.0</td>
<td>12.0</td>
<td>15.0</td>
<td>18.0</td>
<td>21.0</td>
</tr>
<tr>
<td>Anomeric protons plus cytidine H-5 protons</td>
<td>Calculated</td>
<td>12.0</td>
<td>16.0</td>
<td>20.0</td>
<td>23.0</td>
<td>27.0</td>
</tr>
<tr>
<td></td>
<td>Observed</td>
<td>11.9</td>
<td>17.0</td>
<td>19.58</td>
<td>23.1</td>
<td>27.1</td>
</tr>
<tr>
<td>H2'-3-Me of thymidine protons</td>
<td>Calculated</td>
<td>30.0</td>
<td>42.0</td>
<td>51.0</td>
<td>66.0</td>
<td>72.0</td>
</tr>
<tr>
<td></td>
<td>Observed</td>
<td>31.1</td>
<td>46.0</td>
<td>54.6</td>
<td>68.3</td>
<td>72.1</td>
</tr>
</tbody>
</table>

*Note: except H-5 proton of cytidine.

The 'H NMR spectra of the fully unblocked oligonucleotide phosphorothioates showed very good superimposition, and the integrals observed have good consistency with the required values (Table 4.6). The amounts of loss of sulphur in the fully unblocked phosphorothioate blockmers were negligible (below 0.5% in all cases) as indicated by 31P NMR spectroscopy (Figure 4.5).
Chapter 4 Large-scale Synthesis of Oligonucleotide Phosphorothioates in Solution

Figure 4.5 $^1$H and $^{31}$P NMR spectra of the fully unblocked oligonucleotide phosphorothioates in D$_2$O:
a, 9-mer 459 $^1$H NMR spectrum; b, 9-mer 459 $^{31}$P NMR spectrum; c, 12-mer 460 $^1$H NMR spectrum;
d, 12-mer 460 $^{31}$P NMR spectrum; e, 15-mer 461 $^1$H NMR spectrum; f, 15-mer 461 $^{31}$P NMR spectrum;
g, 18-mer 462 $^1$H NMR spectrum; h, 18-mer 462 $^{31}$P NMR spectrum; i, 21-mer 220 $^1$H NMR spectrum;
j, 21-mer 220 $^{31}$P NMR spectrum.

The exact masses of the fully unblocked oligonucleotide phosphorothioates were measured by MALDI-TOF mass spectrometer (Figure 4.6). For all the blocks, the errors are very low and the observed masses are consistent with the expected values (Table 4.7). The isotopic patterns observed are also consistent with the theoretical modes (Figure 4.7).
Figure 4.6 MALDI-TOF mass spectroscopy of the fully unblocked oligonucleotides: a, 21-mer 220; b, 18-mer 462; c, 15-mer 461; d, 12-mer 460; e, 9-mer 459.

Table 4.7 MALDI-TOF results for the fully unblocked oligonucleotide phosphorothioates.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Measured Mass (MH⁺)</th>
<th>Theoretical Mass</th>
<th>Error %</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-Mer 459</td>
<td>2807.6</td>
<td>2807.3</td>
<td>0.011</td>
</tr>
<tr>
<td>12-Mer 460</td>
<td>3753.0</td>
<td>3752.4</td>
<td>0.016</td>
</tr>
<tr>
<td>15-Mer 461</td>
<td>4721.7</td>
<td>4722.4</td>
<td>-0.015</td>
</tr>
<tr>
<td>18-Mer 462</td>
<td>5683.7</td>
<td>5682.5</td>
<td>0.021</td>
</tr>
<tr>
<td>21-Mer 220</td>
<td>6677.9</td>
<td>6677.6</td>
<td>0.004</td>
</tr>
</tbody>
</table>
Chapter 4 Large-scale Synthesis of Oligonucleotide Phosphorothioates in Solution

The fully unblocked oligonucleotide phosphorothioates were also analysed by ion exchange chromatography (Figure 4.8). The 21-mer was compared with the authentic material prepared by solid phase synthesis followed by post-synthetic purification. Both materials eluted at the same retention time within experimental error (32.278 and 31.952 minutes respectively). The percentage of the main peaks in the fully unblocked oligonucleotide phosphorothioates were determined and are shown in Table 4.8. The 18-mer showed significantly lower purity, which may well suggest an incomplete coupling.

Figure 4.7 The observed and theoretical isotope patterns of the fully unblocked oligonucleotides: a, 9-mer 459, observed mode; b, 9-mer 459, theoretical mode; c, 12-mer 460, theoretical mode; d, 12-mer 460, observed mode; e, 15-mer 461, observed mode; f, 15-mer 461, theoretical mode.
Figure 4.8 Ion exchange chromatography profiles of the fully unblocked oligonucleotide phosphorothioates: a, 21-mer 220; b, 21-mer 220 prepared by solid phase synthesis; c, 18-mer 462; d, 15-mer 461; e, 12-mer 460; f, 9-mer 459.

Table 4.8 The percentage of the main peaks in the fully unblocked oligonucleotide phosphorothioates as indicated by the ion exchange chromatography.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Integral of the main peak (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-mer 459</td>
<td>86.79</td>
</tr>
<tr>
<td>12-mer 460</td>
<td>91.80</td>
</tr>
<tr>
<td>15-mer 461</td>
<td>83.71</td>
</tr>
<tr>
<td>18-mer 462</td>
<td>56.89</td>
</tr>
<tr>
<td>21-mer 220</td>
<td>76.64</td>
</tr>
</tbody>
</table>
Finally, capillary gel electrophoresis analysis was carried out (Figure 4.9) and the results are shown in Table 4.9. The 21-mer was compared against the material prepared by solid phase synthesis. The materials both migrated at 21.5–22.1 minutes under the same conditions. The 18-mer showed the lowest purity, which corresponds to the ion exchange analysis.

Figure 4.9 Capillary gel electrophoretograms of the fully unblocked oligonucleotide phosphorothioates: a, 21-mer 220; b, 21-mer 220 prepared by solid phase synthesis; c, 18-mer 462; d, 15-mer 461; e, 12-mer 460; f, 9-mer 459.
Table 4.9 The percentages of the main peaks in the capillary gel electrophoretograms of the fully unblocked oligonucleotide phosphorothioates.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Integral of the main peak (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-mer 459</td>
<td>98.57</td>
</tr>
<tr>
<td>12-mer 460</td>
<td>95.69</td>
</tr>
<tr>
<td>15-mer 461</td>
<td>81.56</td>
</tr>
<tr>
<td>18-mer 462</td>
<td>70.89</td>
</tr>
<tr>
<td>21-mer 220</td>
<td>82.81</td>
</tr>
</tbody>
</table>

In conclusion, the analysis above confirmed the authenticity of the fully unblocked oligonucleotide phosphorothioates. The purities of these materials vary in different analyses. However, if purification had been carried out after the unblocking procedure, much better material would have been obtained. The long retention time impurities in the ion exchange chromatograms, which correspond to the impurities of longer migration time in the capillary gel electrophoretograms, are believed to be symmetrical products of various chain-lengths. In practical terms, it is advantageous to carry out the unblocking procedure with the dimethoxytrityl group on. It would then be easier to separate the desired oligonucleotide phosphorothioates from the truncated sequences by ion exchange chromatography.

4.10 Conclusion

To conclude this chapter, we believe that we have demonstrated a protocol which is suitable for the preparation of oligonucleotide phosphorothioates on a large scale at a temperature not below 0°C. However, more work needs to be done to improve this strategy. In our view, several aspects should be looked into in more detail:

1. The course of the formation of the symmetrical products and the way to suppress them need to be investigated;

2. To ensure the stoichiometry to be as required, it would be advantageous to produce both the H-phosphonate and the 5'-hydroxyl components in a solid state instead of glass;
3. More selective coupling agents need to be evaluated in order to minimise the amount of by-products;

4. The conditions of coupling need to be optimised to ensure complete coupling reactions.
Conversion of Fully-Protected Phosphorothioate Triesters to Phosphate Diesters

5.1 Introduction

While oligonucleotide phosphorothioates have shown great potential in therapeutic applications, with improved stability towards nucleases, an analytical problem arises with their resistance to nucleases. This prevents the effective use of standard enzymatic digestion and sequence analysis methods, complicating the characterisation of these oligonucleotides. Although mass spectrometric sequencing of small oligonucleotides and their analogues appears a promising technique, only up to about 30–50 nucleotides can be routinely sequenced using MALDI or ESI mass spectrometry.189

So far, a few papers have been published on the oxidative desulphurisation of a range of phosphorothioate diesters. In 1979, Burgers and Eckstein190 reported the first desulphurisation of a dinucleotide phosphorothioate with an excess of ethanolic iodine at 0°C. In their study, a dimer (Up(s)A) was treated with iodine in aqueous ethanol (15%) containing 0.5% NaHCO₃ at 0°C for 10 min. After the excess of iodine had been removed by repeated extraction with ether, the aqueous solution was digested with snake venom phosphodiesterase (SVPD) and alkaline phosphatase and analysed by HPLC to give a U:A nucleoside ratio which was close to unity. In later studies, solutions of iodine in pyridine117,191 and iodine in 2,6-lutidine191 were also used for the desulphurisation of oligomers with phosphorothioate internucleotide linkages.

An effective procedure suitable for the desulphurisation of a phosphorodithioate diester 501 was later proposed by Porritt and Reese.192 This led to the complete
desulphurisation of phosphorodithioate dimers and trimers. A solution of iodine in THF–H₂O–1-methylimidazole was used as the reagent and the reactions went to completion in 90 min (Scheme 5.1). The resulting phosphates 502 were purified by reverse phase HPLC and characterised by enzymatic digestion.

![Scheme 5.1](image)

The iodine-promoted desulphurisation was later automated by Wyrzykiewicz and Cole. In their study, oligomers which were still attached to solid supports (LCA/CPG) were subjected to iodine oxidation followed by cleavage from the solid support. 2-Cyanoethyl protected phosphorothioate triesters, which were prepared by phosphoramidite chemistry, were first treated with tert-butylamine in pyridine (1:9 v/v) to remove the 2-cyanoethyl groups, under which conditions the succinoyl ester linkage to the solid support was shown to be stable. Then desulphurisation was effected with a 0.24 M solution of iodine in THF–water–1-methylimidazole, followed by cleavage of the succinoyl ester linkage from the solid support by standard ammonia treatment. The conversion yields were found to be 96.0% with a small amount of internucleotide linkage cleavage (ca. 2.5%) and ca. 2.5% undesulphurised phosphorothioate internucleotide linkages as indicated by ³¹P NMR spectroscopy. The desulphurised material was then purified by polyacrylamide gel electrophoresis (PAGE). A general cycle is demonstrated in Table 5.1.

This solid support based desulphurisation offers a facile and fast approach to the conversion of phosphorothioate triesters to phosphate diesters. However, extensive post-synthetic purification had to be conducted to remove the truncated sequences formed during the solid phase synthesis, and the retention of phosphorothioate diesters cannot be completely avoided.
### Table 5.1 Automated desulphurisation cycle.

<table>
<thead>
<tr>
<th>Function</th>
<th>Reagent</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wash</td>
<td>Acetonitrile (1 ml)</td>
<td></td>
</tr>
<tr>
<td>Removal of cyanoethyl</td>
<td>10% solution of tert-butylamine in pyridine</td>
<td>110</td>
</tr>
<tr>
<td>protecting groups</td>
<td>(10 washes, 0.8 ml each)</td>
<td></td>
</tr>
<tr>
<td>Wash</td>
<td>Acetonitrile (1 ml)</td>
<td></td>
</tr>
<tr>
<td>Desulphurisation</td>
<td>0.24 M solution of iodine in THF–water–1-methyllumidazole (13 portions of the oxidising solution) (0.77 ml each)</td>
<td>130</td>
</tr>
<tr>
<td>Wash</td>
<td>Acetonitrile (1 ml)</td>
<td></td>
</tr>
</tbody>
</table>

An alternative approach involves the replacement of sulphur by oxygen at the phosphorothioate triester stage. Iodine oxidation was found to be ineffective in this approach. In a paper published by Michalski and co-workers,\textsuperscript{a} a procedure involving trifluoroacetic anhydride (TFAA) and pyridine was proposed. Almost quantitative conversion was observed in a dinucleoside phosphorothioate model (Scheme 5.2). By means of diastereoisomeric cyclic phosphorothioates, it was established that this TFAA-promoted desulphurisation proceeds in a stereospecific manner with retention of configuration at the phosphorus centre.

![Scheme 5.2](image)

Although the iodine-promoted desulphurisation procedure has been proved to be effective for phosphorothioate diesters, it is unlikely that pure products can be
obtained by this approach. Indeed, when a hexamer [Ac-Cp(s)Tp(s)Tp(s)Gp(s)Cp(s)G-Lev] (prepared by treating Ac-Cp(s)Tp(s)Tp(s)Gp(s)Cp(s)G-Lev with DBU) was treated with iodine in THF–1-methylimidazole–water, numerous impurities were observed by $^1$H NMR spectroscopy and reverse-phase HPLC. We believed that it should be possible to devise an approach involving oximate treatment of a fully-protected S-(2-cyanoethyl) oligonucleotide phosphorothioate triester to give the corresponding unmodified oligonucleotide, with solely phosphodiester internucleotide linkages, as the only product.

5.2 Oximate Treatment of Phosphate and Phosphorothioate Triesters

Prior to the late 1970s, unblocking of the phosphotriester group was effected by alkaline hydrolysis. Under these conditions, ca. 3% internucleotide cleavage per phosphotriester group occurred.113 A solution to this problem was suggested by Reese and co-workers,32 which involved treatment of the phosphotriester group with the conjugate bases of various oximes. It was shown that treatment of a fully-protected tetranucleotide triphosphate triester 505 with the conjugate base (i.e. 1,1,3,3-tetramethylguanidinium salt) of (E)2-nitrobenzaldoxime 125 (2-NBO), (E)-4-nitrobenzaldoxime 506 (4-NBO) or (E)pyridine-2-carboxaldoxime 126 gave the desired linkage-unprotected tetranucleoside triphosphate diester in high yield, with less than ca. 0.5–1% internucleotide cleavage per phosphotriester group. (E)-2-Nitrobenzaldoxime 125 was found to be particularly fast (with a $t_{1/2}$ of 20 min$^{23}$ in dioxane–water at 20°C) and effective, leading to not more than 0.1% internucleotide cleavage$^{23}$ per phosphotriester group. This method was thereafter widely used for unblocking aryl-protected internucleotide linkages.159,195–197

\[
\begin{align*}
&\text{NO}_2 \\
&\text{N} \quad \text{OH} \\
&125 \\
&\text{H} \\
&\text{N} \quad \text{HO} \\
&126
\end{align*}
\]
It was established\textsuperscript{198} that the unblocking of the phosphotriester group by oximate ions proceeds by a two-step mechanism (Scheme 5.3), and oximes with $pK_a$ values (Table 5.2) close to 10 proved to be the most effective unblocking agents.
Chapter 5 Conversion of Fully-Protected Phosphorothioate Triesters to Phosphate Diesters

Table 5.2 The pKₐ values of some oximes.

<table>
<thead>
<tr>
<th>Oxime</th>
<th>pKₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-4-Nitrobenzaldoxime 506</td>
<td>9.95, 199, 10.19, 200</td>
</tr>
<tr>
<td></td>
<td>10.36 201</td>
</tr>
<tr>
<td>E-2-Nitrobenzaldoxime 125</td>
<td>10.28 200</td>
</tr>
<tr>
<td>E-2,4-Dinitrobenzaldoxime 507</td>
<td>9.64 201</td>
</tr>
<tr>
<td>E-Pyridine-2-carboxaldoxime 126</td>
<td>10.05 199</td>
</tr>
<tr>
<td>Butan-2,3-dione monoxime 508</td>
<td>9.30 202</td>
</tr>
<tr>
<td>2-Oxopropanal-1-oxime 509</td>
<td>8.30 202</td>
</tr>
</tbody>
</table>

5.3 Development of a New Approach for Conversion of S-(2-Cyanoethyl) Phosphorothioate Triesters to Phosphate Diesters

On the basis of these observations, we believed that if conditions were optimised, oximate treatment of S-(2-cyanoethyl) phosphorothioates could yield phosphate diesters exclusively.

Our initial attempt was to unblock the S-(2-cyanoethyl) phosphorothioate triesters with different oximes and bases, using DMTr-Tp(s)T-Lev 451 as a model compound. Some of the results are listed in Table 5.3.
Table 5.3 Treatment of DMTr-Tp(s)T-Lev 451 with different oximate ions.

<table>
<thead>
<tr>
<th>Oxime</th>
<th>Base</th>
<th>Products (%)</th>
<th>Phosphorothioate</th>
<th>Phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-2-Nitrobenzaldoxime 125</td>
<td>TMG 510</td>
<td>ca. 100</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>E-2-Nitrobenzaldoxime 125</td>
<td>DIPEAa</td>
<td>ca. 100</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Butan-2,3-dione monoxime 508</td>
<td>TMG 510</td>
<td>62</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>2-Oxopropanal 1-oxime 509</td>
<td>DIPEA</td>
<td>58</td>
<td>42</td>
<td></td>
</tr>
</tbody>
</table>

Note: a, N,N-diisopropylethylamine.

The above results indicated that the more acidic the oxime is, the more phosphate is formed by oximate treatment. But it seems unlikely that a very acidic oxime could be used for this purpose because its anion would be expected to be weakly nucleophilic thereby resulting in a very long reaction time.

This being the case, our attention was drawn towards a second approach, which involved replacement of the S-(2-cyanoethyl) group by a second protecting group before the oximate treatment was carried out. This approach is indicated in outline in Scheme 5.4. This approach was based on the fact that oximate treatment of S-aryl phosphorothioate triesters produces phosphate diesters virtually quantitatively.
A fully-protected S-(2-cyanoethyl) phosphorothioate triester 172a is treated with DBU to give a phosphorothioate diester 175a, which is then alkylated to form another phosphorothioate triester 511. Oximate treatment is then carried out.

For an S-protecting group to be suitable for this purpose, (i), it must be easy to introduce; (ii), the oximate ion should attack exclusively on phosphorus.

2,4-Dinitrofluorobenzene 512 is a very powerful electrophile and the S-(2,4-dinitrophenyl) group would be expected to behave as an S-aryl group as far as oximate treatment is concerned. But 2,4-dinitrofluorobenzene did not react with the phosphorothioate diester 175a; and no desired phosphorothioate triester 511 was formed.
Substituted benzyl groups 513 have been used\textsuperscript{114} as S-protecting groups and can be removed by nucleophilic attack by thiolate ions (e.g., the conjugate bases of thiophenol and p-thiocresol) on the benzylic CH\(_2\) groups (Figure 5.1). However, concomitant nucleophilic attack\textsuperscript{175,203} occurring on the C-5' carbon atoms adjacent to the internucleotide linkages can lead to internucleotide cleavage.\textsuperscript{204} We found that the installation of a 4-nitrobenzyl group (as in 514a) on the phosphorothioate diester 175a is facile, but treatment of the corresponding product 514a with the conjugate base of butan-2,3-dione monoxime 508, which is rather acidic, results in a mixture of phosphorothioate (27\%) and phosphate (72\%) diesters. By using the conjugate base of an even more acidic oxime, i.e. 2-oxopropanal 1-oxime 509, the formation of phosphorothioate can be suppressed at the expense of the rate of reaction. It was found that treatment of S-(p-nitrobenzyl) phosphorothioate triester 515 with 2-oxopropanal 1-oxime 509 and diisopropylethylamine resulted in the exclusive conversion of \textit{ca.} 18\% of 515 into the corresponding phosphate in 4 hours. This would not be practically useful because it would be too slow if a larger sequence were involved.
Chapter 5 Conversion of Fully-Protected Phosphorothioate Triesters to Phosphate Diesters

It occurred to us that the cyano group is such a strong electron-withdrawing group that if an S-cyanomethyl group were attached to a phosphorothioate internucleotide linkage, the chance for the nucleophilic attack of oximate on the \(-\text{CH}_2\) adjacent to the cyano group would be very small. Conjugate bases of various oximes 509, 516, 517a–c were evaluated for the unblocking of S-cyanomethyl phosphorothioate triesters. In anhydrous solution, only a negligible amount (less than 2%) of phosphorothioate diester was detected by \(^{31}\text{P}\) NMR spectroscopy.

Of the oximes examined, 2-oxopropanal-1-oxime 509 gave the best result. Treatment of an S-cyanomethyl phosphorothioate hexamer triester with 509 and diisopropylethylamine in acetonitrile led to full conversion to phosphate. However, this rather acidic oxime is not effective in unblocking the O-aryl protecting groups of guanine and thymine residues. Ammonia treatment of resulting partially unblocked oligonucleotides would lead to undesirable side reactions.
Again, \((E)\)-2-nitrobenzaldoxime 125 was evaluated and satisfactory results were obtained. Thus, treatment of S-cyanomethyl phosphorothioate triester with 2-nitrobenzaldoxime 125 and TMG in acetonitrile unblocked the S-cyanomethyl groups overnight to give phosphodiester exclusively, and the carbonyl protecting groups on the base residues were also removed completely.

Having established a suitable protecting group and oxime, a serious problem associated with the introduction of the cyanomethyl group onto the sulphur centre was identified.

The problem is outlined in Scheme 5.5. A fully-protected dimer (Ac-\(Gp(s)C\)-Lev, 518) was treated first with DBU in dry dichloromethane to give the corresponding diester 519. The reaction mixture was precipitated from diethyl ether to remove the excess of DBU and then bromoacetonitrile and diisopropylethylamine were added. Alkylation on sulphur was found to be very fast and complete in 1.5 h, but undesired alkylation on the cytosine base residue also occurred and the base-modified dimer (possibly 520) was isolated in 75% yield. Upon the subsequent oximate treatment and ammonolysis of the product, uncharacterised mixtures were obtained. This was also true when \(N\)-benzoyladenine was involved.
We believed that the base alkylation must be due to the ionisation of the exocyclic NH- of the base residue (i.e. 6-N-benzyladenine or 4-N-benzoylecytosine), and this was supported by several observations.

1. Treatment of DMTr-C-Ac 521 and DMTr-A-Ac 522 with bromoacetonitrile in acetonitrile did not produce any product;
2. Treatment of DMTr-C-Ac 521 and DMTr-A-Ac 522 with bromoacetonitrile in the presence of \( N,N \)-diethylaniline (\( pK_a \) ca. 6.6\textsuperscript{205}) in acetonitrile did not produce any product;

3. Treatment of DMTr-C-Ac 521 and DMTr-A-Ac 522 with bromoacetonitrile in the presence of diisopropylethylamine (\( pK_a \) 11.5\textsuperscript{206} 10.1\textsuperscript{207}) in acetonitrile resulted in a higher \( R_f \) product;

4. Treatment of DMTr-C-Ac 521 and DMTr-A-Ac 522 with bromoacetonitrile in the presence of DBU (\( pK_a \) ca. 12.0\textsuperscript{207}) in acetonitrile resulted in a higher \( R_f \) product.

Because 4-N-benzoyl-2'-deoxycytidine and 6-N-benzoyl-2'-deoxyadenosine probably have \( pK_a \) of ca. 10–11 (this estimate is based on the \( pK_a \) values of 4-N-benzoylcytidine and 6-N-benzoyladenosine\textsuperscript{208,209}), a base of \( pK_a \) greater than 11 may well remove the exocyclic NH-proton, and the resulting conjugate base may then undergo alkylation by bromoacetonitrile.

A possible solution would be to avoid the use of strong base in the unblocking of the \( S \)-(2-cyanoethyl) protecting group. A weak base, such as an \( N,N \)-dialkyl derivative of aniline, would be expected to be suitable in the alkylation step but not in the removal of the \( S \)-(2-cyanoethyl) protecting groups. To find an alternative for the DBU in the unblocking step was, however, problematic.

We then thought that if the ionised protected adenine and cytosine base residues could be temporarily protected prior to alkylation with bromoacetonitrile, base modification could probably be suppressed.
If seemed probable that if diester 519 was treated with chlorotrimethylsilane (TMS-Cl) in the presence of $N,N$-dimethylaniline, the $N^3$- (or $N^4$-) position of cytosine (or $N^1$- or the $N^6$- position of adenine in corresponding compounds containing $N$-benzoyladenine residues) would be blocked by a TMS group. The phosphorothioate diester would probably also be protected on sulphur to give 523 (Scheme 5.6). In this way, base modification could be suppressed. However, alkylation with bromoacetonitrile becomes sluggish (Scheme 5.7). Under these conditions, conversion of 523 to 524 takes over 48 hours. This is undesirable and in the case of larger blockmers, exposure of the substrates to bromoacetonitrile over a long period of time is likely to lead to a small amount of base cyanomethylation.
A better approach would be to neutralise the excess of base used in the unblocking of the \textit{S}-(2-cyanoethyl) groups. This proved to work well. Thus, if 10 equivalents of DBU per internucleotide linkage were used in the unblocking of the \textit{S}-(2-cyanoethyl) groups, and 9 equivalents of trifluoroacetic acid were added to the reaction mixture after the unblocking was complete, subsequent alkylation on the sulphur ion was fast and clean. Under these conditions, alkylation of a 21-mer only took overnight and base modification was not observed.

Thus, we believe that we have established a protocol for the conversion of an \textit{S}-(2-cyanoethyl) phosphorothioate triester to phosphate diester (Scheme 5.8).
To start with, when a 5'-O-acetylated S-(2-cyanoethyl) phosphorothioate triester 172a was treated with DBU (10 equivalents per internucleotide linkage) in dichloromethane, the reaction went to completion in 30 min and then 9 equivalents of trifluoroacetic acid were added to neutralize the excess of DBU. After the oligonucleotide 175a had been precipitated, it was collected by centrifugation. It was then treated with bromoacetonitrile (3 equivalents per internucleotide linkage) in the presence of N,N-dimethylaniline in acetonitrile. The alkylation was assumed to finish after standing overnight. No base modified material was observed. The product 525 was isolated by column chromatography and was then treated with (E)-2-nitrobenzaldoxime 125 and TMG in acetonitrile. After ammonolysis, the crude material was passed through an Amberlite ion exchange column to give the sodium salt of the corresponding phosphate diester 175b. Enzymatic digestion of this
desulphurised material was carried out by treatment with *Crotalus adamanteus* snake venom phosphodiesterase followed by *E. coli* alkaline phosphatase.

This protocol was found to work well with a 21-mer phosphorothioate oligonucleotide (ISIS 2922) to give a product containing only 0.2% of phosphorothioate internucleotide linkages (as estimated by $^{31}$P NMR spectroscopy). This percentage of contamination is acceptable, as it may be estimated that, if the phosphodiester analogue of ISIS 2922 were contaminated with 1% of phosphorothioate diester internucleotide linkages, the nucleoside ratio as determined by enzymatic digestion would still be very close to theory. This estimate depends on the assumption that the remaining phosphorothioate diester linkages are randomly distributed in the sequence and are not cleaved by enzymes.

### 5.4 Desulphurisation of Dimer and Trimer Blocks

Using the protocol described above, some fully-protected dimer ($Cp(s)G$, $Gp(s)A$) and trimer ($Gp(s)Cp(s)G$, $Cp(s)Tp(s)T$, $Ap(s)Tp(s)T$) phosphorothioate triesters were converted to their corresponding phosphate diesters. Very satisfactory NMR ($^1$H and $^{31}$P) spectra were recorded and no phosphorothioate diester contamination was observed. Enzymatic digestion of these blocks gave satisfactory nucleoside ratios (*e.g.* A:T = 1.04 : 2.00 for 1.00 : 2.00). The $^1$H and $^{31}$P NMR spectra and reverse phase HPLC traces of the desulphurised trimer derived from Ac-$Ap(s)Tp(s)T$-Lev are illustrated in Figure 5.2.
Figure 5.2 Analysis of desulphurised trimer d(ATT) 526: a, $^1$H NMR spectrum (in D$_2$O) of d(ATT); b, $^{31}$P NMR spectrum (in D$_2$O) of d(ATT); c, reverse-phase HPLC profile of d(ATT); d, reverse-phase HPLC profile of the digest of d(ATT).

The enzymatic digest results for the other dimer and trimer blocks are outlined in Table 5.4.
Table 5.4 Nucleoside ratios of the desulphurised dimers and trimers as determined by enzymatic digest.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ratio</th>
<th>dA</th>
<th>T</th>
<th>dC</th>
<th>dG</th>
</tr>
</thead>
<tbody>
<tr>
<td>d(CG)</td>
<td>Calculated</td>
<td>--</td>
<td>--</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>527</td>
<td>Observed</td>
<td>--</td>
<td>--</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>d(GA)</td>
<td>Calculated</td>
<td>1.00</td>
<td>--</td>
<td>--</td>
<td>1.00</td>
</tr>
<tr>
<td>528</td>
<td>Observed</td>
<td>1.05</td>
<td>--</td>
<td>--</td>
<td>1.00</td>
</tr>
<tr>
<td>d(GCG)</td>
<td>Calculated</td>
<td>--</td>
<td>--</td>
<td>1.00</td>
<td>2.00</td>
</tr>
<tr>
<td>529</td>
<td>Observed</td>
<td>--</td>
<td>--</td>
<td>1.00</td>
<td>1.94</td>
</tr>
<tr>
<td>d(CTT)</td>
<td>Calculated</td>
<td>--</td>
<td>2.00</td>
<td>1.00</td>
<td>--</td>
</tr>
<tr>
<td>530</td>
<td>Observed</td>
<td>--</td>
<td>1.94</td>
<td>1.00</td>
<td>--</td>
</tr>
</tbody>
</table>

5.5 Desulphurisation of Larger Blocks (6-, 12-, and 21-mers)

The same protocol was also applied to the conversion of larger S-(2-cyanoethyl) phosphorothioate triester sequences to the corresponding phosphates. No modification of the procedure was needed compared with the dimer and trimer blocks, and the results were satisfactory. As an example, the ISIS 2922 21-mer was treated according to the conditions described in Scheme 5.8. Only approximately 0.2% of phosphorothioate contamination was recorded by $^{31}$P NMR spectroscopy. The nucleoside ratio as determined by enzymatic digestion was found to be C:G:T = 6.00:5.53:11.23 for 6:5:10.
Chapter 5: Conversion of Fully-Protected Phosphorothioate Triesters to Phosphate Diesters

Figure 5.3 Analysis of the desulphurised ISIS 2922 21-mer 531: a, $^1$H NMR spectrum (in D$_2$O); b, $^{31}$P NMR spectrum (in D$_2$O); c, reverse-phase HPLC profile; d, reverse-phase HPLC profile of enzymatic digest.

The desulphurised 21-mer was compared with the material prepared by standard phosphoramidite solid-phase synthesis. They both eluted at the same retention time on reverse-phase HPLC and co-injection resulted in a single peak.

Two other building blocks (CTT GCG, a hexamer and CTT CTT CTT GCG, a dodecamer) were also converted to their corresponding phosphate diesters using this approach. Satisfactory results were obtained. Nucleoside ratios as determined by enzymatic digestion are indicated in Table 5.5.
Table 5.5 Nucleoside ratios of the desulphurised blocks as determined by enzymatic digest.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ratio</th>
<th>T</th>
<th>dC</th>
<th>dG</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-mer</td>
<td>Calculated</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>532</td>
<td>Observed</td>
<td>2.03</td>
<td>2.00</td>
<td>2.03</td>
</tr>
<tr>
<td>12-mer</td>
<td>Calculated</td>
<td>6.00</td>
<td>4.00</td>
<td>2.00</td>
</tr>
<tr>
<td>533</td>
<td>Observed</td>
<td>5.95</td>
<td>4.00</td>
<td>1.92</td>
</tr>
</tbody>
</table>

In conclusion, we believe that we have demonstrated a protocol for conversion of $S$-(2-cyanoethyl) phosphorothioate triester internucleotide linkages to phosphodiester linkages.
Development of Novel Protecting Groups for vic-Diols

vic-Diols are very important species in synthetic and natural products. A large number of diol-protecting groups of varying stability to a substantial array of reagents have been made available over the past few decades. Among them, 1,3-dioxolanes and 1,3-dioxanes are the most commonly used protecting groups.

In the 1960s, in connection with studies on the solution phase synthesis of oligonucleotides, a protecting group for the 2',3'-cis-diol system of a ribonucleoside that was considerably more acid-labile than the conventional isopropylidene group (as in 601) was required. Despite the fact that it is chiral, the methoxymethylene group (as in 602), which is some two orders of magnitude more labile to acidic hydrolysis than the isopropylidene group, was used. Much more recently, in connection with work on the synthesis of phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P₃] Reese and Gaffney prepared 1-O-stearoyl-sn-glycerol ((S)-1-O-stearoylglycerol) 604 from its 2,3-O-isopropylidene derivative 605. As the acidic conditions required for the removal of the isopropylidene group were relatively drastic, it would have been desirable to have used a more acid-labile protecting group and thereby to have ensured that absolutely no concomitant acyl migration and hence racemisation could have occurred. In this and no doubt in a number of other studies, a chiral protecting group such as methoxymethylene would not have been suitable as its use would almost certainly have led to an undesirable mixture of diastereoisomers.
6.1 vic-Diol Protecting Groups

6.1.1 Acetals

Acetals exhibit almost complete stability to basic conditions but they are labile towards acid. This property stems from participation of a lone pair on the adjacent oxygen atom in the cleavage of a protonated intermediate. Thus, they are unaffected by the basic conditions required for alkylation and acylation; they are also stable to oxidation by CrO₃-pyridine, periodate, Pb(OAc)₄, Ag₂O, alkaline permanganate and under Oppenauer conditions. Also they are stable towards reduction by NaBH₄, LiAlH₄, Na–Hg and, with the exception of benzylidene acetals, to catalytic hydrogenolysis.

The most commonly used protecting groups of this type are isopropylidene acetals and benzylidene acetals. Methylene and ethylidene acetals have also been used frequently.

Isopropylidene acetals (also known as acetonides) have been used more frequently than any other protecting group for the protection of 1,2-diols. These acetals are easily prepared and they are stable to most reaction conditions except protic and Lewis acids.

Benzylidene acetals are frequently used in carbohydrate chemistry. They are stable to most strong bases, mild oxidants and metal hydrides (in the absence of Lewis acids) but they are readily attacked by N-bromosuccinimide and ozone – a fact that can be usefully exploited. Benzylidene acetals are not stable to some strong bases such as alkylthium reagents and they are hydrogenolysed in the presence of Pd or Pt
catalysts. Lewis acids can also decompose them. A further useful feature is that they can be reductively cleaved to give an alcohol and benzyl ether.

The chemistry of cyclohexylidene acetals is very similar to that of isopropylidene acetals but the cyclic derivatives have two advantages over their acyclic counterparts. Firstly, they reduce the water solubility of low molecular weight fragments and therefore facilitate their isolation. Secondly, they also show a greater preference for the formation of 1,3-dioxolanes over 1,3-dioxanes.

6.1.2 Cyclic Ortho Esters

A variety of cyclic ortho esters, including cyclic orthoformates, have been developed to protect cis-1,2-diols. Cyclic ortho esters are more readily cleaved by acidic hydrolysis\textsuperscript{216} (e.g. by acidic phosphate buffer or by 0.005–0.05 M HCl) than are acetonides. Careful hydrolysis or reduction can be used to prepare selectively monoprotected diol derivatives.

Cyclic orthoformates are stable in alkaline media, but undergo acid-catalysed hydrolysis under very mild conditions. Thus the half-time of hydrolysis of 2',3'-O-methoxymethyleneuridine (602; B = uracil-1-yl) was found to be ca. 10 min in 0.01 N hydrochloric acid at 20°C.\textsuperscript{217} The use of methoxy- and ethoxy-methylene protecting groups has so far been restricted mainly to oligoribonucleotide synthesis.

6.2 Mechanism of Acetal Hydrolysis

The hydrolysis of acetals (Scheme 6.1) has been found to be a specifically acid-catalysed reaction with the accepted mechanism involving a fast pre-equilibrium protonation of the acetal followed by a rate-determining decomposition of the protonated intermediate to an alcohol and a resonance-stabilised carbocation.\textsuperscript{218}
This mechanism has been confirmed with the aid of evidence such as the lack of racemisation at the alcohol carbon atom,\textsuperscript{219} the proportionality between the logarithm of the rate constant and the Hammett acidity function,\textsuperscript{220} $H_0$, and the much faster rate of hydrolysis in D$_2$O than in water ($k_{D_2O}/k_{H_2O} = 2.0-3.0$).\textsuperscript{221,222}

Substituent and steric effects were studied to determine their impact on acetal hydrolysis. Fife and Jao\textsuperscript{223} studied the hydrolysis of a series of substituted benzaldehyde diethyl acetals (606) and a series of the corresponding 2-(substituted phenyl)-1,3-dioxolanes (607).

The rates of hydrolysis were measured in 50\% dioxane–water (v/v) or 50\% dioxane–acetate or formate buffer (v/v) with HCl as proton donor. Results are presented in Table 6.1.
Table 6.1 Rates of hydrolysis of substituted benzaldehyde diethyl acetals and 2-(p-substituted phenyl)-1,3-dioxolanes in 50% dioxane-water or 50% dioxane-D₂O.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( k_H ), 1-mole⁻¹.min⁻¹</th>
<th>( k_D ), 1-mole⁻¹.min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>606a</td>
<td>29,200</td>
<td></td>
</tr>
<tr>
<td>606b</td>
<td>3,937</td>
<td></td>
</tr>
<tr>
<td>606c</td>
<td>723.3</td>
<td></td>
</tr>
<tr>
<td>606d</td>
<td>181.3</td>
<td>541.1</td>
</tr>
<tr>
<td>606e</td>
<td>1.84</td>
<td>5.01</td>
</tr>
<tr>
<td>606f</td>
<td>453.1</td>
<td></td>
</tr>
<tr>
<td>606g</td>
<td>1,170</td>
<td></td>
</tr>
<tr>
<td>606h</td>
<td>2.48</td>
<td></td>
</tr>
<tr>
<td>607a</td>
<td>824.1</td>
<td></td>
</tr>
<tr>
<td>607b</td>
<td>119.2</td>
<td>335.9</td>
</tr>
<tr>
<td>607c</td>
<td>25.4</td>
<td>70.1</td>
</tr>
<tr>
<td>607d</td>
<td>6.19</td>
<td>17.8</td>
</tr>
<tr>
<td>607e</td>
<td>0.0541</td>
<td>0.177</td>
</tr>
</tbody>
</table>

\(^a\) Acetate buffer; \(^b\) Formate buffer.

It is noticeable that the rates of hydrolysis drop drastically with the increase of the electron withdrawing effect of the substituents, which agrees with the established mechanism. With the introduction of an electron-withdrawing group, protonation would be impeded which makes departure of the leaving group more difficult, and the carbocation intermediate is destabilised; therefore, the hydrolysis rate drops.

Studies on steric effects in acetal hydrolysis were also carried out by Fife and Hagopian.\(^{224}\) In this case, a series of diethyl acetals/ketals and 2,2-disubstituted 1,3-dioxolane derivatives 608 were studied. Results are listed in Table 6.2.
Chapter 6 Development of Novel Protecting Groups for vic-Diols

608\text{a}; R_1 = C_6H_5, R_2 = CH_3  
608\text{b}; R_1 = C_6H_5, R_2 = C_2H_5  
608\text{c}; R_1 = R_2 = C_6H_5  
608\text{d}; R_1 = CH_3, R_2 = C_2H_5  
608\text{e}; R_1 = R_2 = CH_3  
608\text{f}; R_1 = CH_3, R_2 = CH(CH_3)_2  
608\text{g}; R_1 = CH_3, R_2 = C(CH_3)_3  
608\text{h}; R_1 = R_2 = CH(CH_3)_2

Table 6.2 Rates of hydrolysis of diethyl acetals/ketals and 1,3-dioxolane derivatives in 50% dioxane-water or 50% dioxane-D_2O.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( k_H ), l-mole(^{-1}).min(^{-1} )</th>
<th>( k_D ), l-mole(^{-1}).min(^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzaldehyde diethyl acetal</td>
<td>723.3</td>
<td></td>
</tr>
<tr>
<td>Acetophenone diethyl ketal\text{a}</td>
<td>24,200</td>
<td></td>
</tr>
<tr>
<td>Propiophenone diethyl ketal\text{b}</td>
<td>6,724</td>
<td></td>
</tr>
<tr>
<td>Acetone diethyl ketal\text{b}</td>
<td>82,370</td>
<td></td>
</tr>
<tr>
<td>2-Phenyl-1,3-dioxolane</td>
<td>25.4</td>
<td>70.1</td>
</tr>
<tr>
<td>608\text{a}</td>
<td>5.00</td>
<td>14.76</td>
</tr>
<tr>
<td>608\text{b}</td>
<td>1.04</td>
<td>3.13</td>
</tr>
<tr>
<td>608\text{c}\text{c}</td>
<td>0.216</td>
<td></td>
</tr>
<tr>
<td>608\text{d}</td>
<td>4.53</td>
<td></td>
</tr>
<tr>
<td>608\text{e}</td>
<td>3.92</td>
<td></td>
</tr>
<tr>
<td>608\text{f}</td>
<td>2.90</td>
<td></td>
</tr>
<tr>
<td>608\text{g}</td>
<td>3.06</td>
<td></td>
</tr>
<tr>
<td>608\text{h}</td>
<td>0.765</td>
<td></td>
</tr>
</tbody>
</table>

\text{a} Formate buffer, \text{b} Acetate buffer; \text{c} 0.5 M HCl.

Salomaa and Kankaanperä\textsuperscript{225} also found the relative rates for hydrolysis of methyl-substituted 1,3-dioxolanes, shown in Table 6.3.
Table 6.3 Relative rates of hydrolysis of 1,3-dioxolanes in water at 25°C.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative hydrolysis rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,3-Dioxolane</td>
<td>1.0</td>
</tr>
<tr>
<td>2-Methyl-1,3-dioxolane</td>
<td>5,132</td>
</tr>
<tr>
<td>2,2-Dimethyl-1,3-dioxolane</td>
<td>54,339</td>
</tr>
</tbody>
</table>

The fact that replacement of a hydrogen at the 2 position by one methyl group produced an increase in rate of over 5,000 times, quite similar to the difference in rates of hydrolysis of formaldehyde and acetaldehyde diethyl acetals, while substitution of the second methyl further increased the rate of hydrolysis by only a factor of 10 was interpreted as indicating that the presence of two substituents results in steric retardation of the rate so that the normal rate enhancement due to the inductive and/or hyperconjugative effects of the methyl groups is reduced. Since the rate increase due to substitution with the first methyl group was found to be so large, it was thought that steric hindrance was probably absent in the case of monosubstitution. In explaining these effects, it was assumed that the transition state has a great deal of oxo-carbocation character so that the bond between oxygen and the carbon at the 2-position has partial double-bond character (Figure 6.1). The groups bonded by these atoms would, therefore, approach coplanarity, resulting in steric interaction between one of the substituent groups and the ring. Presumably only the smaller of the two substituent groups would be turned towards the ring so that steric retardation of the rate would only become apparent in the case of disubstitution.

From the data presented in Table 6.2, it can be seen that replacement of the hydrogen atom at the 2-position in 2-phenyl-1,3-dioxolane by an alkyl group results in a large rate decrease. Thus, 2-phenyl-2-methyl-1,3-dioxolane hydrolyses only one-fifth as
fast as 2-phenyl-1,3-dioxolane. However acetophenone diethyl ketal hydrolyses approximately 33 times faster than benzaldehyde diethyl acetal. It is apparent that the observed order of reactivity indicates the presence of a larger rate-retarding effect in the hydrolysis of the 2-phenyl-2-alkyl-1,3-dioxolanes than in the 2,2-dialkyl-1,3-dioxolane series. Of special interest is the very slow rate of hydrolysis of 2,2-diphenyl-1,3-dioxolane. It might have been expected that this compound would hydrolyse more rapidly owing to the ability of the second phenyl group to stabilise a carbocation through a resonance effect. These results were explained as being due to either steric inhibition of resonance in the transition state or the transition state resembling the protonated substrate so that conjugation effects would be unimportant.

### 6.3 Development of New Acid-Labile Protecting Groups

In the case of substituted 5'-O-trityluridine 609, it was found that introduction of each p-methoxy group increased the rate of hydrolysis in 80% acetic acid at room temperature by a factor of approximately 10. Therefore, p-methoxy substituents would be expected to facilitate the acid-catalysed hydrolysis of 2,2-diphenyl-1,3-dioxolane 608c and its derivatives.

![Chemical structure](image)

In the light of this observation, 2',3'-O-[di-(p-anisyl)methylene]uridine 612 was prepared (Scheme 6.2) by reacting uridine 610 with di-(p-anisyl)-dimethoxymethane 611 in the presence of a catalytic amount of (±)-camphor-10-sulphonic acid in acetonitrile solution.
Scheme 6.2 *Reagents and conditions:* i, (+)-camphor-10-sulphonic acid, CH₃CN, room temp.

The rate of hydrolysis of 2',3'-O-[di-(p-anisyl)methylene]uridine 612 was evaluated against 2',3'-O-isopropylideneuridine 613 in trifluoroacetic acid – water – methanol (1:2:7) and 2',3'-O-[di-(p-anisyl)methylene]uridine 612 was found to be ca. two times more labile than 2',3'-O-isopropylideneuridine.²²⁷

The increase in lability of 2',3'-O-[di-(p-anisyl)methylene]uridine 612 to acids can probably be attributed to the mesomeric effect introduced by the para-methoxy groups. In the carbonium ion intermediate 614 in the hydrolysis of 2',3'-O-[di-(p-anisyl)methylene]uridine 612, the positive charge delocalises over the ring and is further stabilised by the unshared electron pair of the oxygen.
This effect can be further exploited if the two benzene rings are fused together through an oxygen atom, *i.e.* if xanthone derivatives 615 are used.

![Diagram of xanthone derivatives](image)

Indeed, it was found that 2',3'-\(O\)-(xanthen-9-ylidene)uridine 616a and 2',3'-\(O\)-(2,7-dimethylxanthen-9-ylidene)uridine 616b are both more labile to acids than 612, and it is particularly noteworthy that 2',3'-\(O\)-(2,7-dimethylxanthen-9-ylidene)uridine 616b undergoes hydrolysis at a rate just over 20 times faster than that of 2',3'-\(O\)-isopropylideneuridine 613 in trifluoroacetic acid – water – methanol (1:2:7), which is due to both the mesomeric effect and the hyperconjugation of the methyl groups with the aromatic rings (as in 617). Clearly, by an appropriate choice of substituents an even more labile xanthen-9-ylidene protecting group could be designed. The main limiting factor would be the ready availability of the corresponding xanthen-9-ones.

![Diagram of xanthen-9-ylidene](image)

Apart from the advantage that the xanthen-9-ylidene protecting groups are much more labile than the isopropylidene group, from the analytical point of view they have another advantage in that they absorb in the ultraviolet. This may not be of importance in nucleoside and nucleotide chemistry but it might well prove to be so in lipid and carbohydrate chemistry.
Chapter 6 Development of Novel Protecting Groups for vic-Diols

Table 6.4 Acidic hydrolysis of 2',3'-protected uridine derivatives.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Half-time ($t_{1/2}$) min</th>
</tr>
</thead>
<tbody>
<tr>
<td>2',3'-O-Isopropylidene uridine 613</td>
<td>178.1</td>
</tr>
<tr>
<td>2',3'-O-[Di-(p-anisyl)methylene]uridine 612</td>
<td>56.9</td>
</tr>
<tr>
<td>2',3'-O-(Xanthen-9-ylidene)uridine 616a</td>
<td>31.7</td>
</tr>
<tr>
<td>2',3'-O-(2,7-Dimethylxanthen-9-ylidene) uridine 616b</td>
<td>8.6</td>
</tr>
</tbody>
</table>

To conclude this section, we have developed three new protecting groups, i.e. di-(p-anisyl)methylene, xanthen-9-ylidene and 2,7-dimethylxanthen-9-ylidene groups (as in 612, 616a, and 616b respectively). The rates of hydrolysis of the corresponding 2',3'-O-protected uridine derivatives were measured under three different sets of conditions. In each case, pseudo first order kinetics were observed and good straight lines were obtained by plotting log$_{10}$(% remaining substrate) against time (see 6.6 Appendix). The half times are presented in Table 6.4.

6.4 Preparation of (R)- and (S)-1,2-O-Xanthen-9-ylideneglycerol

Contemporary asymmetric synthesis is a widely used method for stereo-controlled reaction of C-C bonds in organic molecules. During recent years this approach to organic synthesis has greatly contributed to progress in the directed introduction of various functionalities, and in the highly controlled formation of new centres of chirality. These processes still remain the basic problems in the total synthesis of natural products. Preparation of the latter in an optically pure form by application of chiral starting materials is very advantageous, enabling precise planning and efficient realisation of synthetic pathways. Many monosaccharides and their readily available derivatives are versatile and relatively inexpensive substrates for the synthesis of optically active target molecules.

D-Mannitol 618 and L-ascorbic acid 619, both of which are inexpensive polyhydroxy compounds, are widely used substrates for the synthesis of optically active target
molecules. The corresponding $S$- and $R$- enantiomers of glycerol acetonide (622 and 626) can be prepared according to the outline in Scheme 6.3.228,229

(a)  
OH OH OH
A-

(b)  
O 19 HO OHTO_

OH OH O OH HO', k,, O
618 620 0 621 622

Scheme 6.3

The key reagents required for the preparation of 1,2-$(\text{o}-xanthene-9-ylidene)$ and 1,2-$(\text{o}-2,7$-dimethylxanthene-9-ylidene) derivatives are the 9,9-dichloroxanthenes 627a,b and corresponding 9,9-dimethoxyxanthenes 628a,b. Following a literature procedure,230 9,9-dichloroxanthene 627a was prepared (Scheme 6.4, step i) in virtually quantitative yield by heating commercially available xanthene-9-one 615a with thionyl chloride, under reflux, in the presence of a catalytic amount of DMF. Treatment of 9,9-dichloroxanthene 627a with sodium methoxide in methanol – THF (Scheme 6.4, step ii) gave 9,9-dimethoxyxanthene 628a in 94.5% overall yield for the two steps. In the same way, 9,9-dichloro-2,7-dimethylxanthene 627b and 9,9-dimethoxy-2,7-dimethylxanthene 628b were prepared from 2,7-dimethylxanthene-9-one 615b.
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Scheme 6.4  Reagents and conditions: i, SOCl₂, DMF, reflux; ii, NaOMe, MeOH, THF, 0°C to room temp.

When glycerol 629 was allowed to react with 9,9-dimethoxyxanthene 628a in the presence of a catalytic quantity of (±)-camphor-10-sulphonic acid (CSA) in acetonitrile solution at room temperature (Scheme 6.5), racemic 1,2-O-(xanthen-9-ylidene)glycerol 630a was obtained and isolated as a crystalline solid in 81% yield. In the same way, racemic 1,2-O-(2,7-dimethylxanthen-9-ylidene)glycerol 630b was prepared (Scheme 6.5) and isolated as a crystalline solid in 67% yield.

Scheme 6.5  Reagents and conditions: i, CSA, MeCN, room temp., 4 h.

Like (S)-1,2-O-isopropylideneglycerol (as in Scheme 6.3a), (S)-1,2-O-(xanthen-9-ylidene)glycerol 632a and (S)-1,2-O-(2,7-dimethylxanthen-9-ylidene)glycerol 632b may both be prepared from D-mannitol 618. Treatment of D-mannitol with 9,9-dichloroxanthene 627a in pyridine solution (Scheme 6.6a) gave 1,2:5,6-di-O-(xanthen-9-ylidene)-D-mannitol 631a, which was isolated as a crystalline solid in 84.5% yield. In the same way, D-mannitol 618 reacted with 9,9-dichloro-2,7-dimethylxanthene 627b to give its 1,2:5,6-di-O-(2,7-dimethylxanthen-9-ylidene) derivative 631b in 81% isolated yield. Oxidative cleavage of 1,2:5,6-di-O-(xanthen-9-ylidene)-D-mannitol 631a was effected either with lead (IV) acetate in ethyl acetate or with sodium metaperiodate in aqueous THF. In both cases, the putative intermediate glyceraldehyde derivative was reduced with sodium borohydride.

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(Scheme 6.6a, step iv) to give (S)-1,2-O-(xanthen-9-yldene)glycerol 632a as a crystalline solid in 81 and 94% isolated yield, respectively, based on the D-mannitol derivative 631a. The 1,2:5,6-di-O-(2,7-dimethylxanthen-9-yldene) derivative 631b was similarly treated to produce (S)-1,2-O-(2,7-dimethylxanthen-9-yldene)glycerol 632b in 69% isolated yield. As indicated in Table 6.5, compounds 632a and 632b are both dextrorotatory.

\[ \text{Scheme 6.6 Reagents and conditions: i, CSHSN, 0°C to room temp.; ii, Pb(OAc)_4, NaHCO}_3, \text{EtOAc, room temp.; iii aq. NaIO}_4, \text{NaHCO}_3, \text{THF, H}_2\text{O, room temp., 4 h; iv, NaBH}_4, \text{EtOH; v, CSA, MeCN, reflux, 3 h; vi, (a) Li}_2\text{CO}_3, (b) aq. H}_2\text{O}_2, 0°C to room temp., 16 h.} \]

(R)-1,2-O-(Xanthen-9-yldene)glycerol 634a and (R)-1,2-O-(2,7-dimethylxanthen-9-yldene)glycerol 634b may both be prepared (Scheme 6.6b) from L-ascorbic acid 619. Thus L-ascorbic acid was first heated, under reflux, with a slight excess of 9,9-dimethoxyxanthene 628a in the presence of a catalytic quantity of CSA in dry acetonitrile (Scheme 6.6b, step v) to give its 5,6-O-(xanthen-9-yldene) derivative 633a. After the addition of an excess of lithium carbonate, the products were treated
first with aqueous hydrogen peroxide and then with lead (IV) acetate (step vi and ii, respectively) to give the putative (S)-2,3-O-(xanthen-9-ylidene)glyceraldehydes. Reduction with sodium borohydride (step iv) gave (R)-1,2-O-(xanthen-9-ylidene)glycerol 634a, which was isolated as a crystalline solid in 41.5% overall yield. In the same way, (R)-1,2-O-(2,7-dimethylxanthen-9-ylidene)glycerol 634b was prepared in an overall yield of 32%. Compounds 634a and 634b are both levorotatory (Table 6.5).

Table 6.5 Specific rotation of (R) and (S) enantiomers of glycerol derivatives.

<table>
<thead>
<tr>
<th>Compound</th>
<th>([\alpha]^{20^\circ}_D)</th>
<th>c (ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>632a</td>
<td>+15.7</td>
<td>1.5</td>
</tr>
<tr>
<td>632b</td>
<td>+18.2</td>
<td>1.5</td>
</tr>
<tr>
<td>634a</td>
<td>-15.3</td>
<td>1.5</td>
</tr>
<tr>
<td>634b</td>
<td>-18.2</td>
<td>1.5</td>
</tr>
</tbody>
</table>

6.5 Preparation of (R)- and (S)-1-O-stearoylglycerol

(S)-1-O-Stearoylglycerol 604 (1-O-stearoyl-sn-glycerol) is a very important building block in lipid and carbohydrate chemistry. In the preparation of phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P3] 603 carried out by Gaffney and Reese, (S)-1-O-Stearoylglycerol 604 was prepared as outlined in Scheme 6.7.

![Scheme 6.7 Reagents and conditions: i, DMAP, stearoyl chloride, Et3N, CH2Cl2, 0°C to room temp.; ii, (EtO)3B, TFA, 2,2,2-trifluoroethanol, room temp., 5 h.](image)

It is noteworthy that in this preparation, rather drastic unblocking conditions were used and the product 604 was exposed to these conditions for a fairly long period of time (5 h). As a result, ca. 1% of concomitant acyl migration was observed. This is undesirable and alternative unblocking conditions were sought.
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Racemic 1,2-\(O\)-(xanthen-9-ylidene)- and 1,2-\(O\)-(2,7-dimethylxanthen-9-ylidene)-glycerol 630a and 630b were both examined as potential starting materials for the preparation of racemic \(1-O\) -stearylglycerol 635. Treatment of the xanthen-9-ylidene derivative 630a with stearoyl chloride and 1-methylimidazole in dichloromethane solution (Scheme 6.8, step i) gave the putative stearate ester 636a as the sole product. It was found that the xanthen-9-ylidene protecting group could be removed under very mild conditions indeed. When a ca. 0.15 \(M\) solution of the intermediate stearate ester 636a in dichloromethane was treated with dichloroacetic acid (ca. 4 molar equivalents) and pyrrole (ca. 5 molar equivalents) at room temperature, rapid unblocking occurred and, following work-up of the product after 15 min, racemic \(1-O\)-stearylglycerol 635 was isolated as a pure crystalline solid in 80% overall yield. The other product was identified as 9,9-di-(pyrrole-2-yl)xanthene 647. It seemed desirable that the lipid product 635, which would be expected to undergo acyl migration under mildly basic conditions, should be isolated without recourse to column chromatography. This was achieved by treating a solution of the products (i.e. compounds 635 and 647) with an excess of iron (III) chloride in diethyl ether solution. In this way, the xanthene derivative 647 was quantitatively removed and a dark brown solid precipitate was obtained. Following the same procedure (Scheme 6.8a) racemic 1,2-\(O\)-(2,7-dimethylxanthen-9-ylidene)glycerol 630b was also converted into racemic \(1-O\)-stearylglycerol 635, which was isolated in 85% overall yield. The xanthene by-product 648 was again removed by the iron (III) chloride precipitation method.
It is noteworthy that the 2,7-dimethylxanthen-9-ylidene derivative 636b did not appear to undergo more rapid dichloroacetic acid–pyrrole promoted unblocking than the simple xanthen-9-ylidene derivative 636a. Therefore, if unblocking is to be effected in this way, there appears to be no obvious advantage in using the 2,7-dimethylxanthen-9-ylidene rather than the more easily accessible unsubstituted xanthen-9-ylidene protecting group. For this reason, the enantiomeric [\(\alpha\)]-1-\(O\)-stearoylglycerol 604 and 639 were prepared from the corresponding [\(\beta\)]-1,2-\(O\)-(xanthen-9-ylidene)glycerol 634a and 632a (Scheme 6.8b and 6.8c, respectively) by exactly the same procedure as was used for the preparation of the racemic material 635 (Scheme 6.8a). [\(\alpha\)](+)-1-\(O\)-stearoylglycerol 604 and [\(\beta\)](-)-1-\(O\)-stearoylglycerol 639 were thereby prepared and isolated in 77 and 79% overall yields, respectively. The specific rotation data of the [\(\alpha\)] and [\(\beta\)]-1-\(O\)-stearoylglycerol 604 and 639 are presented in Table 6.6.
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Table 6.6 Rotation of the (S) and (R) enantiomers of 1-O-stearoylglycerol 604, 639.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>[α]_{D}^{20^\circ} \text{ in C}_4\text{H}_4\text{N}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured</td>
</tr>
<tr>
<td>(S)-1-O-stearoylglycerol 604</td>
<td>+3.64 (c 4.1)</td>
</tr>
<tr>
<td>(R)-1-O-stearoylglycerol 639</td>
<td>−3.68 (c 4.1)</td>
</tr>
</tbody>
</table>

(a)

(b)

Scheme 6.9 Reagents: i, pyrrole, Cl\textsubscript{2}CHCO\textsubscript{2}H, CH\textsubscript{2}Cl\textsubscript{2}.

The procedure for the dichloroacetic acid–pyrrole promoted removal of the xanthen-9-ylidene protecting group is essentially the same as the procedure that was recommended some 15 years ago by Reese and co-workers\textsuperscript{182} for the removal of the 9-phenylxanthen-9-yl and related (e.g. 4,4'-dimethoxytrityl) protecting groups from alcoholic hydroxy functions. Thus, when a 9-phenylxanthen-9-yl ether 640 (Scheme 6.9a) is treated with dichloroacetic acid and pyrrole, the 9-phenylxanthen-9-yl cation 641 is generated and then rapidly and irreversibly quenched by pyrrole to give 2-(9-phenylxanthen-9-yl)pyrrole 642. The suggested mechanism for the removal of the xanthen-9-ylidene protecting group, which is illustrated in Scheme 6.9b for an acyclic acetal 643, is somewhat more complicated. The cation 644 generated initially would
perhaps be expected to be as stable as the 9-phenylxanthen-9-yl cation 641 inasmuch as the mesomeric effect of an alkoxy group (OR²) is generally unlikely to be smaller than that of a phenyl group. The monopyrrol-2-yl intermediate 645, formed by the reaction between cation 644 and pyrrole, would be expected to fragment very rapidly indeed under the reaction conditions to give cation 646. This intermediate 646, which is stabilized by the mesomeric effect of the pyrrol-2-yl residue, would be expected to be more stable than the 9-phenylxanthen-9-yl cation 641. Although we have not so far obtained any supporting experimental evidence, the inductive effect of the methyl substituents in the 2,7-dimethylxanthen-9-ylidene protecting group would be expected to facilitate the transformations indicated in Scheme 6.9.

The two di-(pyrrol-2-yl) derivatives 647 and 648 were both obtained as pure crystalline compounds and were fully characterised. Racemic 1-O-stearoyl-2,3-O-(xanthen-9-ylidene)glycerol 636a was treated with an excess each of dichloroacetic acid and pyrrole in dichloromethane solution at room temperature. After the fractionation of the products, the di-(pyrrol-2-yl) derivative 647 was isolated as a colourless crystalline solid in 92% yield. The latter compound was also prepared from 9,9-dimethoxyxanthene 628a and obtained in 74% isolated yield. In the same way, 2,7-dimethyl-9,9-di-(pyrrol-2-yl)xanthene 648 was prepared both from racemic 1-O-stearoyl-2,3-O-(2,7-dimethylxanthen-9-ylidene)glycerol 636b and 9,9-dimethoxy-2,7-dimethylxanthene 628b and isolated in 82 and 64% yield, respectively. When a solution of each of these di-(pyrrol-2-yl) derivatives 647 and 648 was treated with a threefold excess of iron (III) chloride in dry diethyl ether solution, dark coloured solid precipitates were obtained and, in both cases, none of the starting material remained in the ethereal solution. No attempt has so far been made to characterise these precipitated solids.
To conclude, we believe that we have developed a series of new protecting groups which are more acid-labile than the conventionally used isopropylidene group. The appropriately protected enantiomeric isomers of glycerol derivatives were prepared. Their stearoyl esters were also prepared and unblocked under very mild conditions.

6.6 Appendix – Hydrolysis Data of the 2',3'-O-Protected Uridine Derivatives

6.6.1 Hydrolysis of the Protected Uridine Derivatives

Hydrolysis kinetics were examined under three sets of conditions. Using 3'-O-benzoylthymidine as internal standard, rates of disappearance of the substrates were calculated. Half-times of the substrates were calculated in the following way:

$S_0$ is defined as percentage absorbance of substrate at 260 nm at time zero;
$A_0$ is defined as percentage absorbance of internal standard at 260 nm at time zero;
$S_t$ is defined as percentage absorbance of substrate at 260 nm at time $t$;
$A_t$ is defined as percentage absorbance of internal standard at 260 nm at time $t$;

Then $100 \left( \frac{A_S}{A_0} \right)$ is the percentage of substrate remaining at time $t$.

$$\text{Rate} = k[H_3O^+] \left[ \frac{S_S}{A_0} \right];$$

at constant pH, $\text{rate} = k \left[ \frac{S_S}{A_0} \right]$,

then,

$$\frac{d}{dt} \left[ 100 \left( \frac{A_S}{A_0} \right) \right] = k \left( \frac{1}{100 \left( \frac{A_S}{A_0} \right)} \right),$$

$$\int_0^t \frac{1}{100 \left( \frac{A_S}{A_0} \right)} dt \left( \frac{1}{100 \left( \frac{A_S}{A_0} \right)} \right) = \int_0^t k dt$$

$$-\ln \left[ \frac{A_S}{A_0} \right] + \ln \left[ \frac{A_0}{A_S} \right] = kt$$
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\[-\ln\left(\frac{A_0 \cdot S_i}{A_i \cdot S_o}\right) + \ln 100 = kt\]

\[-\frac{\log_{10}\left(\frac{A_0 \cdot S_i}{A_i \cdot S_o}\right)}{\log_{10} e} + \frac{2}{\log_{10} e} = kt\]

\[-\log_{10}\left(\frac{A_0 \cdot S_i}{A_i \cdot S_o}\right) + 2 = (\log_{10} e) \cdot kt\]

\[\log_{10}\left(\frac{A_0 \cdot S_i}{A_i \cdot S_o}\right) + (\log_{10} e) \cdot kt = 2\]

When \[\left[100\left(\frac{A_0 \cdot S_i}{A_i \cdot S_o}\right)\right] = 50\left(\frac{A_0 \cdot S_0}{A_o \cdot S_o}\right),\] which means that fifty percent of substrate remains,

\[\log_{10}(50\left(\frac{A_0 \cdot S_i}{A_o \cdot S_o}\right)) + (\log_{10} e) \cdot k \cdot t_{1/2} = 2, \text{ that is}\]

\[(\log_{10} e) \cdot k \cdot t_{1/2} = \log_{10} 2\]

We therefore have half-time as

\[t_{1/2} = \frac{\log_{10} 2}{(\log_{10} e) k}\]

Therefore, if \[\log_{10}\left(100\left(\frac{A_0 \cdot S_i}{A_i \cdot S_o}\right)\right)\] is plotted against time, gradient = \((\log_{10} e) \cdot k\), we have half-time as:

\[t_{1/2} = \frac{\log_{10} 2}{\text{gradient}}\]
6.6.2 Hydrolysis Charts of the Protected Uridine Derivatives

(a), $2',3'-O$-(Isopropylidene)uridine in McOH:H$_2$O:TFA = 8:1:1 at 40°C ($t_{1/2} = 129.2$ min).

(b), $2',3'-O$-[Di-(p-anisyl)methylene]uridine in MeOH:H$_2$O:TFA = 8:1:1 at 40°C ($t_{1/2} = 52.5$ min).
(c), $2',3'-O$-($\text{Xanthen-9-ylidene}$)uridine in MeOH:H$_2$O:TFA = 8:1:1 at 40°C ($t_{1/2} = 22.8$ min).

\[ y = -0.01320x + 1.98544 \\ R^2 = 0.99893 \]

(d), $2',3'$-O-(2,7-Dimethylxanthen-9-ylidene)uridine in MeOH:H$_2$O:TFA = 8:1:1 at 40°C ($t_{1/2} = 6.7$ min).

\[ y = -0.04508x + 2.02859 \\ R^2 = 0.99478 \]
(e) $2',3'-O$-(Isopropyldene)uridine in MeOH:H$_2$O:TFA = 7:2:1 at 40°C ($t_{1/2} = 51.1$ min).

(f) $2',3'-O$-[Di-(p-anisyl)methylene]uridine in MeOH:H$_2$O:TFA=7:2:1 at 40°C ($t_{1/2} = 22.7$ min).
(g), $2',3'-O$(Xanthen-9-ylidene)uridine in MeOH:H$_2$O:TFA = 7:2:1 at 40°C ($t_{1/2} = 10.1$ min).

(y = -0.02977x + 1.97769
$R^2 = 0.99731$

(h), $2',3'-O$(2,7-Dimethylxanthen-9-ylidene)uridine in MeOH:H$_2$O:TFA = 7:2:1 at 40°C ($t_{1/2} = 2.5$ min).

(y = -0.12060x + 2.03760
$R^2 = 0.99667$
(i), 2',3'-O-[Di-(p-anisyl)methylene]uridine in MeOH:H₂O:TFA=7:2:1 at 30°C (t₁/₂ = 56.9 min).

\[ y = -0.00529x + 2.00479 \]
\[ R² = 0.99840 \]

(j), 2',3'-O-(2,7-Dimethylxanthen-9-yl)uridine in MeOH:H₂O:TFA=7:2:1 at 30°C (t₁/₂ = 8.6 min).

\[ y = -0.03520x + 2.00569 \]
\[ R² = 0.99883 \]
(k), 2',3'-O-Isopropylideneuridine in MeOH:H₂O:TFA=7:2:1 at 30°C ($t_{1/2} = 178.1$ min).

\[ y = -0.00169x + 1.99882 \]
\[ R^2 = 0.99601 \]

(l), 2',3'-O-(Xanthen-9-yl)uridine in MeOH:H₂O:TFA=7:2:1 at 30°C ($t_{1/2} = 31.7$ min).

\[ y = -0.00947x + 1.99385 \]
\[ R^2 = 0.99974 \]
Experimental

7.1 Instrumentation

Melting points were measured with a Büchi melting pointing apparatus and are uncorrected. $^1$H NMR spectra were measured at 360 and 400 MHz with Bruker AM360, AM400 and AV400 spectrometers; tetramethylsilane was used as an internal standard; $J$ values are given in Hz. $^{13}$C NMR spectra were measured at 90.6 and 100.6 MHz with the same spectrometers. $^{31}$P NMR spectra were measured at 145.8 and 161.9 MHz respectively with the same spectrometers, and were referenced to external 85% H$_3$PO$_4$. Chemical shifts are given in ppm. Micro-analysis was carried out by the Analytical Services at the Department of Chemistry, University College London. Mass spectra were recorded on KRATOS MS890MS and JEOL JMS-AX505 mass spectrometers. MALDI Mass spectra were measured with a Micromas TofSpec 2E spectrometer. Capillary gel electrophoresis analysis and ion exchange chromatography were carried out at Avecia Pharmaceuticals.

7.2 Chromatography

Merck silica gel 60 F$_{254}$ TLC plates (Art. 5715 and 5642) were developed in the following solvent systems:

A [dichloromethane–methanol (95:5 v/v)]

B [dichloromethane–methanol (90:10 v/v)]

C [petroleum spirit (bp 60–80°C)–ethyl acetate (80:20 v/v)]

Merck silica gel 60H (Art. 7729 and 9385) was used for short-column chromatography.
Reverse phase HPLC was carried out with the following programmes:

A Hypersil 5 µ ODS (250×4 mm) column which was eluted with acetonitrile–0.1 M aqueous triethylammonium acetate (TEAA):

Programme I: linear gradient 10:90 to 40:60 v/v over 15 min then isocratic elution;

Programme II: linear gradient 5:95 to 15:85 v/v over 10 min then isocratic elution;

or acetonitrile–water:

Programme III: linear gradient 30:70 to 80:20 v/v over 10 min then isocratic elution.

7.3 Solvents

Acetonitrile, 1-methylimidazole, 1-methylpyrrolidine, pyridine and triethylamine were dried by heating, under reflux, over calcium hydride and then distilled. Diethyl ether, tetrahydrofuran (THF) and toluene were refluxed over sodium and distilled. 1,2-Dichloroethane and dichloromethane were dried by heating, under reflux, over phosphorus pentoxide and distilled. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU), N,N-dimethylformamide (DMF), 2,6-lutidine and N,N',N,N'-tetramethylguanidine (TMG) were dried by heating with calcium hydride at 60°C and then distilled under reduced pressure.
7.4 Experimental for Chapter 3

Bis(2-chlorophenyl) phosphorochloridate (347)

\[
\begin{array}{c}
\text{Cl} \\
\text{O} \\
\text{P} \\
\text{Cl} \\
\text{Cl} \\
\text{O} \\
\text{Cl}
\end{array}
\]

2-Chlorophenol (207.2 ml, 2.00 mol), phosphorus oxychloride (93.2 ml, 1.00 mol) and 1-methylimidazole (1 ml) were heated at 180°C for 15 h. Then the reaction mixture was distilled under reduced pressure. The title compound was collected at 172-175°C/1 mmHg as a colourless liquid (265.0 g, 78%).

\[\delta_{\text{H}}(\text{CDCl}_3): 7.23 (2\ H, \text{ m}), 7.25 (2\ H, \text{ m}), 7.47 (2\ H, \text{ m}), 7.58 (2\ H, \text{ m}).\]

\[\delta_{\text{C}}(\text{CDCl}_3): 121.94, 125.89, 127.38, 128.12, 145.76.\]

\[\delta_{\text{P}}(\text{CDCl}_3): -4.95.\]

Ammonium 4-methylphenyl H-phosphonate (342c)

\[
\begin{array}{c}
\text{H}_3\text{C} \\
\text{O} \\
\text{P} \\
\text{O} \\
\text{NH}_4
\end{array}
\]

\[p\text{-Cresol (43.26 g, 0.40 mol) and phosphorus trichloride (17.44 ml, 0.20 mol) were heated together, first under gentle reflux, and then reacted at 160°C. After 3 h, tert-butanol (21 ml) was added with stirring and the reactants were kept at the same temperature for a further period of 30 min. The products were evaporated under reduced pressure. The residue was cooled to 0°C (ice–water bath) and poured into a}\]

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stirred, cooled (ice-water bath) solution of concentrated aqueous ammonia (33%, d 0.88, 125 ml, 2.13 mol) in water (200 ml). The mixture was allowed to warm up to room temperature over a period of 1 h and evaporated under reduced pressure, followed by co-evaporation with absolute ethanol (2×100 ml). The residue was suspended in absolute ethanol (400 ml) and filtered through a bed of Celite (30 g) and the filter bed was washed with ethanol (100 ml). The filtrate and washings were combined and evaporated under reduced pressure to ca. half volume until crystals appeared. Then diethyl ether (400 ml) was added dropwise over a period of 1 h and stirring was continued for a further 2 h. The title compound was collected by filtration and dried in vacuo as white crystals (32.95 g, 87%).

$\begin{align*}
\delta_H[(CD_3)\text{SO}] & : 2.22 (3 \text{ H, s}), 6.81 (1 \text{ H, d}), 6.95 (2 \text{ H, d, } J 8.4), 7.04 (2 \text{ H, d, } J 8.3), 7.49 (4 \text{ H, br}). \\
\delta_C[(CD_3)\text{SO}] & : 20.1, 120.2 (J_{C,P} 4.8), 129.4, 131.1, 150.4 (d, J_{C,P} 6.5). \\
\delta_P[(CD_3)\text{SO}] & : -1.0 (d, J_{P,H} 603.4).
\end{align*}$

**Morpholine-3,5-dione (701)**

Diglycolic acid (266.9 g, 0.20 mol) and ammonium carbonate (19.2 g, 0.20 mol) were powdered and thoroughly mixed together. The mixture, contained in a round-bottomed flask, was heated at 230°C in an atmosphere of argon. Water formed in the reaction was removed continuously. After 5 h, the homogeneous liquid was cooled to 70°C, and water (200 ml) was added. The resulting solution was stirred with activated charcoal (1.0 g) for 10 min and then filtered. The residue was washed with hot water (20 ml). The filtrate and washings were combined and cooled to 0°C (ice-water bath)
to give morpholine-3,5-dione as colourless crystals (15.7 g, plus a second crop of 4.0 g after concentrating the original mother liquor to ca. 20 ml; total yield 19.7 g, 85%). Recrystallisation of this material from ethanol gave colourless needles, mp 141–142°C.

$\delta_{\text{H}}[(\text{CD}_3\text{SO})]: 4.25 (4 \text{ H, s}), 11.40 (1 \text{ H, br}).$

$\delta_{\text{C}}[(\text{CD}_3\text{SO})]: 66.3 (\text{CH}_2), 171.0 (\text{C}).$

**Di-(2-cyanoethyl) disulphide (702)**

\[\begin{array}{c}
\text{NC} - \text{S} - \text{S} - \text{CN}
\end{array}\]

Dichloromethane (400 ml), followed by sodium perborate tetrahydrate (44.1 g, 0.286 mol) were added to a cooled (ice–water bath), stirred solution of S-(2-cyanoethyl)isothiouronium chloride (83.0 g, 0.50 mol) in water (500 ml). A solution of sodium hydroxide (30.0 g, 0.75 mol) in water (250 ml) was then added dropwise over a period of 30 min to the cooled, stirred reaction mixture. After a further period of 5 h, the two layers were separated and the aqueous layer was back-extracted with dichloromethane (3×50 ml). The combined organic layers were dried (MgSO$_4$) and evaporated under reduced pressure. The residue was recrystallised from methanol to give the title compound as colourless needles (38.5 g, 89%), mp 47–48°C.

$\delta_{\text{H}}[\text{CDCl}_3]: 2.91 (4 \text{ H, m}), 3.02 (4 \text{ H, m}).$

$\delta_{\text{C}}[\text{CDCl}_3]: 17.1 (\text{CH}_2), 32.5 (\text{CH}_2), 119.2 (\text{C}).$
4-[(2-Cyanoethyl)sulphanyl]morpholine-3,5-dione (174)

A solution of bromine (4.82 g, 1.55 ml, 30.2 mmol) in dichloromethane (20 ml) was added dropwise over 30 min to a stirred, cooled (ice–water bath) mixture of di-(2-cyanoethyl) disulphide (4.51 g, 26.2 mmol), morpholine-3,5-dione (5.75 g, 50.0 mmol), 2,6-lutidine (17.4 ml, 0.15 mol) and dichloromethane (20 ml). The resulting reactants were stirred for a further period of 1.5 h. Ice-cold methanol (50 ml) was then added dropwise over 30 min. The solid obtained was collected by filtration to give the title compound as an off-white solid (8.23 g, 82%). Recrystallisation of this material from ethyl acetate gave colourless needles, mp 121–122°C.

$\delta_H([\text{CD}_3\text{SO}])$: 2.77 (2 H, t, J 6.9), 3.10 (2 H, t, J 6.9), 4.49 (4 H, s).

$\delta_C([\text{CD}_3\text{SO}])$: 17.9 (CH$_2$), 33.0 (CH$_2$), 68.1 (CH$_2$), 119.4 (C), 170.8 (C).

5'-O-(Dimethoxytrityl)-4-O-phenylthymidine (371)

1-Methylpyrrolidine (52.0 ml, 0.50 mol) and chlorotrimethylsilane (12.7 ml, 0.10 mol) were added to a stirred suspension of 5'-O-(dimethoxytrityl)thymidine (21.78 g,
40.00 mmol) in dry acetonitrile (200 ml) at room temperature. After 1 h, the reactants were cooled to 0°C (ice–water bath), and stirred for 10 min. Phosphorus oxychloride (5.59 ml, 60 mmol) was added, followed after a further period of 20 min by phenol (22.59 g, 0.24 mol). After 3 h, water (10 ml) was added and the reactants were allowed to warm up to room temperature and stirred overnight. The products were evaporated under reduced pressure. The residue was dissolved in dichloromethane (200 ml) and washed with saturated aqueous sodium hydrogen carbonate (2×150 ml). The aqueous layers were back-extracted with dichloromethane (2×30 ml). The combined dichloromethane layers were dried (MgSO₄) and concentrated under reduced pressure. The residue was then purified by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (99:1 v/v) were evaporated to give the title compound as a colourless glass (20.359 g, 82.0%).

δH[(CD3)2SO]: 1.74 (3 H, s), 2.20 (1 H, m), 2.35 (1 H, m), 3.25 (2 H, m), 3.76 (6 H, s), 3.97 (1 H, d, J 3.3), 4.34 (1 H, d, J 4.7), 5.38 (1 H, d, J 4.6, ex), 6.15 (1 H, t, J 6.3), 6.93 (4 H, d, J 8.6), 7.18 (2 H, d, J 8.1), 7.25 – 7.5 (12 H, m), 8.00 (1 H, s).

2'-Deoxy-6-O-(2,5-dichlorophenyl)-5'-O-(4,4'-dimethoxytrityl)-2-N-isobutyrylguanosine (380)

Chlorotrimethylsilane (3.2 ml, 25.2 mmol) was added to a stirred solution of 2'-deoxy-5'-O-(4,4'-dimethoxytrityl)-2-N-isobutyrylguanosine (6.08 g, 9.50 mmol) in dry pyridine (15 ml) at room temperature. After 30 min, the resulting mixture was
concentrated under reduced pressure to ca. half volume and more dry pyridine (10 ml) was added. 2-Mesitylenesulphonylchloride (3.28 g, 15.00 mmol) and 1-methyl pyrrolidine (10 ml, 96 mmol) were then added to the cooled (ice–water bath), stirred solution. After 15 min, 2,5-dichlorophenol (4.89 g, 30.00 mmol) was added and, after a further period of 3 h, triethylamine (5 ml, 35.87 mmol) and water (5 ml) were added. The stirring reactants were then left overnight and poured into saturated aqueous sodium hydrogen carbonate (200 ml). The resulting mixture was extracted with dichloromethane (3×100 ml). The combined organic layers were further washed with saturated aqueous sodium hydrogen carbonate (2×100 ml). The aqueous layers were back extracted with dichloromethane (2×20 ml). The organic layers were dried (MgSO₄) and evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (99:1 v/v) were evaporated under reduced pressure to give 2'-deoxy-6-O-(2,5-dichlorophenyl)-5'-O-(4,4'-dimethoxytrityl)-2-N-isobutyrylguanosine as a colourless glass (7.06 g, 94.7%).

δH[(CD3)2SO]: 0.92 (6 H, t, J 6.4), 2.38 (1 H, m), 2.82 (1 H, m), 2.91 (1 H, m), 3.12 (1 H, dd, J 2.9 and 10.2), 3.72 (6 H, s), 4.00 (1 H, m), 4.57 (1 H, m), 5.33 (1 H, d, J 4.6, ex), 6.42 (1 H, t, J 6.4), 6.74 (2 H, d, J 8.9), 6.79 (2 H, d, J 8.9), 7.19 (7 H, m), 7.32 (2 H, m), 7.47 (1 H, dd, J 2.4 and 8.7), 7.70 (2 H, m), 8.49 (1 H, s), 10.25 (1 H, s, ex).

2'-Deoxy-6-O-(2,5-dichlorophenyl)-2-N-isobutyryl-3'-O-levulinylguanosine (384)
A solution of 2'-deoxy-6-O-(2,5-dichlorophenyl)-5'-O-(4,4'-dimethoxytrityl)-2-N-isobutyrylguanosine (3.76 g, 4.79 mmol) and crude levulinic anhydride (2.14 g, 10.00 mmol) in dry pyridine (15 ml) was stirred at room temperature. After 16 h, the products were concentrated under reduced pressure and the residue was partitioned between dichloromethane (50 ml) and saturated aqueous sodium hydrogen carbonate (50 ml). The organic layer was separated, washed with saturated aqueous sodium hydrogen carbonate (2×50 ml), dried (MgSO₄) and evaporated under reduced pressure. The residue was co-evaporated with toluene (2×20 ml) and then dissolved in dichloromethane (150 ml). Pyrrole (5 ml, 72.07 mmol) and then a solution of dichloroacetic acid (4.14 ml, 50.18 mmol) in dichloromethane (50 ml) were added to the cooled (ice–water bath), stirred solution. After 10 min, the products were extracted with saturated aqueous sodium hydrogen carbonate (100 ml). The aqueous layer was back extracted with dichloromethane (3×50 ml). The combined organic layers were dried (MgSO₄) and evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (98:2, v/v) were evaporated under reduced pressure to give the title compound as a colourless glass (2.52 g, 90.6%).

δH[(CD₃)₂SO]: 0.95 (6 H, d, J 6.7), 2.15 (3 H, s), 2.47–2.56 (3 H, m), 2.76–2.88 (3 H, m), 3.02–3.10 (1 H, m), 3.58–3.69 (2 H, m), 4.09 (1 H, m), 5.09 (1 H, ex), 5.38 (1 H, m), 6.38 (1 H, dd, J 5.9 and 8.6), 7.47 (1 H, dd, J 2.5 and 8.6), 7.68 (1 H, d, J 8.6), 7.77 (1 H, d, J 2.5), 8.63 (1 H, s), 10.36 (1 H, s, ex).

**Triethylammonium 5'-O-dimethoxytrityl-4-O-phenylthymidine 3'-H-phosphonate (382)**
Ammonium 4-methylphenyl \(H\)-phosphonate (5.64 g, 29.82 mmol) and triethylamine (8.36 ml, 59.98 mmol) were co-evaporated with pyridine (20 ml) under reduced pressure. 5'-O-Dimethoxytrityl-4-O-phenylthymidine (6.21 g, 10.00 mmol) was added to the residue and co-evaporated with dry pyridine (2x20 ml). The residue was redissolved in anhydrous pyridine (60 ml) and cooled to \(-20^\circ\text{C}\) (IMS-dry ice bath). Pivaloyl chloride (3.67 ml, 29.80 mmol) was added dropwise to the stirred solution over a period of 5 min. The resulting reactants were stirred at the same temperature for 60 min. Water (15 ml) was added and the mixture was allowed to warm up to room temperature over a period of 1 h. Then it was partitioned between dichloromethane (300 ml) and saturated aqueous sodium hydrogen carbonate (200 ml). The layers were separated and the aqueous layer was back extracted with dichloromethane (2x30 ml). The combined organic layers were washed with triethylammonium phosphate buffer (0.5 \(M\), pH 7.0, 2x100 ml). The aqueous layers were back extracted with dichloromethane (2x50 ml). The dried (MgSO\(_4\)) combined organic layers were concentrated to a volume of ca. 40 ml. Toluene (50 ml) was added to the residue and the mixture was applied to a short silica gel column. Evaporation of the appropriate fractions, which were eluted with methanol–dichloromethane (5:95 v/v), gave the title compound as a colourless glass (7.50 g, 95.4%).

\[\delta_{\text{H}}[(\text{CD}_3)_2\text{SO}]: 1.13 (9 \text{ H, t, } J 7.3), 1.69 (3 \text{ H, s}), 2.24–2.31 (1 \text{ H, m}), 2.42–2.46 (1 \text{ H, m}), 3.00 (6 \text{ H, m}), 3.75 (6 \text{ H, s}), 4.13 (1 \text{ H, d, } J 3.4), 4.76 (1 \text{ H, br}), 5.79 (0.5 \text{ H, s}), 6.13 (1 \text{ H, t, } J 6.3), 6.92 (4 \text{ H, dd, } J 1.9 \text{ and } 8.9), 7.20 (4 \text{ H, d, } J 1.1), 7.30–7.48 (10.5 \text{ H, m}), 8.00 (1 \text{ H, s}), 10.76 (1 \text{ H, br, ex}).\]

\[\delta_{\text{F}}[(\text{CD}_3)_2\text{SO}]: 1.17 (^{1}J_{\text{P,H}} 585.596).\]
Triethylammonium 2'-deoxy-5'-O-dimethoxytrityl-4-N-benzoylcydine 3'-H-phosphonate (381)

![Chemical Structure]

Ammonium 4-methylphenyl H-phosphonate (8.47 g, 44.70 mmol) and triethylamine (12.54 ml, 89.97 mmol) were co-evaporated with dry pyridine (20 ml). 5'-O-Dimethoxytrityl-4-N-benzoyl-2'-deoxycytidine (9.51 g, 15.01 mmol) was added to the residue and co-evaporated with dry pyridine (2×20 ml). The residue was redissolved in dry pyridine (90 ml) and cooled to -20°C (IMS–dry ice bath). Pivaloyl chloride (5.51 ml, 44.74 mmol) was added to the solution over a period of 5 min and the reaction mixture was stirred at the same temperature for 60 min before it was quenched by addition of water (20 ml). The mixture was then allowed to warm up to room temperature over a period of 1 h. The resulting clear solution was partitioned between dichloromethane (450 ml) and saturated aqueous sodium hydrogen carbonate (300 ml). The layers were separated and the aqueous layer was back extracted with dichloromethane (2×40 ml). The combined organic layers were washed with triethylammonium phosphate buffer (0.5 M, pH 7.0, 2×100 ml). The two layers were separated, and the aqueous layers were back extracted with dichloromethane (2×50 ml). The combined organic layers were dried (MgSO₄) and concentrated to a volume of ca. 80 ml. Toluene (100 ml) was added to the residue. The resulting mixture was then fractionated by short column chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (95:5 v/v) gave the title compound as a colourless glass (11.45 g, 95.5%).

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$\delta_{\text{H}}[(\text{CD}_3)_{2}\text{SO}]$: 1.13 (9 H, t), 2.25–2.32 (1 H, m), 2.90–2.96 (6 H, m), 3.75 (6 H, s), 4.12–4.17 (1 H, m), 4.71–4.77 (1 H, m), 5.81 (0.5 H, s), 6.13 (1 H, t, J 5.8), 6.91 (4 H, d, J 8.9), 7.18 (1 H, d, J 7.5), 7.23–7.44 (8.5 H, m), 7.52 (2 H, t, J 7.3), 7.63 (1 H, t, J 7.4), 8.01 (2 H, d, J 7.2), 8.22 (1 H, d, J 7.5), 11.30 (1 H, br, ex).

$\delta_{\text{P}}[(\text{CD}_3)_{2}\text{SO}]$: 1.17 ($^{1}J_{\text{P-H}}$ 585.70).

**Triethylammonium 2'-deoxy-6-O-(2,5-dichlorophenyl)-5'-O-(4,4'-dimethoxytrityl)-2-N-isobutyrylguanosine 3'-H-phosphonate (383)**

![Chemical structure of 383](image)

Ammonium 4-methylphenyl $H$-phosphonate (8.47 g, 44.78 mmol) and triethylamine (12.5 ml, 89.97 mmol) were co-evaporated with dry pyridine (20 ml). 2'-Deoxy-6-O-(2,5-dichlorophenyl)-5'-O-(4,4'-dimethoxytrityl)-2-N-isobutyrylguanosine (11.770 g, 15.00 mmol) was added to the residue and co-evaporated with dry pyridine (2×20 ml). The residue was redissolved in dry pyridine (90 ml) and cooled to $-20^\circ$C (IMS-dry ice bath). Pivaloyl chloride (5.51 ml, 44.75 mmol) was added to the solution over a period of 5 min and the reaction mixture was stirred at the same temperature for 60 min before it was quenched with water (20 ml). The mixture was then allowed to warm up to room temperature over a period of 1 h. The resulting clear solution was partitioned between dichloromethane (450 ml) and saturated aqueous sodium hydrogen carbonate (300 ml). The layers were separated and the aqueous layer was back extracted with dichloromethane (2×40 ml). The combined organic layers were washed with triethylammonium phosphate buffer (0.5 $M$, pH 7.0, 2×100 ml). The
layers were separated, and the aqueous layer was back extracted with dichloromethane (2×50 ml). The combined organic layers were dried (MgSO₄) and concentrated to a volume of ca. 100 ml. Toluene (100 ml) was added to the residue. The mixture was then subjected to short column chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (95:5 v/v) gave the title compound as a colourless glass (13.218 g, 92.8%).

δₚ[(CD₃)₂SO]: 1.07.

**Levulinic anhydride (703)**

Levulinic acid (11.60 g, 0.10 mol) and N,N'-dicyclohexylcarbodi-imide (10.30 g, 0.05 mol) were stirred in dry diethyl ether (100 ml) for 1.5 h at room temperature. Then the reaction mixture was filtered and the residue was washed with diethyl ether (2×20 ml). The filtrate and washings were combined and concentrated under reduced pressure to give the title compound as a pale yellow oil (11.0 g, ca. 100%) which solidified upon storing at -4°C.

δₜ[CDCl₃]: 2.71–2.79 (4 H, m), 2.63–2.68 (4 H, m), 2.13 (6 H, s).

νₘₚ (Nujol) 1717, 1747, 1820 cm⁻¹.
3'-O-Levulinyl-4-O-phenylthymidine (385)

To a solution of 5'-O-dimethoxytrityl-4-O-phenylthymidine (3.16 g, 5.09 mmol), triethylamine (1.75 ml, 12.56 mmol) and 4-dimethylaminopyridine (0.09 g, 0.74 mmol) in anhydrous dichloromethane (25 ml) was added levulinic anhydride (2.14 g, 10 mmol) and stirring was continued for 1 h at room temperature. Then the reaction mixture was poured into saturated aqueous sodium hydrogen carbonate (30 ml). The layers were separated and the aqueous layer was back extracted with dichloromethane (3×20 ml). The combined organic layers were dried (MgSO₄) and evaporated to dryness under reduced pressure. The residue was then dissolved in dichloromethane (50 ml). Pyrrole (3.2 ml, 46.12 mmol) followed by dichloroacetic acid (2.5 ml, 30.30 mmol) was added. After 15 min, the products were poured into saturated aqueous sodium hydrogen carbonate (30 ml). The organic layer was separated and further washed with saturated aqueous sodium hydrogen carbonate (30 ml). The aqueous layers were back extracted with dichloromethane (4×30 ml). The dried (MgSO₄) combined organic layers were evaporated to dryness under reduced pressure. The residue was triturated with ethyl acetate (20 ml). The precipitate obtained was collected by filtration and washed with ethyl acetate (3 ml) to give the title compound as colourless crystals (1.74 g, 82.1%).

δH[(CD3)2SO]: 2.09 (3 H, d, J 0.7), 2.12 (3 H, s), 2.18–2.29 (1 H, m), 2.34–2.40 (1 H, m), 2.74 (2 H, t, J 6.5), 3.61–3.72 (2 H, m), 4.06 (1 H, m), 5.22 (1 H, t), 5.28 (1 H, t, ex, J 5.2), 6.13–6.16 (1 H, m), 7.18–7.20 (2 H, m), 7.26–7.32 (1 H, m), 7.43–7.48 (2 H, m), 8.19 (1 H, d, J 0.9).
\[ \delta_{13C}[(CD_3)_2SO]: 12.08 (CH_3), 27.69 (CH_2), 29.51 (CH_3), 37.40 (CH_2), 37.77 (CH_2), 61.12 (CH_2), 74.68 (CH), 85.20 (CH), 85.72 (CH), 103.32 (C), 122.01 (CH), 125.75 (CH), 129.52 (CH), 142.25 (CH), 151.84 (C), 154.26 (C), 169.94 (C), 172.01 (C), 206.88 (C). \]

**HO-Tp(s)T-Lev (387)**

![Chemical Structure](image)

Triethylammonium 5'-O-dimethoxytrityl-4-O-phenylthymidine 3'-H-phosphonate (1.890 g, 2.40 mmol) and 3'-O-levulinyl-4-O-phenylthymidine (0.833 g, 2.00 mmol) were co-evaporated with anhydrous pyridine (2×8 ml) and then re-dissolved in dry pyridine (30 ml). After the mixture had been cooled to −40°C (IMS–dry ice bath), a solution of bis(2-chlorophenyl) phosphorochloridate (1.15 ml, 5.0 mmol) in dry dichloromethane (1.15 ml) was added dropwise to the stirred solution during a period of 5 min. The mixture was kept at the same temperature for 15 min and 4-[(2-cyanoethyl)sulphanyl]morpholine-3,5-dione (1.20 g, 6.00 mmol) was added. After 15 min, pyridine–water (2 ml, 1:1 v/v) was added and the resulting mixture was stirred at the same temperature for a further 5 min. The reaction mixture was then poured into triethylammonium phosphate buffer (0.5 M, pH 7.00, 100 ml) and extracted with dichloromethane (2×50 ml). The combined organic layers were further washed with saturated aqueous sodium hydrogen carbonate (3×50 ml). The dichloromethane layer was dried (MgSO₄) and concentrated under reduced pressure. The residue was then co-evaporated with toluene (2×15 ml) and redissolved in dry dichloromethane (30 ml). To the cooled [−50°C (IMS–dry ice bath)], stirred solution, a solution of hydrogen chloride in dioxane (5.0 ml, 4 M) was added during 1 min. After 10 min, a
mixture of methanol and pyridine (4 ml, 1:1 v/v) was added and the resulting mixture was maintained at the same temperature for a further 5 min. The reactants were then partitioned between dichloromethane (50 ml) and saturated aqueous sodium hydrogen carbonate (40 ml). The layers were separated and the organic layer was further washed with saturated aqueous sodium hydrogen carbonate (2x20 ml). The aqueous layers were back-extracted with dichloromethane (2x20 ml). The combined dichloromethane layers were dried (MgSO₄) and evaporated under reduced pressure. The residue was then purified by short column chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (96:4 v/v) gave the title compound as a colourless glass (1.620 g, 93.0%).

δp[(CD₃)₂SO]: 27.80, 27.86.

δH[(CD₃)₂SO]: 2.09–2.12 (9 H, m), 2.29–2.43 (3 H, m), 2.50–2.53 (3 H, m), 2.60 (1 H, m), 2.74 (2 H, m), 2.96 (2 H, m), 3.11–3.21 (2 H, m), 3.63–3.71 (2 H, m), 4.22 (1 H, m), 4.28 (1 H, s), 4.35 (2 H, m), 5.12 (1 H, s), 5.23 (1 H, m), 5.32 (1 H, m, ex), 6.15 (2 H, m), 7.16–7.19 (4 H, m), 7.27–7.32 (2 H, m), 7.41–7.46 (4 H, m), 8.01 (1 H, d, J 5.5), 8.15 (1 H, d, J 4.7).

Rt (programme III): 12.22 min.

**DMTr-Cp(s)Tp(s)T-Lev (396)**

HO-Tp(s)T-Lev (4.330 g, 5.00 mmol) and triethylammonium 5’-O-dimethoxytrityl-4-N-benzoyl-2’-deoxycytidine 3’-H-phosphonate (4.790 g, 6.00 mmol) were co-evaporated with dry pyridine (2x15 ml). The residue was then taken up with anhydrous pyridine (75 ml) and cooled to −40°C (IMS–dry ice bath). A solution of bis(2-chlorophenyl) phosphorochloridate (2.88 ml, 12.50 mmol) in dichloromethane (2.88 ml) was added over a period of 5 min. After 15 min, 4-[(2-cyanoethyl)sulphanyl]morpholine-3,5-dione (3.00 g, 15.00 mmol) was added. The reaction mixture was kept at the same temperature for 15 min, and then quenched with pyridine–water (5 ml, 1:1 v/v). After 5 min, it was partitioned between triethylammonium phosphate buffer (0.5 M, pH 7.00, 200 ml) and dichloromethane
The layers were separated and the aqueous layer was back extracted with dichloromethane (150 ml). The combined organic layers were then washed with saturated aqueous sodium hydrogen carbonate (3x100 ml). The dried (MgSO₄) combined organic layers were concentrated under reduced pressure. The residue was then purified by chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane-methanol (96:4 v/v) gave the title compound as a colourless foam (7.75 g, 95%).

δ[D(3)2SO]: 27.97, 28.02, 28.07, 28.16, 28.32, 28.34.

Rt (Programme III): 17.57, 17.82 min.

**DMTr-Cp(s)Tp(s)T-OH (397)**

To a cooled (ice-water bath), solution of DMTr-Cp(s)Tp(s)T-Lev (4.89 g, 3.00 mmol) in pyridine (30.0 ml), a mixture of pyridine (60.0 ml), acetic acid (15.0 ml), hydrazine hydrate (0.93 ml, 30.0 mmol) and water (3.0 ml) was added. The reactants were stirred at the same temperature for 10 min. Then 2,4-pentanedione (4.4 ml) was added. The products were stirred for 15 min and then partitioned between water (200 ml) and dichloromethane (200 ml) with care. The two layers were separated and the organic layer was further washed with saturated aqueous sodium hydrogen carbonate (3x100 ml), dried (MgSO₄) and evaporated to dryness under reduced pressure. The residue was then dissolved in dichloromethane (10 ml) and added dropwise to diethyl ether (300 ml) with stirring. After 30 min, the title compound was collected by filtration as a colourless solid (4.40 g, 95.6%).

δ[D(3)2SO]: 27.99, 28.04, 28.10, 28.18, 28.33, 28.35.

Rt (programme III): 16.66, 16.96 min.
HO-Cp(s)Tp(s)T-Lev (395)

HO-Tp(s)T-Lev (2.600 g, 3.00 mmol) and triethylammonium 5'-O-dimethoxytrityl-4-N-benzoyl-2'-deoxycytidine 3'-H-phosphonate (2.870 g, 3.60 mmol) were co-evaporated with dry pyridine (2x10 ml). The residue was redissolved in anhydrous pyridine (45 ml) and cooled to -40°C (IMS-dry ice bath). A solution of bis(2-chlorophenyl) phosphorochloridate (1.73 ml, 7.50 mmol) in dichloromethane (1.73 ml) was added to the reactants during a period of 5 min. After 15 min, 4-[(2-cyanoethyl)sulphanyl]morpholine-3,5-dione (1.800 g, 9.00 mmol) was added. The reaction mixture was stirred at the same temperature for 15 min, and then quenched with pyridine-water (3 ml, 1:1 v/v). After 5 min, it was partitioned between triethylammonium phosphate buffer (0.5 M, pH 7.00, 200 ml) and dichloromethane (2x100 ml). The two layers were separated and the organic layer was further washed with saturated aqueous sodium hydrogen carbonate (3x80 ml). The dried (MgSO₄) organic layer was concentrated under reduced pressure. The residue was co-evaporated with toluene (2x20 ml) and dissolved in dichloromethane (40 ml). After this solution had been cooled to -50°C (IMS-dry ice bath), a solution of hydrogen chloride in dioxane (4 M, 7.5 ml) was added during 1 min. After 10 min, pyridine-methanol (4 ml, 1:1 v/v) was added and the reaction mixture was maintained at the same temperature for 5 min. Then it was poured into saturated aqueous sodium hydrogen carbonate (100 ml). The layers were separated and the organic layer further washed with saturated aqueous sodium hydrogen carbonate (3x100 ml). The dried (MgSO₄) organic layer was concentrated under reduced pressure. The residue was then purified by short column chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (95:5 v/v) gave the title compound as a colourless foam (3.68 g, 92.3%).

δₚ[(CD₃)₂SO]: 27.91, 27.96, 28.15, 28.33.

Rt (Programme III): 12.63 min.

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DMTr-Cp(s)Tp(s)Tp(H) (398)

Ammonium 4-methylphenyl H-phosphonate (1.630 g, 8.61 mmol) was co-evaporated with methanol (15 ml) and triethylamine (2.40 ml, 17.2 mmol) under reduced pressure. DMTr-Cp(s)Tp(s)T-OH (4.400 g, 2.87 mmol) was added to the residue and co-evaporated with dry pyridine (2x10 ml). The residue was then redissolved in anhydrous pyridine (50 ml) and cooled to −40°C (IMS–dry ice bath). Pivaloyl chloride (1.06 ml, 8.61 mmol) was added over a period of 5 min and the mixture was maintained at the same temperature for 1 h. Water (20 ml) was added and the reactants were allowed to warm up to room temperature over a period of 1 h. They were then partitioned between triethylammonium phosphate buffer (0.5 M, pH 7.00, 200 ml) and dichloromethane (200 ml). The layers were separated, and the organic layer was further washed with the same buffer (3x100 ml). The dichloromethane layer was dried (MgSO₄) and evaporated under reduced pressure. Toluene (100 ml) was added to the residue and the mixture was purified by short column chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (80:20 v/v) gave a colourless glass. This material was dissolved in dichloromethane (45 ml) and washed with triethylammonium phosphate buffer (0.5 M, pH 7.00, 35 ml). The layers were separated and the dried (MgSO₄) organic layer was concentrated under reduced pressure to give the title compound as a colourless froth (4.50 g, 92.4%).

δ[(CD₃)₂SO]: −0.16 (1 P), 27.95, 28.02, 28.04, 28.08, 28.25, 28.30 (2 P).

Rt (Programme III): 10.50, 10.62 min.

DMTr-Cp(s)Tp(s)Tp(s)Cp(s)Tp(s)T-Lev (3105)

DMTr-Cp(s)Tp(s)Tp(H) (3.000 g, 1.77 mmol) and HO-Cp(s)Tp(s)T-Lev (1.960 g, 1.47 mmol) were co-evaporated with dry pyridine (2x5 ml) and the residue was taken up with dry pyridine (20 ml). After the solution had been cooled to −40°C (IMS–dry ice), a solution of bis(2-chlorophenyl) phosphorochloridate in dichloromethane (2.1 ml, 1:1 v/v, 4.41 mmol) was added over a period of 5 min. After 15 min, 4-[(2-
cyanoethyl)sulphanyl)morpholine-3,5-dione (0.880 g, 4.41 mmol) was added and the reactants were maintained at the same temperature for another 15 min. Pyridine–water (2 ml, 1:1 v/v) was added and the resulting reaction mixture was stirred at the same temperature for 5 min. The reactants were then partitioned between saturated aqueous sodium hydrogen carbonate (100 ml) and dichloromethane (100 ml). The two layers were separated and the organic layer was further washed with saturated aqueous sodium hydrogen carbonate (3×50 ml). The dried (MgSO₄) organic layer was concentrated under reduced pressure. The residue was purified by short column chromatography on silica gel. The appropriate fractions, which were eluted with methanol–dichloromethane (5:95–6:94 v/v) were combined and evaporated to give the title compound as a colourless glass (4.00 g, 90.8%).

δ_p[(CD₃)₂SO]: 27.96, 28.06, 28.15, 28.31, 28.37.

Rt (Programme III): 20.51, 20.95 min.

DMTr-Cp(s)Tp(s)Tp(s)Cp(s)Tp(s)T-OH (3106)

To a solution of DMTr-Cp(s)Tp(s)Tp(s)Cp(s)Tp(s)T-Lev (3.890 g, 1.30 mmol) in pyridine (13 ml) cooled to 0°C (ice–water bath), a mixture of pyridine (26 ml), acetic acid (6.5 ml), water (1 ml) and hydrazine hydrate (0.42 ml, 13 mmol) was added. After 15 min, 2,4-pentanedione (1.9 ml) was added and the reaction mixture was stirred for a further 20 min. Then it was partitioned between dichloromethane (100 ml) and saturated aqueous sodium hydrogen carbonate (100 ml) with care. The layers were separated and the organic layer was further washed with saturated aqueous sodium hydrogen carbonate (3×100 ml), dried (MgSO₄) and concentrated under reduced pressure to dryness. The residue was dissolved in dichloromethane (5 ml) and added dropwise to diethyl ether (150 ml) with stirring. The title compound was collected as a solid (3.76 g, 92.0%) by filtration.

δ_p[(CD₃)₂SO]: 27.952, 28.050, 28.126, 28.305.

Rt (Programme III): 19.34, 19.70 min.
DMTr-Cp(s)Tp(s)Tp(s)Cp(s)Tp(s)Tp(s)Tp(H) (3107)

Ammonium 4-methylphenyl $H$-phosphonate (0.670 g, 3.52 mmol) was co-evaporated with methanol (5 ml) and triethylamine (7 ml) under reduced pressure. DMTr-Cp(s)Tp(s)Tp(s)Cp(s)Tp(s)Tp(s)T-OH (3.400 g, 1.17 mmol) was added to the residue and co-evaporated with dry pyridine (2×5 ml). The residue was then taken up with pyridine (15 ml) and cooled to $-35^\circ$C (IMS–dry ice bath). Pivaloyl chloride (0.43 ml, 3.52 mmol) was added to the cooled solution over a period of 5 min. After 1 h, water (5 ml) was added and the mixture was allowed to warm up to room temperature over a period of 1 h. Then the reactants were partitioned between dichloromethane (100 ml) and triethylammonium phosphate buffer (0.5 $M$, pH 7.00, 100 ml). The layers were separated and the organic layer was further washed with triethylammonium phosphate buffer (0.5 $M$, pH 7.00, 3×100 ml). To the dried (MgSO$_4$) solution, toluene (100 ml) was added and the resulting mixture was purified by short column chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with methanol–dichloromethane (20:80 v/v), gave an off-white glass. It was then dissolved in dichloromethane (50 ml) and washed with triethylammonium phosphate buffer (0.5 $M$, pH 7.00, 15 ml) and dried (MgSO$_4$). Solvent was removed under reduced pressure to give the title compound as a colourless froth (3.250 g, 90.8%).

$\delta_p[(\mathrm{CD}_3)_2\mathrm{SO}]: 0.969$ (1 P), 27.959, 28.053, 28.223, 28.279 (5 P).

HO-Cp(s)G-Lev (386)

\[ \text{HO-Cp(s)G-Lev (386)} \]
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DMTr-Cp(H) (4.601 g, 5.76 mmol) and HO-G-Lev (2.786 g, 4.80 mmol) were co-evaporated with dry pyridine (2×5 ml) and the residue redissolved in anhydrous pyridine (60 ml). After this solution had been cooled to −50°C (IMS—dry ice bath), a solution of bis(2-chlorophenyl) phosphorochloridate in dichloromethane (6.86 ml, 1:1 v/v, 14.4 mmol) was added over a period of 5 min. The reactants were stirred for 15 min followed by addition of 4-[(2-cyanoethyl)sulphanyl]morpholine-3,5-dione (1.920 g, 9.6 mmol). After 15 min, pyridine–water (3 ml, 1:1 v/v) was added and the resulting mixture was maintained at the same temperature for 5 min. The clear solution was then partitioned between dichloromethane (250 ml) and saturated aqueous sodium hydrogen carbonate (250 ml). The organic layer was separated, dried (MgSO₄) and concentrated under reduced pressure. The residue was co-evaporated with toluene (3×50 ml) and dissolved in dichloromethane (150 ml). After this solution had been cooled to −50°C (IMS—dry ice bath), a solution of hydrogen chloride in dioxane (12.0 ml, 4 M) was added during 1 min. The reactants were kept at the same temperature for 15 min, and pyridine–methanol (10 ml, 1:1 v/v) was added. After 5 min, the products were poured into saturated aqueous sodium hydrogen carbonate (200 ml). The layers were separated and the organic layer was washed with saturated aqueous sodium hydrogen carbonate (2×100 ml). The dried (MgSO₄) organic layer was concentrated under reduced pressure and the residue was purified by short column chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (97:3 v/v) gave the title compound as a colourless froth (4.995 g, 92.8%).

δ_p[(CD₃)₂SO]: 27.23, 27.40, 27.49.

Rt (programme III): 12.84 min.

HO-Gp(s)Cp(s)G-Lev (389)

Triethylammonium 2'-deoxy-6-O-(2,5-dichlorophenyl)-5'-O-(4,4'-dimethoxytrityl)-2-N-isobutryrylguanosine 3'-H-phosphonate (2.895 g, 3.05 mmol) and HO-Cp(s)G-Lev (2.650 g, 2.54 mmol) were co-evaporated with dry pyridine (2×5 ml) and the residue was redissolved in anhydrous pyridine (30 ml). The solution was cooled to
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-40°C (IMS–dry ice bath) followed by addition of a solution of bis(2-chlorophenyl) phosphorochloridate in dichloromethane (3.63 ml, 1:1 v/v, 7.62 mmol) over a period of 5 min. After 15 min, 4-[(2-cyanoethyl)sulphanyl]morpholine-3,5-dione (1.520 g, 7.62 mmol) was added and stirring was continued for 15 min at the same temperature. Then pyridine–water (2 ml, 1:1 v/v) was added and the reaction mixture was stirred for 5 min. The resulting clear solution was partitioned between dichloromethane (120 ml) and saturated aqueous sodium hydrogen carbonate (120 ml). The organic layer was separated, dried (MgSO₄) and concentrated under reduced pressure. The residue was co-evaporated with toluene (3×30 ml) and dissolved in dichloromethane (60 ml). After this solution had been cooled to -50°C (IMS–dry ice bath), a solution of hydrogen chloride in dioxane (6.35 ml, 4 M) was added during 1 min. After the reaction mixture had been stirred at the same temperature for 15 min, pyridine–methanol (2 ml, 1:1 v/v) was added. After 5 min, the products were poured into saturated aqueous sodium hydrogen carbonate (100 ml). The two layers were separated and the organic layer was further washed with saturated aqueous sodium hydrogen carbonate (2×100 ml). The dried (MgSO₄) organic layer was evaporated under reduced pressure. The residue was purified by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane – methanol (97:3 v/v), were concentrated under reduced pressure to give the title compound as a colourless glass (4.207 g, 91.0%).

δₚ[(CD₃)₂SO]: 27.70, 27.77, 27.84, 27.98.

Rt (programme III): 12.43 min.

**HO-Cp(s)Tp(s)Tp(s)Gp(s)Cp(s)G-Lev (3108)**

DMTr-Cp(s)Tp(s)Tp(H) (2.600 g, 1.53 mmol) and HO-Gp(s)Cp(s)G-Lev (2.110 g, 1.280 mmol) were co-evaporated with dry pyridine (2×2 ml). The residue was redissolved in anhydrous pyridine (15 ml) and cooled to -40°C (IMS–dry ice bath). A solution of bis(2-chlorophenyl) phosphorochloridate in dichloromethane (1.83 ml, 1:1 v/v, 3.840 mmol) was then added over a period of 5 min. After 15 min, 4-[(2-cyanoethyl)sulphanyl]morpholine-3,5-dione (0.770 g, 3.84 mmol) was added. The
resulting mixture was maintained at the same temperature for 15 min, followed by addition of pyridine–water (2 ml, 1:1 v/v). The reactants were stirred for 5 min and then partitioned between dichloromethane (100 ml) and saturated aqueous sodium hydrogen carbonate (100 ml). The layers were separated and the organic layer was dried (MgSO₄) and concentrated under reduced pressure. The residue was co-evaporated with toluene (3×30 ml) and then dissolved in dichloromethane (30 ml). After this solution had been cooled to −50°C (IMS–dry ice bath), a solution of hydrogen chloride in dioxane (3.2 ml, 4 M) was added over 1 min. The resulting solution was maintained at the same temperature for a period of 15 min and then pyridine–methanol (3 ml, 1:1 v/v) was added. The reactants were stirred for 5 min and then poured into saturated aqueous sodium hydrogen carbonate (80 ml). The layers were separated and the organic layer was washed with saturated aqueous sodium hydrogen carbonate (2×50 ml). The dried (MgSO₄) organic layer was concentrated under reduced pressure. The residue was then purified by short column chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (95:5 v/v) gave the title compound as a colourless glass (3.420 g, 88.5%).

δp[(CD₃)₂SO]: 27.11, 27.15, 27.23, 27.29, 27.33, 27.38, 27.50, 27.61, 27.69.

Rt (Programme III): 16.15.

HO-Cp(s)Tp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Gp(s)Cp(s)G-Lev (HO-12-Lev) (3109)

DMTr-Cp(s)Tp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(H) (2.290 g, 0.749 mmol) and HO-Cp(s)Tp(s)Tp(s)Cp(s)Tp(s)Cp(s)Gp(s)Cp(s)G-Lev (1.880 g, 0.624 mmol) were co-evaporated with dry pyridine (2×2 ml) and the residue was redissolved in anhydrous pyridine (20 ml). After this solution had been cooled to −40°C (IMS–dry ice bath), a solution of bis(2-chlorophenyl) phosphorochloridate in dichloromethane (1.10 ml, 1:1 v/v, 2.25 mmol) was added over a period of 5 min. The resulting solution was stirred at the same temperature for 15 min and then 4-[(2-cyanoethyl)sulphanyl]morpholine-3,5-dione (0.450 g, 2.25 mmol) was added. After the reactants had been maintained at the same
temperature for 15 min, pyridine–water (2 ml, 1:1 v/v) was added. After 5 min, the reaction mixture was partitioned between dichloromethane (100 ml) and saturated aqueous sodium hydrogen carbonate (100 ml). The layers were separated and the organic layer was dried (MgSO₄) and concentrated under reduced pressure. The residue was co-evaporated with toluene (3×40 ml) and dissolved in dichloromethane (50 ml). After it had been cooled to −50°C (IMS–dry ice bath), a solution of hydrogen chloride in dioxane (4 M, 1.60 ml) was added during 1 min. The resulting solution was maintained at the same temperature for a further period of 15 min before pyridine–methanol (2 ml, 1:1 v/v) was added. After 5 min, the products were poured into saturated aqueous sodium hydrogen carbonate (80 ml). The layers were separated and the dichloromethane layer was further washed with saturated aqueous sodium hydrogen carbonate (2×50 ml). The dried (MgSO₄) organic layer was concentrated under reduced pressure and the residue was purified by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (93:7 v/v) were concentrated under reduced pressure to give the title compound as a colourless froth (2.990 g, 83.5%).

δ_p[(CD₃)₂SO]: 27.79, 27.92, 27.99, 28.06, 28.13, 28.33.

Rt (programme III): 17.47 min.

HO-Cp(s)T-Lev (388)
5'-O-Dimethoxytrityl-4-N-benzoyl-2'-deoxycytidine 3'-H-phosphonate (2.160 g, 2.70 mmol) and 3'-O-levulinyl-4-O-phenylthymidine (0.938 g, 2.25 mmol) were co-evaporated with anhydrous pyridine (2x5 ml). The residue was redissolved in dry pyridine (30 ml) and cooled down to -40°C (IMS-dry ice bath). A solution of bis(2-chlorophenyl) phosphorochloridate in dichloromethane (3.20 ml, 1:1 v/v, 6.75 mmol) was added over a period of 5 min, followed after 15 min by addition of 4-[(2-cyanoethyl)sulphanyl]morpholine-3,5-dione (1.350 g, 6.75 mmol). After 15 min, pyridine–water (2 ml, 1:1 v/v) was added. The reaction mixture was maintained at the same temperature for 5 min and then partitioned between dichloromethane (100 ml) and saturated aqueous sodium hydrogen carbonate (100 ml). The layers were separated and the organic layer was washed with saturated aqueous sodium hydrogen carbonate (3x50 ml). The dried (MgSO₄) organic layer was concentrated under reduced pressure. The residue was co-evaporated with toluene (2x10 ml), dissolved in dichloromethane (30 ml) and cooled to -50°C (IMS–dry ice bath). A solution of hydrogen chloride in dioxane (4 M, 5.63 ml) was added. After a period of 15 min, pyridine–methanol (3 ml, 1:1 v/v) was added. The reactants were stirred at the same temperature for 5 more min and then partitioned between saturated aqueous sodium hydrogen carbonate (100 ml) and dichloromethane (100 ml). The layers were separated and the organic layer was further washed with saturated aqueous sodium hydrogen carbonate (3x50 ml). The dried (MgSO₄) dichloromethane layer was evaporated under reduced pressure. The residue was purified by short column chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (97:3 v/v) gave the title compound as a colourless froth (1.860 g, 94.0%).

δH[(CD₃)₂SO]: 2.08 (6 H, m), 2.30 (3 H, m), 2.70 (3 H, m), 2.95 (2 H, m), 3.15 (2 H, m), 3.64 (2 H, br), 4.25 (2 H, m), 4.40 (2 H, m), 5.15 (1 H, m), 5.25 (2 H, m, including 1 ex H), 6.2 (2 H, m), 7.15 (2 H, d, J 8.2), 7.25–7.55 (7 H, m), 7.61 (1 H, t, J 7.38), 7.97 (3 H, m), 8.32 (1 H, br), 11.28 (1 H, s, ex).

δp[(CD₃)₂SO]: 27.10, 27.17.
DMTr-\textit{Gp}(s)\textit{Cp}(s)T-\text{Lev} (399)

DMTr-\textit{Gp}(H) (2.300 g, 2.400 mmol) and HO-\textit{Cp}(s)T-\text{Lev} (1.760 g, 2.0 mmol) were co-evaporated with dry pyridine (2\times2 ml) and the residue was redissolved in pyridine (25 ml). After this solution had been cooled to \(-40^\circ\text{C}\) (IMS–dry ice bath), a solution of bis(2-chlorophenyl) phosphorochloridate in dichloromethane (2.85 ml, 1:1 v/v, 6.00 mmol) was added over a period of 5 min. The resulting solution was maintained at the same temperature for 15 min and then 4-[(2-cyanoethyl)sulphanyl]morpholine-3,5-dione (1.200 g, 6.00 mol) was added. After 15 min, pyridine–water (2 ml, 1:1 v/v) was added and the reactants were stirred at the same temperature for 5 min and then partitioned between dichloromethane (100 ml) and saturated aqueous sodium hydrogen carbonate (100 ml). The layers were separated and the organic layer was washed with saturated aqueous sodium hydrogen carbonate (2\times50 ml). The dried (MgSO\(_4\)) organic layer was concentrated under reduced pressure. The residue was purified by chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (96:4 v/v) gave the title compound as a colourless froth (3.440 g, 96.0%).

\(\delta_p[(\text{CD}_3)_2\text{SO}]: 27.73, 28.20, 28.31.\)

Rt (Programme III): 19.08, 19.41 min.

\textbf{DMTr-\textit{Gp}(s)\textit{Cp}(s)T-OH} (3100)

To a cooled (ice–water bath) solution of DMTr-\textit{Gp}(s)\textit{Cp}(s)T-\text{Lev} (2.790 g, 1.55 mmol) in pyridine (16 ml), a mixture of pyridine (32 ml), acetic acid (8 ml), water (1.5 ml) and hydrazine hydrate (0.50 ml, 15.5 mmol) was added. After 15 min, 2,4-pentanedione (2.3 ml) was added and the resulting mixture was allowed to warm up to room temperature over a period of 15 min. The products were then poured into saturated aqueous sodium hydrogen carbonate (100 ml) with care and extracted with dichloromethane (150 ml). The layers were separated and the organic layer was washed with saturated aqueous sodium hydrogen carbonate (3\times100 ml). The dried (MgSO\(_4\)) organic layer was evaporated under reduced pressure. The residue was
dissolved in dichloromethane (4 ml) and added dropwise to diethyl ether (150 ml) with stirring. The precipitate obtained was then collected by filtration and dried in vacuo to give the title compound as a colourless solid (2.420 g, 92.6%).

\[ \delta_p[(CD_3)_2SO] : 28.32, 28.18, 27.76, 27.70. \]

Rt (Programme III): 18.09 min.

**DMTr-Gp(s)Cp(s)Tp(H) (3101)**

Ammonium 4-methylphenyl H-phosphonate (0.720 g, 3.80 mmol), methanol (5 ml) and triethylamine (1.06 ml, 7.60 mmol) were co-evaporated under reduced pressure. DMTr-Gp(s)Cp(s)T-OH (2.150 g, 1.26 mmol) was added to the residue and the mixture was co-evaporated with dry pyridine (2x5 ml). The residue was re-dissolved in anhydrous pyridine (15 ml) and cooled to \(-30^\circ C\) (IMS–dry ice bath). Pivaloyl chloride (0.47 ml, 3.80 mmol) was added during 5 min. The resulting reaction mixture was then maintained at the same temperature for 1 h. Water (5 ml) was added and the reactants were allowed to warm up to room temperature over a period of 1 h. The clear solution was then partitioned between dichloromethane (100 ml) and triethylammonium phosphate buffer (0.5 \(M\), pH 7.00, 80 ml). The layers were separated and the organic layer was washed with triethylammonium phosphate buffer (0.5 \(M\), pH 7.00, 3x80 ml). The dried (MgSO\(_4\)) organic layer was mixed with toluene (50 ml) and the resulting mixture was purified by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (80:20 \(v/v\)) were concentrated under reduced pressure and the residue was re-dissolved in dichloromethane (50 ml) and washed with triethylammonium phosphate buffer (0.5 \(M\), pH 7.00, 15 ml). The layers were separated, and the dried (MgSO\(_4\)) organic layer was concentrated under reduced pressure to give DMTr-Gp(s)Cp(s)Tp(H) as a colourless froth (2.100 g, 89.5%).

\[ \delta_p[(CD_3)_2SO] : 1.45 (1 P, J_{P-H} 578.9), 27.52, 27.57, 27.67, 27.92, 28.09 (2 P). \]

Rt (Programme III): 10.43 min.

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HO-Gp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Gp(s)Cp(s)G-Lev (HO-15-Lev, 3110)

DMTr-Gp(s)Cp(s)Tp(s)H (0.932 g, 0.501 mmol) and HO-Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Gp(s)Cp(s)G-Lev (HO-12-Lev, 2.300 g, 0.400 mmol) were co-evaporated with dry pyridine (2x3 ml) and the residue was redissolved in anhydrous pyridine (15 ml). After this solution had been cooled to -40°C (IMS–dry ice bath), a solution of bis(2-chlorophenyl) phosphorochloridate in dichloromethane (0.57 ml, 1:1 v/v, 1.20 mmol) was added over a period of 5 min. After 15 min, 4-[(2-cyanoethyl)sulphonyl]morpholine-3,5-dione (0.240 g, 1.20 mmol) was added and the resulting mixture was stirred at the same temperature for a further period of 15 min. Pyridine–water (2 ml, 1:1 v/v) was then added. After 5 min, the products were partitioned between dichloromethane (80 ml) and saturated aqueous sodium hydrogen carbonate (80 ml). The layers were separated and the dried (MgSO₄) organic layer was concentrated under reduced pressure. The residue was co-evaporated with toluene (3x30 ml) and then dissolved in dichloromethane (40 ml). After this solution had been cooled to -50°C (IMS–dry ice bath), a solution of hydrogen chloride in dioxane (4 M, 1.0 ml) was added during 1 min. The reactants were kept at the same temperature for a period of 20 min followed by addition of pyridine–methanol (2 ml, 1:1 v/v). After 5 min, the reaction mixture was poured into saturated aqueous sodium hydrogen carbonate (60 ml). The organic layer was separated and further washed with saturated aqueous sodium hydrogen carbonate (2x50 ml). The dried (MgSO₄) organic layer was concentrated under reduced pressure and the residue was purified by short column chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (92:8 v/v) gave the title compound as a colourless froth (2.570 g, 88.5%).

δp[(CD₃)₂SO]: 27.79, 27.89, 28.05, 28.36.

DMTr-Tp(s)Tp(s)T-Lev (3102)

Triethylammonium 5'-O-dimethoxytrityl-4-O-phenylthymidine 3'-H-phosphonate (1.318 g, 1.680 mmol) and HO-Tp(s)T-Lev (1.210 g, 1.33 mmol) were co-evaporated
with dry pyridine (2×3 ml) and the residue was redissolved in anhydrous pyridine (20 ml) and cooled to -40°C (IMS–dry ice bath). A solution of bis(2-chlorophenyl) phosphorochloridate in dichloromethane (1.98 ml, 1:1 v/v, 4.20 mmol) was added over a period of 5 min. After 15 min, 4-[(2-cyanoethyl)sulphanyl]morpholine-3,5-dione (0.840 g, 4.20 mmol) was added and the reactants were kept at the same temperature for 15 min. Pyridine–water (2 ml, 1:1 v/v) was added and after 5 min, the reaction mixture was partitioned between dichloromethane (100 ml) and saturated aqueous sodium hydrogen carbonate (100 ml). The layers were separated. The organic layer was further washed with saturated aqueous sodium hydrogen carbonate (2×100 ml), dried (MgSO₄) and concentrated under reduced pressure. The residue was then purified by short column chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (97:3 v/v) gave the title compound as a colourless glass (1.971 g, 91.6%).

δₚ[(CD₃)₂SO]: 27.99, 28.03, 28.12, 28.30.

DMTr-Tp(s)Tp(s)T-OH (3103)

DMTr-Tp(s)Tp(s)T-Lev (1.520 g, 1.00 mmol) was dissolved in pyridine (5 ml) and cooled to 0°C (ice–water bath). A solution of hydrazine monohydrate (0.90 ml, 18.55 mmol), acetic acid (9.2 ml, 0.161 mol) and water (2 ml) in pyridine (8.5 ml) was added to the cooled solution. After 10 min, 2,4-pentanedione (5 ml, 48.69 mmol) was added and the resulting mixture was allowed to warm up to room temperature over a period of 15 min. The reactants were then poured into saturated aqueous sodium hydrogen carbonate (90 ml) with care and extracted with dichloromethane (2×60 ml). The combined organic layers were further washed with saturated aqueous sodium hydrogen carbonate (2×100 ml); the aqueous layers were back extracted with dichloromethane (2×50 ml). The combined organic layers were dried (MgSO₄) and concentrated under reduced pressure. The residue was dissolved in dichloromethane (5 ml) and added dropwise to diethyl ether (200 ml) with stirring. After 30 min, the precipitates were collected by filtration and washed with diethyl ether (30 ml) to give the title compound as a colourless solid (2.679 g, 95.1%).
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\[ \delta_{\text{p}}[(\text{CD}_3)_2\text{SO}]: 27.99, 28.04, 28.12, 28.28. \]

**DMTr-\text{Tp}(\text{s})\text{Tp}(\text{s})\text{Tp}(\text{H}) (3104)**

Ammonium 4-methylphenyl \( H \)-phosphonate (0.520 g, 2.76 mmol), methanol (3 ml) and triethylamine (0.77 ml, 5.52 mmol) were co-evaporated and to the residue was added DMTr-\text{Tp}(\text{s})\text{Tp}(\text{s})T-OH (1.403 g, 0.920 mmol). The mixture was then co-evaporated with dry pyridine (2×5 ml). The residue was redissolved in anhydrous pyridine (10 ml) and cooled to \(-30^\circ\text{C}\) (IMS–dry ice bath). Pivaloyl chloride (0.34 ml, 2.76 mmol) was added over a period of 5 min. The resulting mixture was stirred for 1 h at the same temperature followed by addition of water (4 ml). The reactants were allowed to warm up to room temperature over a period of 1 h and then partitioned between dichloromethane (70 ml) and triethylammonium phosphate buffer (0.5 \( M \), pH 7.00, 50 ml). The organic layer was separated and further washed with triethylammonium phosphate buffer (0.5 \( M \), pH 7.00, 2×50 ml). The dried \((\text{MgSO}_4)\) organic layer was mixed with toluene and the mixture was purified by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (80:20 v/v) were evaporated under reduced pressure to give a colourless froth. This material was dissolved in dichloromethane (20 ml) and washed with triethylammonium phosphate buffer (0.5 \( M \), pH 7.00, 20 ml). The dichloromethane layer was separated, dried \((\text{MgSO}_4)\) and concentrated under reduced pressure to give the title compound as a colourless glass (1.406 g, 90.7%).

\[ \delta_{\text{p}}[(\text{CD}_3)_2\text{SO}]: 1.52 (1 \text{ P}), 27.97, 28.03, 28.06, 28.13 (2 \text{ P}). \]

**HO-\text{Tp}(\text{s})\text{Tp}(\text{s})\text{Gp}(\text{s})\text{Cp}(\text{s})\text{Tp}(\text{s})\text{Cp}(\text{s})\text{Tp}(\text{s})\text{Cp}(\text{s})\text{Tp}(\text{s})\text{Cp}(\text{s})\text{Tp}(\text{s})\text{Tp}(\text{s})\text{Gp}(\text{s})\text{Cp}(\text{s})\text{G-Lev} (\text{HO-18-Lev}, 3111)**

DMTr-\text{Tp}(\text{s})\text{Tp}(\text{s})\text{Tp}(\text{H}) (0.561 g, 0.33 mmol) and HO-Gp(\text{s})\text{Cp}(\text{s})\text{Tp}(\text{s})\text{Cp}(\text{s})\text{Tp}(\text{s})\text{Cp}(\text{s})\text{Tp}(\text{s})\text{Cp}(\text{s})\text{Tp}(\text{s})\text{Cp}(\text{s})\text{G-Lev} (\text{HO-15-Lev}, 1.860 g, 0.256 mmol) were co-evaporated with dry pyridine (2×2 ml) and the residue redissolved in anhydrous pyridine (10 ml). After this solution had been cooled to \(-40^\circ\text{C}\) (IMS–dry ice bath), a solution of bis(2-chlorophenyl) phosphorochloridate in dichloromethane

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(0.37 ml, 1:1 v/v, 0.768 mmol) was added over a period of 4 min. After 15 min, 4-[(2-cyanoethyl)sulphanyl]morpholine-3,5-dione (0.154 g, 0.768 mmol) was added and the resulting mixture was maintained at the same temperature for 15 min. Water-pyridine (1 ml, 1:1 v/v) was then added. After 5 min, the products were partitioned between dichloromethane (60 ml) and saturated aqueous sodium hydrogen carbonate (60 ml). The organic layer was separated, dried (MgSO₄) and concentrated under reduced pressure. The residue was co-evaporated with toluene (3×20 ml) and dissolved in dichloromethane (30 ml). After this solution had been cooled to -50°C (IMS-dry ice bath), a solution of hydrogen chloride in dioxane (4 M, 0.64 ml) was added during 1 min and the reactants maintained at the same temperature for 15 min. Then pyridine-methanol (2 ml, 1:1 v/v) was added and the resulting solution was stirred for 5 more min. Then it was poured into saturated aqueous sodium hydrogen carbonate (50 ml). The organic layer was separated and further washed with saturated aqueous sodium hydrogen carbonate (2×50 ml), dried (MgSO₄) and concentrated under reduced pressure. The residue was then purified by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane-methanol (92:8 v/v), were evaporated under reduced pressure to give the title compound as a colourless glass (1.900 g, 86.1%).

δp[(CD₃)₂SO]: 27.83, 27.94, 28.08, 28.15, 28.32, 28.38.

DMTr-Gp(s)Cp(s)G-Lev (390)

Triethylammonium 2'-deoxy-6-O-(2,5-dichlorophenyl)-5'-O-(4,4'-dimethoxytrityl)-2-N-isobutyrylguanosine 3'-H-phosphonate (3.651 g, 3.844 mmol) and HO-Cp(s)G-Lev (3.340 g, 3.203 mmol) were co-evaporated with dry pyridine (2×5 ml) and the residue was redissolved in anhydrous pyridine (40 ml). After this solution had been cooled to -40°C (IMS-dry ice bath), a solution of bis(2-chlorophenyl) phosphorochloridate in dichloromethane (4.47 ml, 1:1 v/v, 9.609 mmol) was added over a period of 5 min. After 15 min, 4-[(2-cyanoethyl)sulphanyl]morpholine-3,5-dione (1.924 g, 9.609 mmol) was added and the resulting mixture was stirred for 15 min. Pyridine-water (3 ml, 1:1 v/v) was added and the reactants were kept for 5 more min. Then the products were partitioned between dichloromethane (180 ml) and
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saturated aqueous sodium hydrogen carbonate (180 ml). The layers were separated and the organic layer was further washed with saturated aqueous sodium hydrogen carbonate (2x150 ml). The dried (MgSO₄) organic layer was concentrated under reduced pressure. The residue was purified by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (97:3 v/v) were evaporated under reduced pressure to give the title compound as a colourless froth (5.916 g, 94.3%).

δ_p[(CD₃)₂SO]: 2.82 (1 P), 28.08, 28.19, 28.38, 28.45.

DMTr-Gp(s)Cp(s)G-OH (391)

DMTr-Gp(s)Cp(s)G-Lev (2.300 g, 1.20 mmol) was dissolved in pyridine (6 ml) and cooled to 0°C (ice–water bath). To this solution was added a mixture of pyridine (10 ml), acetic acid (6 ml), hydrazine hydrate (0.62 ml, 12.0 mmol) and water (1.3 ml). After 15 min, 2,4-pentanedione (4.3 ml) was added and the resulting solution was allowed to warm up to room temperature over a period of 15 min. The products were then partitioned between dichloromethane (100 ml) and saturated aqueous sodium hydrogen carbonate (100 ml). The aqueous layer was back extracted with dichloromethane (50 ml). The combined organic layers were further washed with saturated aqueous sodium hydrogen carbonate (3x100 ml). The aqueous layers were back extracted with dichloromethane (50 ml). The combined organic layers were dried (MgSO₄) and concentrated under reduced pressure to dryness. The residue was dissolved in dichloromethane (5 ml) and added dropwise to diethyl ether (220 ml) with stirring. After 30 min, the precipitate were collected by filtration and dried in vacuo to give the title compound as a colourless solid (2.121 g, 95.0%).

δ_p[(CD₃)₂SO]: 27.68, 27.74, 27.86, 27.88.

DMTr-Gp(s)Cp(s)Gp(H) (392)

Ammonium 4-methylphenyl H-phosphonate (0.482 g, 2.55 mmol), methanol (3 ml) and triethylamine (0.71 ml, 5.1 mmol) were co-evaporated and to the residue was
added DMTr-Gp(s)Cp(s)G-OH (1.581 g, 0.850 mmol). The mixture was co-
evaporated with dry pyridine (2×5 ml). The residue was redissolved in anhydrous pyridine (15 ml) and cooled to −30°C (IMS–dry ice bath). Pivaloyl chloride (0.31 ml, 2.55 mmol) was added over 5 min. The resulting mixture was then stirred at the same temperature for 1 h. Water (5 ml) was added and the reactants were allowed to warm up to room temperature over a period of 1 h. Then the clear solution was partitioned between dichloromethane (90 ml) and triethylammonium phosphate buffer (0.5 M, pH 7.00, 80 ml). The organic layer was separated and further washed with triethylammonium phosphate buffer (0.5 M, pH 7.00, 2×80 ml). The dried (MgSO₄) organic layer was mixed with toluene (50 ml) and the mixture was purified by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (80:20 v/v) were evaporated under reduced pressure to give a colourless froth. This material was dissolved in dichloromethane (30 ml) and washed with triethylammonium phosphate buffer (0.5 M, pH 7.00, 25 ml). The organic layer was separated, dried (MgSO₄) and concentrated under reduced pressure to yield the title compound as a colourless glass (1.543 g, 89.6%).

δH[(CD₃)₂SO]: 2.82 (1 P, Jp,H 507.7), 28.08, 28.19, 28.38, 28.45 (2 P).

HO-Gp(s)Cp(s)Gp(s)Tp(s)Tp(s)Gp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)G-Lev (HO-21-Lev, 3112)

DMTr-Gp(s)Cp(s)Gp(H) (0.273 g, 0.135 mmol) and HO-Tp(s)Tp(s)Tp(s)Gp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)G-Lev (HO-18-Lev, 0.861 g, 0.100 mmol) were co-evaporated with dry pyridine (2 ml) and the residue was redissolved in anhydrous pyridine (10 ml). After this solution had been cooled to −40°C (IMS–dry ice bath), a solution of bis(2-chlorophenyl) phosphorochloridate in dichloromethane (0.19 ml, 1:1 v/v, 0.400 mmol) was added during 3 min. The reactants were then kept at the same temperature for 15 min, and then 4-[(2-cyanoethyl)sulphanyl]morpholine-3,5-dione (0.060 g, 0.300 mmol) was added. After 15 min, pyridine–water (1 ml) was added and the reaction mixture was maintained at the same temperature for a further 5 min. The clear solution was partitioned between dichloromethane (50 ml) and saturated aqueous sodium hydrogen carbonate (50 ml).
The organic layer was separated, dried (MgSO₄) and concentrated under reduced pressure. The residue was co-evaporated with toluene (3×20 ml) and dissolved in dichloromethane (30 ml). After this solution had been cooled to −50°C (IMS—dry ice bath), a solution of hydrogen chloride in dioxane (4 M, 0.30 ml) was added. After 15 min, pyridine—methanol (2 ml, 1:1 v/v) was added and the reactants were maintained at the same temperature. After 5 min, the products were poured into saturated aqueous sodium hydrogen carbonate (40 ml). The organic layer was separated and further washed with saturated aqueous sodium hydrogen carbonate (2×30 ml). The dried (MgSO₄) organic layer was concentrated under reduced pressure and the residue was purified by short column chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane—methanol (92:8 v/v) gave the title compound as a colourless froth (0.878 g, 85.2%).

δP[(CD₃)₂SO]: 27.10, 27.37, 27.61, 27.66.

7.5 Experimental for Chapter 4

N-[(2-Cyanoethyl)sulphonyl]succinimide (420a)

\[
\begin{align*}
\text{N-[(2-Cyanoethyl)sulphonyl]succinimide (420a)}
\end{align*}
\]

\[420a\]

N-Bromosuccinimide (17.8 g, 0.1 mol) and di-(2-cyanoethyl) disulphide (17.2 g, 97.2 mmol) were heated, under reflux, in dry dichloroethane (30 ml) under argon for 2 h. After the reaction mixture had been cooled down to room temperature, n-hexane (300 ml) was added and the mixture was stirred for 10 min. The upper layer was decanted, and the oily residue was triturated with ethyl acetate (20 ml) for 10 min. The solid obtained was collected by filtration and washed with diethyl ether (70 ml) to give the title compound as an off-white solid (12.4 g, 67.3%). (Found, in material
recrystallised from absolute ethanol: C, 45.8; H, 4.4; N, 15.2. C$_7$H$_8$N$_2$O$_2$S requires: C, 45.64; H, 4.38; N, 15.21%, mp 110–112°C.

δ$_\text{H}[(\text{CD}_3)_2\text{SO}]$: 2.71 (4 H, s), 2.75 (2 H, t, $J$ 6.9), 3.02 (2 H, t, $J$ 6.9).

δ$_\text{C}[(\text{CD}_3)_2\text{SO}]$: 17.9 (CH$_2$), 28.7 (CH$_2$), 32.8 (CH$_2$), 119.2 (C), 177.9 (C).

HO-Cp(s)$\text{G-Lev}$ (386)

Triethyl ammonium 5'-O-dimethoxytrityl-4-N-benzoyl-2'-deoxyctydine 3'-H-phosphonate (1.917 g, 2.40 mmol) and 2'-deoxy-6-O-(2,5-dichlorophenyl)-2-N-isobutyryl-3'-O-levuliny1guanosine (1.161 g, 2.0 mmol) were co-evaporated with dry pyridine (2 ml) and the residue was redissolved in dry pyridine (10 ml) at room temperature. To the solution was added dropwise, over a period of 5 min, a solution of $N$-[(2-cyanoethyl)sulphanyllsuccinimide (0.921 g, 5.00 mmol) and diphenyl phosphorochloridate (1.04 ml, 5.02 mmol) in dry pyridine (12 ml). After 15 min, water (2 ml) was added. The reaction mixture was stirred for a further 5 min and then partitioned between dichloromethane (100 ml) and saturated aqueous sodium hydrogen carbonate (100 ml). The organic layer was separated and further washed with saturated aqueous sodium hydrogen carbonate (2×60 ml). The dried (MgSO$_4$) organic layer was concentrated under reduced pressure. The residue was co-evaporated with toluene (2×40 ml) and dissolved in dichloromethane (50 ml). After this solution had been cooled to 0°C (ice–water bath), pyrrole (1.65 ml, 23.78 mmol) was added followed by addition of a solution of dichloroacetic acid (1.65 ml, 20.00 mmol) in dichloromethane (20 ml). After 10 min, the reaction mixture was poured into saturated aqueous sodium hydrogen carbonate (100 ml). The organic layer was separated and further washed with saturated aqueous sodium hydrogen carbonate (100 ml). The dried (MgSO$_4$) organic layer was concentrated under reduced pressure. The residue was fractionated by short column chromatography on silica gel: evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (97:3 v/v) gave HO-Cp(s)G-Lev as a colourless glass (1.968 g, 94.4%).

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δₜ[(CD₃)₂SO]: 0.95 (6 H, m), 2.14 (3 H, s), 2.27–2.33 (1 H, m), 3.59–3.62 (2 H, m),
4.19–4.22 (1 H, m), 4.33–4.37 (1 H, m), 4.43–4.48 (2 H, m), 5.06–5.09 (1 H, m),
5.22–5.25 (1 H, m, ex), 5.52 (1 H, m), 6.17 (1 H, m), 6.46 (1 H, m), 7.38–7.53 (4 H,
m), 7.60–7.72 (3 H, m), 8.01 (2 H, t), 8.30–8.36 (1 H, m), 8.56 (1 H, d, J 2.8), 10.35
(1 H, s, ex), 11.26 (1 H, s, ex).

δₚ[(CD₃)₂SO]: 27.21, 27.48.

Rₜ (System A): 0.21.

**HO-Gp(s)Cp(s)G-Lev (389)**

Triethylammonium 2'-deoxy-6-O-(2,5-dichlorophenyl)-5'-O-(4,4'-dimethoxytrityl)-
2-N-isobutyrylguanosine 3'-H-phosphonate (2.660 g, 2.80 mmol) and HO-Cp(s)G-
Lev (2.085 g, 2.00 mmol) were co-evaporated with dry pyridine (2 ml). The residue
was taken up with dry pyridine (10 ml). To the solution was added dropwise, over a
period of 5 min, a solution of diphenyl phosphorochloridate (1.04 ml, 5.02 mmol) and
N-[(2-cyanoethyl)sulphanyl]succinimide (0.921 g, 5.00 mmol) in pyridine (30 ml) at
room temperature. After 20 min, water (2 ml) was added and the reaction mixture
was allowed to stir for a further 5 min. The products were then poured into saturated
aqueous sodium hydrogen carbonate (100 ml), and extracted with dichloromethane
(100 ml). The organic layer was separated and further washed with saturated aqueous
sodium hydrogen carbonate (2×60 ml). The dried (MgSO₄) organic layer was
concentrated under reduced pressure. The residue was co-evaporated with toluene
(2×50 ml) and then redissolved in dichloromethane (50 ml). After the solution had
been cooled to 0°C (ice–water bath), pyrrole (1.65 ml, 23.78 mmol) was added,
followed by the addition of a solution of dichloroacetic acid (1.65 ml, 20.00 mmol) in
dichloromethane (20 ml). The reaction mixture was allowed to stir at the same
temperature for a further 15 min and then poured into saturated aqueous sodium
hydrogen carbonate (100 ml). The organic layer was separated and further washed
with saturated aqueous sodium hydrogen carbonate (100 ml). The dried (MgSO₄)
organic layer was concentrated under reduced pressure. The residue was purified by
short column chromatography on silica gel. Evaporation of the appropriate fractions,
which were eluted with dichloromethane-methanol (97:3 v/v) gave the title compound as a colourless foam (3.153 g, 95.2%).

δ_F{(CD_3)_2SO}: 27.70, 27.77, 27.83, 27.96.

R_{f} (System A): 0.18.

**HO-Tp(s)T-Lev (387)**

Triethylammonium 5'-O-dimethoxytrityl-4-O-phenylthymidine 3'-H-phosphonate (2.829 g, 3.6 mmol) and 3'-O-levulinyl-4-O-phenylthymidine (1.249 g, 3.0 mmol) were co-evaporated with anhydrous pyridine (2 ml) and the residue was redissolved in dry pyridine (15 ml). To this solution was added, dropwise over a period of 5 min, a solution of N-[(2-cyanoethyl)sulphanyl]succinimide (1.382 g, 7.50 mmol) and diphenyl phosphorochloridate (1.55 ml, 7.48 mmol) in dry pyridine (18 ml) at room temperature. After 15 min, water (2 ml) was added and the resulting mixture was stirred for a further 5 min. The products were poured into saturated aqueous sodium hydrogen carbonate (150 ml), and extracted with dichloromethane (150 ml). The organic layer was separated and further washed with saturated aqueous sodium hydrogen carbonate (2×90 ml). The dried (MgSO_4) organic layer was concentrated under reduced pressure. The residue was co-evaporated with toluene (2×60 ml) and dissolved in dichloromethane (80 ml). After this solution had been cooled to 0°C (ice-water bath), pyrrole (2.48 ml, 35.7 mmol) was added followed by addition of a solution of dichloroacetic acid (2.48 ml, 30.0 mmol) in dichloromethane (30 ml). After 10 min, the reaction mixture was poured into saturated aqueous sodium hydrogen carbonate (150 ml). The organic layer was separated and further washed with saturated aqueous sodium hydrogen carbonate (150 ml), dried (MgSO_4) and concentrated under reduced pressure. The residue was fractionated by short column chromatography on silica gel: evaporation of the appropriate fractions, which were eluted with dichloromethane-methanol (97:3 v/v) gave HO-Tp(s)T-Lev as a colourless foam (2.404 g, 92.5%).
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$\delta_H[(CD_3)_2SO]: 2.10 \text{ (9 H, m), 2.28-2.44 (3 H, m), 2.57-2.62 (1 H, m), 2.73 (2H, m), 2.91-2.97 (2 H, m), 3.10-3.20 (2 H, m), 3.65-3.71 (2 H, m), 4.21-4.33 (2 H, m), 4.36-4.40 (2 H, m), 5.12 (1 H, br), 5.23-5.29 (1 H, m), 5.30-5.33 (1 H, m, ex), 6.14-6.19 (2 H, m), 7.16-7.19 (4 H, m), 7.26-7.31 (2 H, m), 7.40-7.47 (4 H, m), 8.00 \text{ (1 H, dd, } J 0.9 \text{ and } 5.3), 8.15 \text{ (1 H, dd, } J 0.9 \text{ and } 4.7)$. 

$\delta_P[(CD_3)_2SO]: 27.81, 27.86$. 

$R_f$ (System A): 0.22. 

**DMTr-Cp(s)Tp(s)T-Lev (396)**

Triethylammonium $5'-O$-dimethoxytrityl-$4'-N$-benzoyl-$2'$-deoxycytidine $3'$-$H$-phosphonate (3.355 g, 4.20 mmol) and HO-$Tp(s)T$-Lev (2.598 g, 3.00 mmol) were co-evaporated with dry pyridine (2 ml). The residue was then redissolved in anhydrous pyridine (15 ml). To the solution was added dropwise, over a period of 5 min at room temperature, a solution of diphenyl phosphorochloridate (1.55 ml, 7.48 mmol) and $N$-[(2-cyanoethyl)sulphonyl]succinimide (1.382 g, 7.50 mmol) in pyridine (18 ml). After a period of 20 min, water (2 ml) was added and the resulting mixture was stirred for 5 min. Then the reaction mixture was partitioned between dichloromethane (150 ml) and saturated aqueous sodium hydrogen carbonate (150 ml). The two layers were separated; the organic layer was further washed with saturated aqueous sodium hydrogen carbonate (2×100 ml). The dried (MgSO$_4$) organic layer was concentrated under reduced pressure. The residue was then fractionated by short column on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (97:3 v/v) gave DMTr-Cp(s)Tp(s)T-Lev as a colourless foam (4.526 g, 92.5%).

$\delta_P[(CD_3)_2SO]: 27.97, 28.02, 28.07, 28.16, 28.32, 28.34$. 

$R_f$ (System A): 0.24.
DMTr-Cp(s)Tp(s)T-OH (397)

To a cooled (ice–water bath) solution of DMTr-Cp(s)Tp(s)T-Lev (8.153 g, 5.00 mmol) in pyridine (30 ml) was added a solution of hydrazine monohydrate (2.44 ml, 50.30 mmol), acetic acid (25.0 ml, 0.437 mol) and water (5 ml) in pyridine (20 ml). After 10 min, 2,4-pentanedione (13.2 ml, 0.128 mol) was added. The reaction mixture was allowed to warm up to room temperature. After 15 min, it was poured into saturated aqueous sodium hydrogen carbonate (200 ml) with care and extracted with dichloromethane (2×130 ml). The combined organic layers were further washed with saturated aqueous sodium hydrogen carbonate (2×100 ml). The aqueous layers were back extracted with dichloromethane (2×50 ml). The combined dried (MgSO₄) organic layers were evaporated to dryness under reduced pressure. The residue was dissolved in dichloromethane (10 ml) and added dropwise to diethyl ether (350 ml) with stirring. After 30 min, the solid obtained was collected by filtration and washed with diethyl ether (30 ml) to give the title compound as a colourless solid (7.387 g, 96.4%).

δ_p[(CD₃)₂SO]: 27.98, 28.03, 28.09, 28.16, 28.31.

R_f (System A): 0.17.

DMTr-Cp(s)Tp(s)Tp(H) (398)

Ammonium 4-methylphenyl H-phosphonate (2.270 g, 12.00 mmol) and triethylamine (4.60 ml, 33.00 mmol) were co-evaporated with anhydrous pyridine (10 ml). DMTr-Cp(s)Tp(s)T-OH (6.13 g, 4.0 mmol) was added to the residue and the mixture was co-evaporated with dry pyridine (10 ml). The residue was then redissolved in pyridine (50 ml) and cooled to −20°C (IMS–dry ice bath). Pivaloyl chloride (1.48 ml, 12.02 mmol) was added to the cooled solution over a period of 5 min. After 1 h, water (10 ml) was added and the reactants were allowed to warm up to room temperature over a period of 1 h. The resulting clear solution was then partitioned between dichloromethane (150 ml) and saturated aqueous sodium hydrogen carbonate (90 ml). The organic layer was separated and the aqueous layer was back extracted with
dichloromethane (2x30 ml). The combined organic layers were washed with triethylammonium phosphate buffer (2x100 ml, 0.5 M, pH 7.0), dried (MgSO₄) and concentrated to a volume of ca. 30 ml under reduced pressure. Toluene (40 ml) was added and the mixture was then purified by short column chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (80:20 v/v), gave a colourless foam. This material was then dissolved in dichloromethane (100 ml) and washed with triethylammonium phosphate buffer (50 ml, 0.5 M, pH 7.0). The dichloromethane layer was separated, dried (MgSO₄) and concentrated under reduced pressure to give the title compound as a colourless glass (6.240 g, 91.9%).

$$\delta_{p[(CD_3)_2SO]}: 1.21 \ (1 \ P, J_{HH} 586.280), \ 27.91, \ 27.94, \ 27.99, \ 28.03, \ 28.05, \ 28.14 \ (2 \ P).$$

**HO-Cp(s)Tp(s)Tp(s)Gp(s)Cp(s)G-Lev (HO-6-Lev, 3108)**

DMTr-Cp(s)Tp(s)Tp(H) (4.076 g, 2.40 mmol) and HO-Gp(s)Cp(s)G-Lev (3.312 g, 2.00 mmol) were co-evaporated with dry pyridine (2 ml). The residue was then redissolved in dry pyridine (20 ml) and cooled to 0°C (ice–water bath). To this solution was added, over a period of 30 sec a solution of pivaloyl chloride (0.62 ml, 5.03 mmol) in dichloromethane (0.62 ml). After 15 min, well powdered N-[(2-cyanoethyl)sulphanyl]succinimide (0.921 g, 5.00 mmol) was added. The reaction mixture was then allowed to warm up to room temperature over a period of 25 min and then partitioned between dichloromethane (100 ml) and saturated aqueous sodium hydrogen carbonate (100 ml). Triethylammonium phosphate buffer (10 ml, 0.5 M, pH 7.0) was added if emulsion persisted. The organic layer was separated and further washed with triethylammonium phosphate buffer (100 ml, 0.5 M, pH 7.0). The dried (MgSO₄) organic layer was concentrated under reduced pressure. The residue was co-evaporated with toluene (3x50 ml) and taken up with dichloromethane (60 ml) and cooled to 0°C (ice–water bath). Pyrrole (1.66 ml, 23.93 mmol), followed by the a solution of dichloroacetic acid (3.32 ml, 40.24 mmol) in dichloromethane (20 ml) was added. After 15 min, the reaction mixture was washed with saturated aqueous sodium hydrogen carbonate (2x100 ml). The organic layer was separated,
dried (MgSO₄) and concentrated under reduced pressure. The residue was fractionated by short column chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (94:6 v/v) gave the title compound as a colourless glass (5.820 g, 96.4%).

δ_H[(CD₃)₂SO]: ratio of anomeric protons: (C+T)/G=2.04 (required: 2.00).

δ_H[(CD₃)₂SO]: 27.80, 27.84, 27.92, 27.98, 28.02, 28.08, 28.12, 28.19, 28.30, 28.38.

R_f (System B): 0.50.

HO-Cp(s)Tp(s)Tp(s)Cp(s)Tp(s)Tp(s)Gp(s)Cp(s)G-Lev (HO-9-Lev, 458)

DMTr-Cp(s)Tp(s)Tp(H) (2.123 g, 1.25 mmol) and HO-Cp(s)Tp(s)Tp(s)Gp(s)Cp(s)G-Lev (3.018 g, 1.0 mmol) were co-evaporated with dry pyridine (2 ml) and the residue was redissolved in pyridine (11 ml) and cooled to 0°C (ice–water bath). To this cooled solution was added, over a period of 30 sec, pivaloyl chloride (0.31 ml, 2.52 mmol). After 15 min, well powdered N-[(2-cyanoethyl)sulphanyl]succinimide (0.461 g, 2.50 mmol) was added. The resulting reactants were then allowed to warm up to room temperature over 20 min and then partitioned between dichloromethane (80 ml) and saturated aqueous sodium hydrogen carbonate (80 ml). The two layers were separated and the aqueous layer was back extracted with dichloromethane (2×20 ml). The combined organic layers were washed with triethylammonium phosphate buffer (50 ml, 0.5 M, pH 7.0), dried (MgSO₄) and evaporated under reduced pressure. The residue was co-evaporated with toluene (4×40 ml).

Another coupling was carried out on a 1.993 mmol scale based on the 5'-OH compound.

The combined materials from the above couplings were then dissolved in dichloromethane (120 ml) and cooled to 0°C (ice–water bath). Pyrrole (2.45 ml, 35.31 mmol) followed by dichloroacetic acid (4.98 ml, 60.37 mmol) was added. After 45 min, the reaction solution was poured into saturated aqueous sodium hydrogen carbonate (150 ml) with care. The two layers were separated and the aqueous layer
was back extracted with dichloromethane (2×30 ml). The combined organic layers were further washed with triethylammonium phosphate buffer (80 ml, 0.5 M, pH 7.0), dried (MgSO₄) and evaporated under reduced pressure. The residue was then fractionated by short column chromatography on silica gel: evaporation of appropriate fractions, which were eluted with dichloromethane–methanol (96:4 v/v) gave the title compound as a colourless glass (12.533 g, 95.6%).

\[ \delta_{\text{H}}[(\text{CD}_3)_2\text{SO}]: \text{aromatic protons: } 50 \text{ H (required: 53 H)}, \text{ratio of anomeric protons:} \ (\text{C}+\text{T}):\text{G} = 3.63 \text{ (required 3.50), T:G (calculation based on the methyl groups of T and isobutyryl protecting group on G) } = 1.98 \text{ (required 2.00).} \]

\[ \delta_{\text{p}}[(\text{CD}_3)_2\text{SO}]: 27.81, 27.87, 27.92, 27.99, 28.06, 28.13, 28.20, 28.27, 28.33. \]

\[ R_f \text{ (System B): 0.51.} \]

\[ \text{HO-Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Gp(s)Cp(s)G-Lev (HO-12-Lev, 3109)} \]

DMTr-Cp(s)Tp(s)Tp(H) (3.374 g, 1.987 mmol) and HO-Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Gp(s)Cp(s)G-Lev (6.691 g, 1.528 mmol) were co-evaporated with dry pyridine (2 ml) and the residue was dissolved in pyridine (22 ml). The resulting solution was then cooled to 0°C (ice–water bath). To this solution was added pivaloyl chloride (0.53 ml, 4.30 mmol) over 30 sec. The reactants were stirred at the same temperature for 15 min. Well-powdered \[ N\-[(2\text{-cyanoethyl)sulphonyl)succinimide (0.788 g, 4.28 \text{ mmol) was added. The reaction mixture was allowed to warm up to room temperature over a period of 20 min and then partitioned between dichloromethane (100 ml) and saturated aqueous sodium hydrogen carbonate (100 ml). The two layers were separated and the aqueous layer was back extracted with dichloromethane (2×30 ml). The combined organic layers were further washed with triethylammonium phosphate buffer (80 ml, 0.5 M, pH 7.0), dried (MgSO₄) and concentrated under reduced pressure. The residue was then co-evaporated with toluene (4×50 ml).} \]
A second coupling was carried out on a 1.300 mmol scale based on the 5'-OH compound at the same stoichiometry.

The combined materials obtained from the above couplings were dissolved in dichloromethane (130 ml) and cooled to 0°C (ice–water bath). To the cooled solution was added pyrrole (2.74 ml, 39.49 mmol) follow by dichloroacetic acid (5.48 ml, 66.43 mmol). After 45 min, the reactants were poured into saturated aqueous sodium hydrogen carbonate (150 ml) with care. The two layers were separated and the aqueous layer was back extracted with dichloromethane (2×30 ml). The combined organic layers were then washed with triethylammonium phosphate buffer (80 ml, 0.5 M, pH 7.0), dried (MgSO₄) and evaporated under reduced pressure. The residue was then fractionated by short column chromatography on silica gel: evaporation of the appropriate fraction, which were eluted with dichloromethane–methanol (94:6 v/v) gave the title compound as a colourless glass (14.772 g, 91.0%).

δ_H[(CD₃)₂SO]: aromatic protons: 70 H (required: 72 H); ratio of anomic protons: (C+T):G = 5.18 (required: 5.00); T:G (calculation based on the methyl groups of T and isobutyryl protecting group on G) = 2.87 (required: 3.00).

δ_P[(CD₃)₂SO]: 27.80, 27.92, 27.99, 28.06, 28.13, 28.33.

R_f (System B): 0.50.

HO-Cp(s)T-Lev (388)

Triethylammonium 5'-O-dimethoxytrityl-4'-N-benzoyl-2'-deoxycytidine 3'-H-phosphonate (3.835 g, 4.80 mmol) and 3'-O-levulinyl-4'-0-phenylthymidine (1.666 g, 4.00 mmol) were co-evaporated with anhydrous pyridine (3 ml) and the residue was taken up with dry pyridine (20 ml). To this solution was added dropwise, over a period of 5 min, a solution of N-[(2-cyanoethyl)sulphonyl]succinimide (1.843 g, 10.0 mmol) and diphenyl phosphorochloridate (2.07 ml, 10.0 mmol) in dry pyridine (24 ml). After 15 min, water (3 ml) was added and the resulting mixture was stirred for 5 min. Then it was partitioned between saturated aqueous sodium hydrogen carbonate
(200 ml) and dichloromethane (200 ml). The organic layer was separated and further washed with saturated aqueous sodium hydrogen carbonate (2×100 ml). The organic layer was dried (MgSO₄) and concentrated under reduced pressure. The residue was co-evaporated with toluene (2×60 ml) and dissolved in dichloromethane (110 ml). After this solution had been cooled to 0°C (ice-water bath), pyrrole (3.31 ml, 47.71 mmol) was added followed by addition of a solution of dichloroacetic acid (3.31 ml, 40.12 mmol) in dichloromethane (40 ml). After 10 min, the reaction mixture was poured into saturated aqueous sodium hydrogen carbonate (200 ml). The organic layer was separated and further washed with saturated aqueous sodium hydrogen carbonate (200 ml), dried (MgSO₄) and concentrated under reduced pressure. The residue was then fractionated by short column chromatography on silica gel: evaporation of the appropriate fractions, which were eluted with dichloromethane-methanol (97:3 v/v) gave HO-Cp(s)T-Lev as a colourless foam (3.410 g, 97.0%).

δH[(CD3)2SO]: 2.10 (6 H, m), 2.40 (3 H, m), 2.75 (2 H, m), 2.95 (2 H, m), 3.66 (2 H, br), 4.30 (2 H, br), 4.45 (2 H, m), 5.15 (1 H, br), 5.30 (2 H, m, including 1 ex H), 6.20 (2 H, m), 7.18 (2 H, m), 7.27 (1 H, m), 7.35–7.55 (5 H, m), 7.63 (1 H, m), 8.01 (3 H, m), 8.35 (1 H, m), 11.28 (1 H, s, ex).

δF[(CD3)2SO]: 27.81, 27.88.

Rf (System A): 0.22.

DMTr-Gp(s)Cp(s)T-Lev (399)

Triethyl ammonium 2'-deoxy-6-O-(2,5-dichlorophenyl)-5'-O-(4,4'-dimethoxytrityl)-2-N-isobutrylguanosine 3'-H-phosphonate (3.041 g, 3.20 mmol) and HO-Cp(s)T-Lev (2.010 g, 2.29 mmol) were co-evaporated with dry pyridine (2 ml). The residue was then redissolved in anhydrous pyridine (11.5 ml). To this solution was added dropwise, over a period of 5 min, a solution of diphenyl phosphorochloridate (1.19 ml, 5.74 mmol) and N-[(2-cyanoethyl)sulphanyl]succinimide (1.054 g, 5.72 mmol) in pyridine (14 ml) at room temperature. After 20 min, water (2 ml) was added. After 5 min, the products were partitioned between dichloromethane (150 ml) and saturated
aqueous sodium hydrogen carbonate (120 ml). The organic layer was separated and further washed with saturated aqueous sodium hydrogen carbonate (2×100 ml). The dried (MgSO₄) organic layer was concentrated under reduced pressure. The residue was fractionated by short column chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (97:3 v/v) gave DMTr-Gp(s)Cp(s)T-Lev as a colourless glass (3.829 g, 93.2%).

\[\delta_\text{p}[(\text{CD}_3)_2\text{SO}]: 27.68, 27.72, 28.15, 28.28.\]

R_f (System A): 0.25.

**DMTr-Gp(s)Cp(s)T-OH (3100)**

DMTr-Gp(s)Cp(s)T-Lev (6.681 g, 3.72 mmol) was dissolved in pyridine (22 ml) and cooled to 0°C (ice–water bath). To the solution was added a mixture of hydrazine monohydrate (1.82 ml, 37.52 mmol), acetic acid (18.6 ml, 0.325 mol) and water (3.7 ml) in pyridine (15 ml). After 10 min, 2,4-pentanedione (9.8 ml, 95.44 mmol) was added. The resulting mixture was allowed to warm up to room temperature over a period of 15 min. Then it was poured into saturated aqueous sodium hydrogen carbonate (160 ml) with care and extracted with dichloromethane (2×100 ml). The combined organic layers were further washed with saturated aqueous sodium hydrogen carbonate (2×80 ml); the aqueous layers were back extracted with dichloromethane (2×50 ml). The combined organic layers were dried (MgSO₄) and evaporated to dryness under reduced pressure. The residue was dissolved in dichloromethane (7 ml) and added dropwise to diethyl ether (280 ml) with stirring. After 30 min, the precipitate obtained was collected by filtration and washed with diethyl ether (30 ml) to give the *title compound* as a colourless solid (6.017 g, 95.1%).

\[\delta_\text{p}[(\text{CD}_3)_2\text{SO}]: 27.70, 27.76, 28.18, 28.32.\]

R_f (System A): 0.20.
Ammonium 4-methylphenyl $H$-phosphonate (1.870 g, 9.885 mmol) and triethylamine (3.8 ml, 27.26 mmol) were co-evaporated with dry pyridine (20 ml). DMTr-Gp(s)Cp(s)Tp(H) (3.770 g, 2.02 mmol) and HO-Cp(s)Tp(s)Cp(s)T(s)Cp(s)T(s)Cp(s)T(s)Cp(s)T(s)Gp(s)Cp(s)G-Lev (HO-15-Lev, 3110) were co-evaporated with dry pyridine (2×5 ml). The residue was then redissolved in pyridine (30 ml) and cooled to 0°C (ice–water bath). Pivaloyl chloride (0.56 ml, 4.55

\[
\delta_p[(CD_3)_2SO]: 1.59 (1 P, J_{P-H} 584.822), 27.54, 27.59, 27.65, 27.70, 27.76, 27.84, 27.91, 27.90, 28.02, 28.08.
\]

DMTr-Gp(s)Cp(s)Tp(H) (3.770 g, 2.02 mmol) and HO-Cp(s)Tp(s)Cp(s)T(s)Cp(s)T(s)Cp(s)T(s)Cp(s)T(s)Gp(s)Cp(s)G-Lev (HO-12-Lev, 8.610 g, 1.50 mmol) were co-evaporated with dry pyridine (2×10 ml). The residue was then redissolved in pyridine (40 ml) and cooled to −20°C (IMS–dry ice bath). To the cooled solution was added, over a period of 5 min, pivaloyl chloride (1.22 ml, 9.905 mmol). The reactants were stirred at the same temperature for 1 h. Water (10 ml) was then added and the resulting mixture was allowed to warm up to room temperature and stirred for 1 h. The clear solution was then poured into saturated aqueous sodium hydrogen carbonate (50 ml) and extracted with dichloromethane (3×60 ml). The combined organic layers were then washed with triethylammonium phosphate buffer (2×50 ml, 0.5 M, pH 7.0). The aqueous layers were extracted with dichloromethane (2×30 ml). The combined organic layers were dried (MgSO₄) and concentrated to one sixth of the original volume. Toluene (50 ml) was added. The mixture was then purified by short column chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (80:20 v/v), gave a colourless foam. This material was then dissolved in dichloromethane (100 ml) and washed with triethylammonium phosphate buffer (50 ml, 0.5 M, pH 7.0). The dichloromethane layer was separated, dried (MgSO₄) and concentrated under reduced pressure to give the title compound as a colourless glass (5.695 g, 92.8%).
mmol) was added over 30 sec. After 15 min, N-[(2-cyanoethyl)sulphonyl]succinimide (0.829 g, 4.5 mmol) was added as a well-powdered solid and the resulting reactants were allowed to warm up to room temperature over 20 min. Water (1 ml) was added and the reaction mixture was stirred at the same temperature for a further 5 min. The products were partitioned between dichloromethane (100 ml) and saturated aqueous sodium hydrogen carbonate (100 ml). The two layers were separated and the aqueous layer was back extracted with dichloromethane (20 ml). The combined organic layers were further washed with triethylammonium phosphate buffer (50 ml, 0.5 M, pH 7.0), dried (MgSO₄) and evaporated under reduced pressure. The residue was then co-evaporated with toluene (4×50 ml).

Another coupling was carried out on a 1.0 mmol scale at the same stoichiometry.

The combined materials obtained from the above two couplings were then dissolved in dichloromethane (100 ml) and cooled to 0°C (ice–water bath). To the cooled solution were added pyrrole (2.05 ml, 29.55 mmol) and dichloroacetic acid (4.15 ml, 50.30 mmol). After 50 min, the reactants were poured into saturated aqueous sodium hydrogen carbonate (100 ml) with care. The organic layer was separated and the aqueous layer was back extracted with dichloromethane (30 ml). The combined organic layers were further washed with triethylammonium phosphate buffer (50 ml, 0.5 M, pH 7.0), dried (MgSO₄) and evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel: evaporation of the appropriate fractions, eluted with dichloromethane–methanol (94:6 v/v) gave the title compound as a colourless glass (16.389 g, 90.2%).

δ_H[(CD₃)₂SO]: aromatic protons: 83.3 H (required: 89 H), ratio of anomeric protons (C+T):G = 4.04 (required 4.00), T:G (calculation based on the methyl groups of T and isobutryl protecting group on G) = 2.16 (required 2.33).

δ_p[(CD₃)₂SO]: 27.80, 27.89, 28.01, 28.06, 28.13, 28.29, 28.36, 28.38.

R_f (System B): 0.51.
**DMTr-TP(s)TP(s)Lev (3102)**

Triethylammonium 5'-O-dimethoxytrityl-4-O-phenylthymidine 3'-H-phosphonate (2.200 g, 2.8 mmol) and HO-TP(s)TP(s)Lev (1.732 g, 2.0 mmol) were co-evaporated with dry pyridine (2 ml) and the residue was redissolved in anhydrous pyridine (10 ml). To this solution was added, over 5 min, a solution of diphenyl phosphorochloridate (1.04 ml, 5.02 mmol) and N-[(2-cyanoethyl)sulphanyl]succinimide (0.921 g, 5.00 mmol) in pyridine (12 ml) at room temperature. After 20 min, water (2 ml) was added. After a further period of 5 min, the reactants were partitioned between dichloromethane (100 ml) and saturated aqueous sodium hydrogen carbonate (100 ml). The two layers were separated and the organic layer was further washed with saturated aqueous sodium hydrogen carbonate (100 ml). The dried (MgSO₄) organic layer was concentrated under reduced pressure. The residue was fractionated with short column chromatography on silica gel: evaporation of the appropriate fractions, which were eluted with dichloromethane-methanol (97:3, v/v) gave the title compound as a colourless glass (3.200 g, 98.9%).

δ₀[(CD₃)₂SO]: 27.99, 28.03, 28.12, 28.30.

Rᵣ (System A): 0.24.

**DMTr-TP(s)TP(s)OH (3103)**

DMTr-TP(s)TP(s)Lev (3.000 g, 1.85 mmol) was dissolved in pyridine (10 ml) and cooled to 0°C (ice-water bath). A solution of hydrazine monohydrate (0.90 ml, 18.55 mmol), acetic acid (9.2 ml, 0.161 mol) and water (2 ml) in pyridine (8.5 ml) was added to the cooled solution. After 10 min, 2,4-pentanedione (5 ml, 48.69 mmol) was added and the resulting mixture was allowed to warm up to room temperature over a period of 15 min. The reactants were then poured into saturated aqueous sodium hydrogen carbonate (90 ml) with care and extracted with dichloromethane (2×60 ml). The combined organic layers were further washed with saturated aqueous sodium hydrogen carbonate (2×100 ml). The aqueous layers were back extracted with dichloromethane (2×50 ml). The combined organic layers were dried (MgSO₄) and
concentrated under reduced pressure. The residue was dissolved in dichloromethane (5 ml) and added dropwise to diethyl ether (200 ml) with stirring. After 30 min, the precipitates were collected by filtration and washed with diethyl ether (30 ml) to give the title compound as a colourless solid (2.679 g, 95.1%).

$$\delta_{\text{pp}} ([\text{CD}_3]_2\text{SO}) : 27.99, 28.04, 28.12, 28.28.$$  

$R_f$ (System A): 0.17.

**DMTr-Tp(s)Tp(s)Tp(H) (3104)**

Ammonium 4-methylphenyl $H$-phosphonate (1.986 g, 10.50 mmol) and triethylamine (4.0 ml, 28.70 mmol) were co-evaporated with dry pyridine (20 ml). DMTr-Tp(s)Tp(s)Tp(s)T-OH (5.318 g, 3.50 mmol) was added to the residue and co-evaporated with pyridine (2×10 ml). The residue was redissolved in anhydrous pyridine (45 ml) and cooled to –20°C (IMS–dry ice bath). To this cooled solution was then added dropwise, over a period of 5 min, pivaloyl chloride (1.3 ml, 10.55 mmol). After the reaction mixture had been stirred for 1 h at the same temperature, water (15 ml) was added. The resulting mixture was allowed to warm up to room temperature over a period of 1 h. The clear solution was then poured into saturated aqueous sodium hydrogen carbonate (60 ml) and extracted with dichloromethane (2×80 ml). The combined organic layers were washed with triethylammonium phosphate buffer (2×50 ml, 0.5 $M$, pH 7.0). The aqueous layers were back extracted with dichloromethane (2×30 ml). The combined organic layers were dried (MgSO$_4$) and concentrated to a volume of ca. 30 ml under reduced pressure. Toluene (50 ml) was added and the mixture was subjected to short column chromatography on silica gel: evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (80:20 v/v) gave a colourless glass. This material was then redissolved in dichloromethane (100 ml) and washed with triethylammonium phosphate buffer (50 ml, 0.5 $M$, pH 7.0). The dried (MgSO$_4$) organic layer was concentrated under reduced pressure to give the title compound as a colourless glass (5.496 g, 93.2%).

$$\delta_{\text{pp}} ([\text{CD}_3]_2\text{SO}) : 1.53 (1 \text{ P}, {^1J_{P-H}} 584.644), 28.00 (2 \text{ P}).$$
Chapter 7 Experimental

HO-Tp(s)Tp(s)Tp(s)Gp(s)Cp(s)Tp(s)Cp(s)Tp(s)Gp(s)Cp(s)G-Lev (HO-18-Lev, 3111)

DMTr-Tp(s)Tp(s)Tp(s)Tp(s)Gp(s)Cp(s)Tp(s)Cl(s)Tl(s)Tp(s)Cp(s)Tp(s)Tp(s)Gp(s)Cp(s)Tp(s)G-Lev (HO-15-Lev, 7.570 g, 1.04 mmol) were co-evaporated with dry pyridine (2 ml). The residue was then redissolved in anhydrous pyridine (25 ml) and cooled to 0°C (ice-water bath). To the cooled solution was added pivaloyl chloride (0.40 ml, 3.25 mmol) over 30 sec. After 15 min, N-[(2-cyanoethyl)sulphanyl]succinimide (0.589 g, 3.20 mmol) was added and the resulting mixture was allowed to warm up to room temperature over a period of 20 min. Water (0.5 ml) was then added to the reactants. After 5 min, the reaction mixture was partitioned between dichloromethane (80 ml) and saturated aqueous sodium hydrogen carbonate (80 ml). The organic layer was separated and the aqueous layer was back extracted with dichloromethane (20 ml). The combined organic layers were washed with triethyl ammonium phosphate buffer (50 ml, 0.5 M, pH 7.0). The organic layer was separated and the aqueous layer was back extracted with dichloromethane (20 ml). The combined organic layers were then dried (MgSO4) and concentrated under reduced pressure. The residue was co-evaporated with toluene (4×50 ml).

A second coupling was carried out on a 1.152 mmol scale based on the 5'-OH compound at the same stoichiometry.

The combined materials obtained from the above couplings were dissolved in dichloromethane (90 ml) and cooled to 0°C (ice–water bath). Pyrrole (1.76 ml, 25.37 mmol) followed by dichloroacetic acid (3.57 ml, 43.28 mmol) was added. After 70 min, the reactants were poured into saturated aqueous sodium hydrogen carbonate (100 ml) with care. The two layers were separated and the aqueous layer was back extracted with dichloromethane (30 ml). The combined organic layers were then washed with triethylammonium phosphate buffer (50 ml, 0.5 M, pH 7.0). The organic layer was separated, dried (MgSO4) and concentrated under reduced pressure. The residue was purified by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (94:6 v/v), were
concentrated under reduced pressure to give the title compound as a colourless glass (16.932 g, 90.0%).

$\delta_{H}(\text{CD}_3\text{CN}):$ Aromatic protons: 100.7 H (required: 107 H), ratio of anomeric protons: (C+T):G = 5.10 (required: 5.00), T:G (calculation based on the methyl groups of T and isobutyryl protecting group on G) = 3.22 (required: 3.33).

$\delta_{P}(\text{CD}_3\text{SO}):$ 27.80, 27.90, 27.95, 28.05, 28.12, 28.29, 28.35.

$R_f$ (System B): 0.51.

**DMTr-Gp(s)Cp(s)G-Lev (390)**

Triethylammonium 2'-deoxy-6-O-(2,5-dichlorophenyl)-5'-O-(4,4'-dimethoxytrityl)-2-N-isobutyrylguanosine 3'-H-phosphonate (3.989 g, 4.2 mmol) and HO-Cp(s)G-Lev (3.129 g, 3.0 mmol) were co-evaporated with dry pyridine (2 ml) and then redissolved in anhydrous pyridine (15 ml). A solution of diphenyl phosphorochloridate (1.55 ml, 7.48 mmol) and $N$-[(2-cyanoethyl)sulphanyl] succinimide (1.382 g, 7.50 mmol) in pyridine (18 ml) was added dropwise, over a period of 5 min at room temperature. After the resulting solution had been stirred for 20 min, water (2 ml) was added. After 5 min, the products were partitioned between dichloromethane (150 ml) and saturated aqueous sodium hydrogen carbonate (150 ml). The organic layer was separated and further washed with saturated aqueous sodium hydrogen carbonate (100 ml). The dried (MgSO$_4$) organic layer was concentrated under reduced pressure. The residue was purified by short column chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (97:3 v/v) gave the title compound as a colourless glass (5.476 g, 93.2%).

$\delta_{P}(\text{CD}_3\text{SO}):$ 27.64, 27.67, 27.74, 27.91.

$R_f$ (System A): 0.33.
DMTr-Gp(s)Cp(s)G-OH (391)

DMTr-Gp(s)Cp(s)G-Lev (6.855 g, 3.5 mmol) was dissolved in pyridine (20 ml) and cooled to 0°C (ice-water bath). A solution of hydrazine monohydrate (1.70 ml, 35.04 mmol), acetic acid (17.4 ml, 0.304 mol) and water (3.5 ml) in pyridine (15 ml) was added to the cooled solution. After 10 min, 2,4-pentanedione (9.5 ml, 92.51 mmol) was added and the reactants were allowed to warm up to room temperature over 15 min. The products were then poured into saturated aqueous sodium hydrogen carbonate (150 ml) with care and extracted with dichloromethane (2×100 ml). The combined organic layers were further washed with saturated aqueous sodium hydrogen carbonate (2×150 ml). The aqueous layers were back extracted with dichloromethane (2×60 ml). The combined dried (MgSO₄) organic layers were concentrated to dryness under reduced pressure. The residue was then dissolved in dichloromethane (7 ml) and added dropwise to diethyl ether (300 ml) with stirring. The precipitate obtained was collected by filtration and washed with diethyl ether (50 ml) to give the title compound as a colourless solid (6.300 g, 96.7%).

δp[(CD₃)₂SO]: 27.68, 27.74, 27.86, 27.88.

Rf (System A): 0.30.

DMTr-Gp(s)Cp(s)Gp(H) (392)

Ammonium 4-methylphenyl H-phosphonate (1.677 g, 8.868 mmol) and triethylamine (3.4 ml, 24.39 mmol) were co-evaporated with dry pyridine (15 ml). DMTr-Gp(s)Cp(s)G-OH (5.500 g, 2.956 mmol) was added to the residue and co-evaporated with pyridine (2×10 ml). The residue was redissolved in anhydrous pyridine (40 ml) and cooled to −20°C (IMS–dry ice bath). To this cooled solution was then added dropwise, over a period of 5 min, pivaloyl chloride (1.1 ml, 8.93 mmol). After the reaction mixture had been stirred for 1 h at the same temperature, water (15 ml) was added and the resulting mixture allowed to warm up to room temperature over a period of 1 h. The clear solution was then poured into saturated aqueous sodium hydrogen carbonate (60 ml) and extracted with dichloromethane (2×80 ml). The
combined organic layers were washed with triethylammonium phosphate buffer (2×50 ml, 0.5 M, pH 7.0). The layers were separated and the aqueous layers were back extracted with dichloromethane (2×30 ml). The combined organic layers were dried (MgSO₄) and concentrated to ca. one fourth of the original volume under reduced pressure. Toluene (50 ml) was added to the residue and the mixture was purified by short column chromatography on silica gel: evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (80:20, v/v) gave a colourless glass. This material was redissolved in dichloromethane (100 ml) and washed with triethylammonium phosphate buffer (50 ml, 0.5 M, pH 7.0). The dried (MgSO₄) organic layer was concentrated under reduced pressure to give the title compound as a colourless glass (5.601 g, 93.5%).

\[ \delta_{[\text{CD}_3\text{SO}]}: 1.55 \ (1 \text{ P, } J_{\text{P-H}} 588.264), 27.51, 27.65, 27.77 \ (2 \text{ P}), \]

\text{HO-Gp(s)Cp(s)Gp(s)Tp(s)Tp(s)Gp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Gp(s)Cp(s)Tp(s)Cp(s)Gp(s)Cp(s)Gp(H)} \ (2.937 \text{ g, } 1.45 \text{ mmol}) \text{ and } \text{HO-Tp(s)Tp(s)Tp(s)Gp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Gp(s)Cp(s)Tp(s)Cp(s)Gp(s)Cp(s)} \text{ DMTr-Gp(s)Cp(s)Gp(H)} \ (8.616 \text{ g, } 1.00 \text{ mmol}) \text{ were co-evaporated with dry pyridine (2×5 ml). The residue was then taken up with pyridine (30 ml) and cooled to 0°C (ice–water bath). To the cooled solution was added, over a period of 30 sec, pivaloyl chloride (0.42 ml, 3.41 mmol). The reactants were stirred at the same temperature for 15 min and then } N-[(2-cyanoethyl)sulphanyl]succinimide \ (0.626 \text{ g, } 3.40 \text{ mmol}) \text{ was added. The resulting mixture was allowed to warm up to room temperature and stirred for a further 20 min. Water (0.5 ml) was added to the reactants. After 5 min, the products were partitioned between dichloromethane (80 ml) and saturated aqueous sodium hydrogen carbonate (80 ml). The layers were separated and the aqueous layer was back extracted with dichloromethane (20 ml). The combined organic layers were washed with triethylammonium phosphate buffer (50 ml, 0.5 M, pH 7.0). The two layers were separated, and the aqueous layer extracted with dichloromethane (20 ml). The combined dried (MgSO₄) organic layers were concentrated under reduced pressure. The residue was co-evaporated with toluene (4×50 ml).}
A second coupling was carried out on a smaller scale (0.891 mmol of HO-18-Lev) at the same stoichiometry.

The combined materials obtained from the above two batches of coupling were then dissolved in dichloromethane (80 ml) and cooled to 0°C (ice-water bath). To this cooled solution were added pyrrole (1.55 ml, 22.34 mmol) and dichloroacetic acid (6.00 ml, 72.73 mmol). After 1 h, the reactants were poured into saturated aqueous sodium hydrogen carbonate (100 ml) with care. The two layers were separated and the aqueous layer was back extracted with dichloromethane (30 ml). The combined organic layers were washed with triethylammonium phosphate buffer (50 ml, 0.5 M, pH 7.0). The organic layer was separated and the aqueous layer was back extracted with dichloromethane (30 ml). The combined dried (MgSO₄) organic layers were concentrated under reduced pressure. The residue was then purified by short column chromatography on silica gel. Evaporation of the appropriate fractions, which were eluted with dichloromethane–methanol (93:7 v/v), gave the title compound as a slightly yellow coloured glass (17.559 g, 90.1%).

\[ \delta_H[(CD_3)_2SO]: \text{(ratio of anomeric protons)} (T+C):G = 3.12 \text{ (required: 3.20)}, \text{aromatic protons} (108 \text{ H, required: } 122 \text{ H)}, \text{T:G (calculation based on the methyl groups of T and isobutyryl protecting group on G)} = 1.80 \text{ (required 2.00)}. \]

\[ \delta_P[(CD_3)_2SO]: 28.34, 28.12, 28.05, 27.85, 27.79, 27.70. \]

\[ R_f \text{ (System B): 0.51.} \]

**HO-Cp(s)Tp(s)Tp(s)Cp(s)Tp(s)T(s)Gp(s)Cp(s)G-OH (459)**

Ac-9-Lev (0.200 g, 45.6 ìmol) was co-evaporated with dry pyridine (2×1 ml) and the residue dissolved in dry acetonitrile (2.0 ml). To this solution was added chlorotrimethylsilane (0.162 ml, 1.276 mmol) and DBU (0.54 ml, 3.65 mmol) at room temperature. After 30 min, the reactants were concentrated under reduced pressure. The residue was redissolved in acetonitrile (3.6 ml), followed by addition of 2-nitrobenzaldoxime (0.318 g, 1.915 mmol). After 12 h, the products were
concentrated under reduced pressure. The residue was dissolved in methanol (5.0 ml) and precipitated with ethyl acetate (120 ml). The solid was collected by centrifugation and redissolved in methanol. Ethyl acetate (120 ml) was added and the resulting suspension was centrifuged. The solid was dried in vacuo and then incubated with aqueous ammonia (33%, $d$ 0.88, 3.6 ml) and 2-mercaptoethanol (0.4 ml) at 50°C for 15 h. After it had been cooled to room temperature, the reaction mixture was concentrated under reduced pressure, followed by co-evaporation with ethanol (2×2 ml). The residue was dissolved in methanol (3 ml) and precipitated with ethyl acetate (40 ml). This was repeated two more times and the solid was collected by centrifugation. The dried solid residue was dissolved in water (3 ml) and passed through an Amberlite (IR120, Na+ form) ion exchange column (1×15 cm). The appropriate fractions were concentrated under reduced pressure to a small volume (ca. 3 ml). Upon freeze drying, the title compound was obtained as a colourless powder (0.220 g, 88.4%).

\[ \delta_{[D_2O]} 55.90, 56.17, 56.28, 56.33. \]

MS (MALDI): 2807.6, required 2807.3.

**HO-Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Gp(s)Cp(s)G-OH (460)**

Ac-12-Lev (0.360 g, 62.26 µmol) was co-evaporated with dry pyridine (2×2 ml) and the residue dissolved in dry acetonitrile (3.6 ml). To this solution was added chlorotrimethylsilane (0.162 ml, 1.276 mmol) and DBU (0.64 ml, 4.280 mmol) at room temperature. After 30 min, the reactants were concentrated under reduced pressure. The residue was redissolved in acetonitrile (3.6 ml), followed by addition of 2-nitrobenzaldoxime (0.284 g, 1.709 mmol). After 12 h, the products were concentrated under reduced pressure. The residue was dissolved in methanol (5.0 ml) and precipitated with ethyl acetate (120 ml). The solid was collected by centrifugation and redissolved in methanol. Ethyl acetate (120 ml) was added and the resulting suspension was centrifuged. The solid was dried in vacuo and then incubated with aqueous ammonia (33%, $d$ 0.88, 6.5 ml, 0.111 mol) and 2-mercaptoethanol (0.65 ml, 9.268 mmol) at 50°C for 15 h. After it had been cooled to room temperature, the
reaction mixture was concentrated under reduced pressure. The residue was co-evaporated with ethanol (2×5 ml) and then dissolved in methanol (6 ml). Ethyl acetate (120 ml) was added and the solid collected by centrifugation. This material was redissolved in methanol (6.0 ml, use a sonicator if necessary) and precipitated with ethyl acetate (120 ml). The suspension was centrifuged and the precipitate was collected by centrifugation. This was repeated one more time and the dried solid residue was dissolved in water (5 ml) and passed through an Amberlite (IR120, Na⁺ form) ion exchange column (3×20 cm). The appropriate fractions were concentrated under reduced pressure to a small volume (ca. 5 ml). Upon freeze drying, the title compound was obtained as a colourless powder (0.220 g, 88.4%).

δp[D2O] 56.243.

MS (MALDI): 3753.0, required 3752.4.

\[ \text{HO-Gp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Gp(s)Cp(s)G-OH (461)} \]

Ac-15-Lev (0.200 g, 27.34 μmol) was co-evaporated with dry pyridine (2×2 ml) and the residue dissolved in dry acetonitrile (2 ml). To this solution was added chlorotrimethylsilane (68 μl, 0.551 mmol) and DBU (0.36 ml, 2.407 mmol) at room temperature. After 30 min, the reactants were concentrated under reduced pressure. The residue was redissolved in acetonitrile (2.0 ml), followed by addition of 2-nitrobenzaldoxime (0.170 g, 1.026 mmol). After 12 h, the products were concentrated under reduced pressure and the residue was incubated with aqueous ammonia (33%, d 0.88, 3.6 ml, 61.39 mmol) and 2-mercaptoethanol (0.4 ml, 5.703 mmol) at 50°C for 15 h. After it had been cooled to room temperature, the reaction mixture was concentrated under reduced pressure. The residue was co-evaporated with ethanol (2×5 ml) and then dissolved in methanol (2 ml). Ethyl acetate (40 ml) was added and the solid collected by centrifugation. This material was redissolved in methanol (4.0 ml, use a sonicator if necessary) and precipitated with ethyl acetate (40 ml). The suspension was centrifuged and the precipitate was collected by centrifugation. This was repeated one more time and the dried solid residue was dissolved in water (3 ml)
and passed through an Amberlite (IR120, Na⁺ form) ion exchange column (1×15 cm). The appropriate fractions were concentrated under reduced pressure to a small volume (ca. 3 ml). Upon freeze drying, the title compound was obtained as a colourless powder (0.110 g, 85%).

δ_T[D₂O] 56.38.

MS (MALDI): 4721.7, required 4722.4.

HO-Tp(s)Tp(s)Tp(s)Gp(s)Cp(s)Tp(s)Cp(s)Tp(s)Tp(s)Cp(s)Tp(s)Cp(s)Tp(s)Tp(s)Tp(s)Gp(s)Cp(s)G-OH (462)

Ac-18-Lev (0.100 g, 11.6 μmol) was co-evaporated with dry pyridine (2×2 ml) and the residue dissolved in dry acetonitrile (2 ml). To this solution was added chlorotrimethylsilane (68 μl, 0.551 mmol) and DBU (0.294 ml, 1.972 mmol) at room temperature. After 30 min, the reactants were concentrated under reduced pressure. The residue was redissolved in acetonitrile (3.0 ml), followed by addition of 2-nitrobenzaldoxime (0.167 g, 1.009 mmol). After 12 h, the products were concentrated under reduced pressure and the residue was incubated with aqueous ammonia (33%, d 0.88, 2.5 ml) and 2-mercaptoethanol (0.25 ml) at 50°C for 15 h. After it had been cooled to room temperature, the reaction mixture was concentrated under reduced pressure. The residue was co-evaporated with ethanol (2×1 ml) and then dissolved in methanol (2 ml). Ethyl acetate (40 ml) was added and the solid collected by centrifugation. This material was redissolved in methanol (3.0 ml, use a sonicator if necessary) and precipitated with ethyl acetate (40 ml). The suspension was centrifuged and the precipitate was collected by centrifugation. This was repeated one more time and the dried solid residue was dissolved in water (3 ml) and passed through an Amberlite (IR120, Na⁺ form) ion exchange column (1×15 cm). The appropriate fractions were concentrated under reduced pressure to a small volume (ca. 3 ml). Upon freeze drying, the title compound was obtained as a colourless powder (50 mg, 75.7%).

δ_T[D₂O] 56.243.
HO-Gp(s)Cp(s)Gp(s)Tp(s)T(p(s))Gp(s)Cp(s)Tp(s)Cp(s)Tp(s)Gs(s)Cp(s)G-OH (220)

Ac-2I-Lev (0.700 g, 67.65 μmol) was co-evaporated with dry pyridine (2×5 ml). The residue was dissolved in freshly distilled anhydrous acetonitrile (10 ml). To this solution was added chlorotrimethylsilane (0.18 ml, 1.42 mmol) followed by addition of DBU (1.26 ml, 8.42 mmol) at room temperature. After 30 min, the reactants were evaporated under reduced pressure and the residue was redissolved in dry acetonitrile (10 ml). 2-Nitrobenzaldoxime (0.581 g, 3.50 mmol) was added and the resulting solution was stirred overnight at room temperature. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in methanol (7.0 ml). Ethyl acetate (200 ml) was added and the resulting suspension was centrifuged. The solid obtained was then incubated with aqueous ammonia solution (33%, d 0.88, 12.0 ml, 0.205 mol) and 2-mercaptoethanol (1.20 ml, 17.11 mmol) at 50°C for 15 h. After the reaction mixture had been cooled, it was evaporated under reduced pressure and the residue was co-evaporated with ethanol (2×5 ml). Then it was dissolved in methanol (7 ml) and precipitated with ethyl acetate (200 ml). The solid obtained was collected by centrifugation followed by washing with ethyl acetate (2×200 ml). After this material had been dried in vacuo, it was dissolved in water (10 ml) and passed through an Amberlite (IR120, Na⁺ form) ion exchange column (3×30 cm). The appropriate fractions were concentrated under reduced pressure to a small volume (ca. 10 ml) and freeze dried to give an off-white powder (450 mg, 93.4%).

δp[D2O] 56.243.

MS (MALDI): 6677.9, required 6677.6.
7.6 Experimental for Chapter 5

HO-CpG-OH (527)

Ac-Cp(5)G-Lev (50 mg, 46.1 µmol) was dissolved in dichloromethane (1.0 ml) followed by addition of DBU (68.9 µl, 0.461 mmol) at room temperature. After 30 min, a solution of trifluoroacetic acid in dichloromethane (10% w/v, 0.415 mmol, 0.473 ml) was added. After 5 min, the products were precipitated with diethyl ether (30 ml). The solid was collected by centrifugation and redissolved in dichloromethane (1.5 ml) and precipitated with diethyl ether (30 ml). This was repeated two more times and the residue was taken up with dichloromethane (0.8 ml) followed by addition of N,N-dimethylaniline (17.5 µl, 0.138 mmol) and bromoacetonitrile (9.6 µl, 0.138 mmol). The resulting solution was stirred at room temperature for 12 h and then concentrated under reduced pressure. The residue was purified by chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane – methanol (97:3 v/v) were combined and concentrated under reduced pressure to give a colourless froth (45 mg). This material was dissolved in acetonitrile (0.5 ml) followed by addition of 2-NBO (69.8 mg, 0.420 mmol) and TMG (47.4 µl, 0.378 mmol). After 15 h, the products were concentrated under reduced pressure and the residue was incubated in aqueous ammonia solution (1.0 ml, 33%, d 0.88) at 55°C for 15 h. The products were cooled and concentrated under reduced pressure followed by azeotroping with ethanol (2×1 ml). The residue was taken up with methanol (1.0 ml) and precipitated with ethyl acetate (30 ml). The resulting solid was collected by centrifugation. This was repeated two more times and
the residue was dried *in vacuo*. The solid residue was then dissolved in water (3 ml) and passed through an Amberlite (IR120, Na⁺ form) ion exchange column (1.0×10 cm). The appropriate fractions were concentrated under reduced pressure to give the *title compound* as a colourless solid (28 mg).

δ[D₂O] 0.232.

δ[D₂O] 1.55 (1 H, m), 2.20 (1 H, m), 2.40 (1 H, m), 2.70 (1 H, m), 3.52 (2 H, m), 3.93 (3 H, m), 4.07 (1 H, t, J 2.9), 4.5 (1 H, m), 5.81 (1 H, d, J 7.5), 5.95 (1 H, dd, J 5.9 and 8.0), 6.09 (1 H, t, J 6.8), 7.46 (1 H, d, J 7.6), 7.90 (1 H, s).

Rt (programme II) 5.50 min.

**HO-GpA-OH (528)**

Ac-Gp(s)A-Lev (60 mg, ca. 49 μmol, A is a mixture of N-benzyol and N-acetyl-N-benzyol-2'-deoxyadenosine) was dissolved in dichloromethane (1.0 ml) followed by addition of DBU (74.2 μl, 0.496 mmol) at room temperature. After 30 min, a solution of trifluoroacetic acid in dichloromethane (10% w/v, 0.508 ml, 0.446 mmol) was added. After 5 min, the products were precipitated with diethyl ether (30 ml) and the solid was collected by centrifugation. The solid collected was redissolved in dichloromethane (1.5 ml) and precipitated with diethyl ether (30 ml). After this had been repeated two more times, the residual solid was dissolved in dichloromethane (0.5 ml). *N,N*-Dimethylaniline (18.9 μl, 0.149 mmol), followed by bromoacetonitrile
(10.4 µl, 0.149 mmol) was added. The reaction mixture was stirred at room temperature overnight and the solvents were removed under reduced pressure. The residue was purified by chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (97:3 v/v) were combined and evaporated under reduced pressure to give a colourless froth (47 mg). This material was then dissolved in dry acetonitrile (0.5 ml) followed by addition of 2-NBO (71.4 mg, 0.430 mmol, 5 eq./function) and TMG (43.2 µl, 0.344 mmol, 4 eq./function). After 12 h, the reaction mixture was concentrated under reduced pressure and the residue was incubated in aqueous ammonia (1.0 ml, 33%, d 0.88) at 55°C for 15 h. After the reaction mixture had been cooled, it was concentrated under reduced pressure followed by azeotroping with ethanol (2×2 ml). The residue was dissolved in methanol (1.0 ml) and precipitated with ethyl acetate (30 ml). The solid obtained was collected by centrifugation. This was repeated two more times and the colourless solid obtained was dried in vacuo. This material was dissolved in water (3 ml) and passed through an Amberlite (IR120, Na⁺ form) ion exchange column (1.5×10 cm). The nucleotide-containing fractions were pooled and concentrated under reduced pressure to a volume of ca. 2 ml. Upon freeze drying, a colourless powder was obtained (20 mg) which was identified as fully unblocked phosphate diester.

δ$_p$[D$_2$O] 0.147.

δ$_H$[H$_2$O]: 1.82–2.02 (1 H, m), 2.18–2.24 (1 H, m), 2.44–2.50 (1 H, m), 2.70–2.77 (1 H, m), 3.58 (2 H, d, J 3.5), 4.00–4.06 (3 H, m), 4.15 (1 H, d, J 3.0), 5.74 (1 H, dd, J 5.8 and 9.8), 6.22 (1 H, t, J 7.8), 7.60 (1 H, s), 7.83 (1 H, s), 8.22 (1 H, s).

Rt (programme I) 3.28 min.

HO-GpCpG-OH (529)

Ac-Gp(s)Cp(s)G-Lev (80 mg, 47.12 µmol) was dissolved in dichloromethane (1.0 ml) followed by addition of DBU (0.141 ml, 0.942 mmol) at room temperature. After 30 min, a solution of trifluoroacetic acid in dichloromethane (10% w/v, 0.848 mmol, 0.967 ml) was added. After 5 min, the products were precipitated with diethyl ether.
The solid was collected by centrifugation and redissolved in dichloromethane (1.5 ml) and precipitated with diethyl ether (30 ml). This was repeated two more times and the residue was taken up with dichloromethane (1.0 ml) followed by addition of \( N,N \)-dimethylaniline (35.9 \( \mu l \), 0.283 mmol) and bromoacetonitrile (19.7 \( \mu l \), 0.283 mmol). The resulting solution was stirred at room temperature for 12 h and then concentrated under reduced pressure. The residue was purified by chromatography. The appropriate fractions, which were eluted with dichloromethane–methanol (97:3 v/v) were combined and concentrated under reduced pressure to give a colourless froth (40 mg). This material was dissolved in acetonitrile (0.5 ml) followed by addition of 2-nitrobenzaldoxime (0.159 mg, 0.958 mmol) and TMG (96.2 \( \mu l \), 0.767 mmol). After 15 h, the products were concentrated under reduced pressure and the residue was incubated in aqueous ammonia solution (1.0 ml, 33\%, d 0.88) at 55°C for 15 h. The products were cooled and concentrated under reduced pressure followed by azeotroping with ethanol (2×2 ml). The residue was taken up with methanol (1.0 ml) and precipitated with ethyl acetate (30 ml). The resulting solid was collected by centrifugation. This was repeated two more times and the residue was dried under reduced pressure. The solid residue was then dissolved in water (3 ml) and passed through an Amberlite (IR120, Na\(^+\) form) ion exchange column (1.0×10 cm). The appropriate fractions were concentrated under reduced pressure to give the title compound as a colourless solid (17 mg).

\[ \delta_p[H_2O] 0.20. \]

Rt (programme II) 7.04 min.

**HO-ApTpT-OH (526)**

\( \text{Ac-Ap(s)Tp(s)T-Lev (70 mg, ca. 49 \( \mu \text{mol}, A \) is a mixture of N-benzoyl and N-benzoyl-N-acetyl-2'-deoxyadenosine) was dissolved in dichloromethane (1.0 ml) followed by addition of DBU (0.148 ml, 0.990 mmol) at room temperature. After 30 min, a solution of trifluoroacetic acid in dichloromethane (10\% w/v, 1.016 ml, 0.891 mmol) was added and stirred for 5 min. The reaction mixture was precipitated with diethyl ether (40 ml). The solid was collected by centrifugation and redissolved in} \]
dichloromethane (1.5 ml) and precipitated with diethyl ether (30 ml). After this had been repeated two more times, the solid residue was dissolved in dichloromethane (1.0 ml) followed by addition of *N*,*N*-dimethylaniline (37.6 µl, 0.297 mmol) and bromoacetonitrile (20.7 µl, 0.297 mmol). The reaction mixture was stirred at room temperature overnight and the solvents were removed under reduced pressure. The residue was purified by chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (97:3 v/v) were combined and evaporated under reduced pressure to give a colourless froth (50 mg). The material obtained was then dissolved in dry acetonitrile (1.0 ml) followed by addition of 2-NBO (0.120 g, 0.720 mmol, 5 eq./function) and TMG (72.3 µl, 0.576 mmol, 4 eq./function). After 15 h, the reaction mixture was concentrated under reduced pressure and the residue was incubated in aqueous ammonia (1.0 ml, 33%, d 0.88) at 55°C for 15 h. After the reaction mixture had been cooled, it was concentrated under reduced pressure, followed by azeotroping with ethanol (2×2 ml). The residue was dissolved in methanol (1.0 ml) and precipitated with ethyl acetate (30 ml). The solid obtained was collected by centrifugation. This was repeated two more times and the colourless solid was then dried *in vacuo*. The material was dissolved in water (3 ml) and passed through an Amberlite (IR120, Na⁺ form) ion exchange column (1.5×10 cm). The nucleotide-containing fractions were pooled and concentrated under reduced pressure to a volume of ca. 2 ml. Upon freeze drying, a colourless powder was obtained (30 mg) which was identified as fully unblocked phosphate diester.

δ[H₂O] 0.090, 0.0069.

Rt (programme II): 10.60 min.

**HO-CpTpT-OH (530)**

Ac-Cp(s)Tp(s)T-Lev (45 mg, 32.86 µmol) was dissolved in dichloromethane (0.5 ml) followed by addition of DBU (98.2 µl, 0.657 mmol) at room temperature. After 30 min, a solution of trifluoroacetic acid in dichloromethane (10% w/v, 0.674 ml, 0.591 mmol) was added and stirred for 5 min. The reaction mixture was precipitated with diethyl ether (30 ml). The solid was collected by centrifugation and redissolved in
dichloromethane (1.0 ml) and precipitated with diethyl ether (30 ml). After this had been repeated two more times, the solid residue was dissolved in dichloromethane (0.5 ml) followed by addition of N,N-dimethylaniline (25.0 μl, 0.197 mmol) and bromoacetonitrile (13.7 μl, 0.197 mmol). The reaction mixture was stirred at room temperature overnight and the solvents were removed under reduced pressure. The residue was purified by chromatography on silica gel. The oligonucleotide-containing fractions, which were eluted with dichloromethane–methanol (96:4 v/v) were combined and evaporated under reduced pressure to give a colourless froth (25 mg). The material obtained was then dissolved in dry acetonitrile (0.5 ml) followed by addition of 2-NBO (0.062 g, 0.373 mmol, 5 eq./function) and TMG (37.4 μl, 0.298 mmol, 4 eq./function). After 15 h, the reaction mixture was concentrated under reduced pressure and the residue was then incubated in aqueous ammonia (1.0 ml, 33%, d 0.88) at 55°C for 15 h. After the reaction mixture had been cooled, it was concentrated under reduced pressure, followed by azeotroping with ethanol (2x2 ml). The residue was dissolved in methanol (1.0 ml) and precipitated with ethyl acetate (30 ml). The solid obtained was collected by centrifugation. This was repeated two more times and the colourless solid was dried in vacuo. The material was then dissolved in water (3 ml) and passed through an Amberlite (IR120, Na+ form) ion exchange column (1.5×10 cm). The nucleotide containing fractions were pooled and concentrated under reduced pressure to a volume of ca. 2 ml. Upon freeze drying, a colourless powder was obtained (16 mg) which was identified as fully unblocked phosphate diester.

\[ \delta_p [\text{H}_2\text{O}] = 0.116. \]

Rt (programme I): 3.74 min.

**HO-CpTpTpGpCpG-OH (532)**

Ac-Cp(s)Tp(s)Tp(s)Gp(s)Cp(s)G-Lev (50 mg, 16.34 μmol) was dissolved in dichloromethane (1.0 ml) followed by addition of DBU (0.122 ml, 0.817 mmol) at room temperature. After 30 min, a solution of trifluoroacetic acid in dichloromethane (10% w/v, 0.838 ml, 0.735 mmol) was added. After 5 min, the reaction mixture was
precipitated by diethyl ether (30 ml). The solid was collected by centrifugation and redissolved in dichloromethane (1.0 ml) and precipitated with diethyl ether (30 ml). After this process had been repeated two more times, the solid material was dried and redissolved in dichloromethane (1.0 ml) followed by addition of \(N,N\)-dimethylaniline (30.5 \(\mu\)l, 0.241 mmol) and bromoacetonitrile (16.8 \(\mu\)l, 0.241 mmol) at room temperature. After 15 h, the solvents were removed under reduced pressure and the residue was purified by chromatography. The appropriate fractions, which were eluted with dichloromethane–methanol (95:5 v/v), were combined and concentrated under reduced pressure to give a colourless froth (20 mg). This material was dissolved in dry acetonitrile (0.5 ml). 2-NBO (50.0 mg, 0.301 mmol) and TMG (30.2 \(\mu\)l, 0.241 mmol) were added. The reaction mixture was stirred at room temperature overnight and then concentrated under reduced pressure. The residue was heated in aqueous ammonia (0.6 ml, 33%, \(d\) 0.88) at 55°C for 15 h. After the reaction mixture had been cooled, it was concentrated under reduced pressure, followed by azeotroping with ethanol (2×2 ml). The residue was dissolved in methanol (1.0 ml) and precipitated with ethyl acetate (30 ml) and the solid obtained was collected by centrifugation. This was repeated two more times and the colourless solid was dried \textit{in vacuo}. The material was then dissolved in water (3 ml) and was passed through an Amberlite (IR120, Na\(^+\) form) ion exchange column (1.5×10 cm). The nucleotide-containing fractions were pooled and concentrated under reduced pressure to a volume of ca. 2 ml. Upon freeze drying, a colourless powder was obtained (10 mg) which was identified as fully unblocked phosphate diester.

\[\delta_{\text{H}[\text{H}_2\text{O}]}\]\begin{align*} & 0.585, 0.255, -0.041, -0.120, -0.625. \end{align*}

\text{Rt (programme II): 9.64 min.}


Ac-I2-Lev (50 mg, 8.648 \(\mu\)mol) was dissolved in dichloromethane (1.0 ml) followed by addition of DBU (0.142 ml, 0.951 mmol) at room temperature. After 30 min, a solution of trifluoroacetic acid in dichloromethane (10% w/v, 0.976 ml, 0.856 mmol) was added and stirred for 5 min. The reaction mixture was precipitated by diethyl
ether (30 ml). The solid was collected by centrifugation and redissolved in dichloromethane (1.5 ml) and precipitated with diethyl ether (30 ml). After this process had been repeated two more times, the solid material was dried in vacuo and redissolved in dichloromethane (1.0 ml), followed by addition of \(N,N\)-dimethylaniline (60.3 \(\mu\)l, 0.476 mmol) and bromoacetonitrile (33.2 \(\mu\)l, 0.476 mmol). The resulting solution was stirred at room temperature overnight and concentrated under reduced pressure. The residue was purified by chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (96:4 v/v) were combined and concentrated under reduced pressure to give a colourless glass (47 mg).

This material was dissolved in acetonitrile (1.0 ml) followed by addition of 2-NBO (0.132 g, 0.793 mmol) and TMG (79.7 \(\mu\)l, 0.635 mmol), and the reaction mixture was stirred at room temperature for 15 h. The solvent was removed under reduced pressure and the residue was heated in conc. aqueous ammonia (1.5 ml, 33\%, \(d\) 0.88) at 55\(^\circ\)C for 15 h. After the reaction mixture had been chilled, it was concentrated under reduced pressure, followed by co-evaporation with ethanol (2\(\times\)2 ml). The residue was dissolved in methanol (2.0 ml) and precipitated with ethyl acetate (40 ml). The solid was collected by centrifugation and this was repeated two more times. The solid material obtained was then dried in vacuo. Water (3 ml) was added and the solution was run through an Amberlite (IR120, Na\(^+\) form) ion exchange column (1.0\(\times\)10 cm). The appropriate fractions were concentrated under reduced pressure to give the title compound as a colourless solid (19 mg).

\[\delta_p[H_2O]\ 0.245, 0.188, 0.104, 0.015, -0.096, -0.223.\]

Rt (programme I): 10.40 min.

\[\text{HO-GpCpGpTpTpGpCpTpCpTcTpCpTcTpCpTcTpCpGpCpG-OH} \ (21\text{-mer, 531})\]

Ac-21-Lev (70 mg, 6.76 \(\mu\)mol) was dissolved in dichloromethane (1.0 ml) followed by addition of DBU (0.202 ml, 1.353 mmol) at room temperature. After 30 min, a solution of trifluoroacetic acid in dichloromethane (10\% w/v, 1.39 ml, 1.217 mmol) was added and stirred for 5 min. Diethyl ether (40 ml) was added. The solid was
collected by centrifugation and redissolved in dichloromethane (1.5 ml) and precipitated with diethyl ether (30 ml). This was repeated two more times and the solid obtained was dried *in vacuo*. This material was dissolved in dichloromethane (1.0 ml) followed by addition of *N*,*N*-dimethylaniline (103 µl, 0.811 mmol) and bromoacetonitrile (56.5 µl, 0.811 mmol) at room temperature. After 15 h, the reaction mixture was concentrated under reduced pressure and the residue was purified by chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (92:8 v/v) were combined and concentrated under reduced pressure to give a colourless froth (37 mg). This material was dissolved in acetonitrile (1.0 ml), followed by addition of 2-nitrobenzaldoxime (107 mg, 0.643 mmol) and TMG (64.5 µl, 0.514 mmol). The reaction mixture was stirred at room temperature overnight and then concentrated under reduced pressure. The residue was then heated in conc. aqueous ammonia (33%, *d* 0.88, 1.5 ml) at 55°C for 15 h. After it had been chilled, the reaction mixture was concentrated under reduced pressure followed by azeotroping with ethanol (2×2 ml). The residue was dissolved in methanol (3 ml) and precipitated with ethyl acetate (40 ml). The precipitate was collected by centrifugation and this was repeated two more times. The solid obtained was dried *in vacuo* and dissolved in water (4 ml). The resulting solution was passed through an Amberlite (IR120, Na⁺ form) ion exchange column (3×20 cm). The appropriate fractions were concentrated under reduced pressure to give the *title compound* as a colourless solid (20 mg).

δ<sub>t</sub>[H<sub>2</sub>O] 1.323, 0.233, 0.201, 0.172, 0.106, 0.088, 0.002, −0.114, −0.244, −0.486.

Rt (programme I): 3.12 min.
4,4'-Dimethoxybenzophenone (8 g, 0.033 mol) was heated, under reflux, in thionyl chloride (37 ml) and DMF (0.2 ml) for 4 h. The excess of thionyl chloride was removed under reduced pressure. The residue was co-evaporated with dry toluene (2×10 ml) and further dried with an oil pump. The resulting off-white solid was dissolved in anhydrous THF (80 ml) and added dropwise to cooled (ice-water bath), stirred methanolic sodium methoxide (27%, 35 ml) over a period of 30 min. The reactants were allowed to warm up to room temperature and stirring was continued for a further 1 h. The products were then evaporated to dryness under reduced pressure. The residue was dissolved in dichloromethane (100 ml) and washed with saturated aqueous sodium hydrogen carbonate (3×100 ml). The dried (MgSO₄) organic layer was concentrated under reduced pressure to give the title compound as a yellow solid (7.85 g, 82%), mp 104–106°C (ethanol).

δ_H[CDCl₃]: 3.09 (6 H, s), 3.77 (6 H, s), 6.82 (4 H, d), 7.38 (4 H, d).

δ_C[CDCl₃]: 49.18 (CH₃), 55.24 (CH₃), 102.78 (C), 113.29 (CH), 128.17 (CH), 135.05 (C), 158.83 (C).

9,9-Dichloroxanthene (627a)
Xanthone (19.62 g, 0.1 mol) in thionyl chloride (40 ml) was heated under reflux for 4 h. The excess of thionyl chloride was removed by distillation under atmospheric pressure and then evaporation under reduced pressure (water pump). The residue was co-evaporated with dry toluene (240 ml) and was then further dried at 60°C with an oil pump for 1 h to give the title compound as a light yellow solid (25.50 g, ca. 100%). No attempt has been made to characterise this compound.

9,9-Dimethoxyxanthene (628a)

![9,9-Dimethoxyxanthene (628a)]

To a methanolic solution of sodium methoxide (25%, 76.3 ml, 2.83 mol) cooled to 0°C, a solution of 9,9-dichloroxanthene (25.5 g, 0.1 mol) in tetrahydrofuran (100 ml) was added dropwise during a period of 30 min under nitrogen. After the addition was complete, the reaction mixture was allowed to warm up to room temperature and stirred for a further 1 h. The products were then concentrated to dryness under reduced pressure. The residue was dissolved in dichloromethane (150 ml) and washed with saturated aqueous sodium hydrogen carbonate (3×100 ml). The dried organic layer (MgSO₄) was concentrated under reduced pressure to give the title compound (Found, in material recrystallized from ethanol–triethylamine: C, 74.19; H, 5.64%. C₁₅H₁₄O₃ requires: C, 74.36; H, 5.82) as a pale yellow solid (22.9 g, 94.5%), mp 59–61°C.

δ_H[CDCl₃]: 2.94 (6 H, s), 7.23 (4 H, m), 7.42 (2 H, m), 7.74 (2 H, m).

δ_C[CDCl₃]: 51.81 (CH₃), 96.60 (C), 116.51 (CH), 118.77 (C), 123.38 (CH), 127.36 (CH), 130.20 (CH), 153.23 (C).
2,7-Dimethylxanthone (615b)

Oxaloyl chloride (12.5 g, 8.6 ml, 0.098 mol), followed by well-powdered aluminium chloride (5 g, 37.5 mmol) was added to a cooled (ice – water bath), stirred solution of di-p-tolyl ether (5 g, 0.0252 mol) in carbon disulphide (37 ml). After 2 h, more aluminium chloride (4.0 g, 30.0 mmol) was added and the reactants were allowed to warm up to room temperature. After 16 h, the products were added slowly to cooled (ice–water bath), stirred hydrochloric acid (ca. 3.2 M, 120 ml). After 30 min, the organic layer was separated and the aqueous layer was back-extracted with dichloromethane (5×50 ml). The combined organic layers were dried (MgSO₄) and concentrated under reduced pressure. The residue was stirred with saturated aqueous sodium hydrogen carbonate (100 ml) at 60°C for 20 min. The precipitated solid was collected by filtration and recrystallized from ethanol to give the title compound as colourless needles (5.1 g, 90%), mp 139–141°C.

δ_H[CDCl₃]: 2.46 (6 H, s), 7.37 (2 H, d, J 8.6), 7.51 (2 H, dd, J 2.2 and 8.5), 8.11 (2 H, d, J 1.3).

δ_C[CDCl₃]: 20.84 (CH₃), 117.71 (CH), 121.38 (C), 125.96 (CH), 133.43 (C), 135.89 (CH), 154.37 (C), 177.32 (C).
2,7-Dimethyl-9,9-dichloroxanthene (627b)

2,7-Dimethylxanthone (5 g, 0.022 mol) was heated, under reflux, in thionyl chloride (25 ml) and DMF (ca. 0.2 ml) for 4 h. the products were evaporated to dryness under reduced pressure and the residue was co-evaporated with dry toluene (2×15 ml) to give the title compound as a pink solid (6.1 g, ca. 100%). No attempt has been made to characterise this compound.

2,7-Dimethyl-9,9-dimethoxyxanthene (628b)

A solution of 2,7-dimethyl-9,9-dichloroxanthene (6.1 g, 0.022 mol) in anhydrous THF (50 ml) was added dropwise to a cooled (ice-water bath), stirred solution of sodium methoxide in methanol (27%, 25 ml, ca. 0.11 mol) over a period of 30 min. The reactants were then allowed to warm up to room temperature and, after a further period of 1 h, the products were concentrated to dryness under reduced pressure. The residue was dissolved in dichloromethane (100 ml) and washed with saturated aqueous sodium hydrogen carbonate (3×100 ml). The aqueous layer was back extracted with dichloromethane (2×100 ml). The combined organic layers were dried (MgSO₄) and evaporated under reduced pressure to give the title compound (Found, in material recrystallized from ethanol-triethylamine: C, 75.54; H, 6.66%. C₁₇H₁₈O₃ requires: C, 75.53; H, 6.71%) as a yellow solid (5.5 g, 91%), mp 81–82.5°C.
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δ_H[DCl_3]: 2.40 (6 H, s), 2.92 (6 H, s), 7.08 (2 H, d, J 8.3), 7.20 (2 H, d, J 8.3), 7.50 (2 H, s).

δ_C[DCl_3]: 153.23 (C), 132.64 (C), 131.09 (CH), 126.98 (CH), 118.16 (C), 116.21 (CH), 96.94 (C), 51.84 (CH_3), 20.91 (CH_3).

2',3'-O-[Di-(p-anisyl)methylene]uridine (612)

Uridine (0.3 g, 1.23 mmol) and dimethoxydi-(p-anisyl)methane (0.459 g, 1.59 mmol) were co-evaporated with dry acetonitrile (2×10 ml). The residue was then suspended in dry acetonitrile (20 ml) followed by addition of (±)-10-camphorsulphonic acid (20 mg). After the reactants had been stirred at room temperature for 2 h, triethylamine (0.5 ml) was added. The products were then concentrated under reduced pressure. The residue was partitioned between dichloromethane (20 ml) and saturated aqueous sodium hydrogen carbonate (2×20 ml). The layers were separated and the dried (MgSO_4) organic layer was evaporated under reduced pressure. The residue was purified by chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (9:1 v/v) were combined and concentrated under reduced pressure to give the title compound (Found, in material recrystallized from dichloromethane: C, 61.1; H, 5.2; N, 5.65. C_{24}H_{24}N_2O_8·0.2 H_2O requires C, 61.06; H, 5.21; N, 5.93%; mp 112–114°C) as a colourless solid (0.415 g, 95%).

δ_H[(CD_3)_2SO]: 3.55–3.61 (2 H, m), 3.75 (3 H, s), 3.76 (3 H, s), 4.26 (1 H, m), 4.69 (1 H, dd, J 3.2 and 6.6), 4.90 (1 H, dd, J 2.6 and 6.6), 5.11 (1 H, t, ex, J 5.4), 5.62 (1 H,
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\[ \text{d, } J 2.6 \), 6.00 (1 H, d, \text{ J } 2.6 \), 6.88–6.96 (4 H, m), 7.29–7.37 (4 H, m), 7.81 (1 H, d, \text{ J } 8.1 \), 11.40 (1 H, s, ex).

\[ \delta_{c [(\text{CD}_3)_2\text{SO}]}: 55.12 (\text{CH}_3), 61.35 (\text{CH}_2), 81.58 (\text{CH}), 84.14 (\text{CH}), 86.27 (\text{CH}), 91.38 (\text{CH}), 101.72 (\text{CH}), 113.34 (\text{CH}), 113.62 (\text{CH}), 127.34 (\text{CH}), 127.62 (\text{CH}), 133.34 (\text{C}), 142.30 (\text{C}), 150.33 (\text{C}), 159.14 (\text{C}), 159.28 (\text{C}), 163.17 (\text{C}). \]

\[ R_f \text{(System B): } 0.53. \]

2',3'-O-(Xanthen-9-ylidene)uridine (616a)

\[ \text{Uridine (0.3 g, 1.23 mmol) and 9,9-dimethoxyxanthene (0.44 g, 1.84 mmol) were co-evaporated with acetonitrile (2×10 ml) and then suspended in acetonitrile (20 ml) followed by addition of (±)-10-camphorsulphonic acid (20 mg). After the reaction mixture had been stirred for 2 h under argon, triethylamine (0.5 ml) was added. The products were concentrated under reduced pressure and the residue was partitioned between dichloromethane (20 ml) and saturated aqueous sodium hydrogen carbonate (2×20 ml). The layers were separated and the dried (MgSO}_4 \text{) organic layer was concentrated under reduced pressure. The residue was purified by chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane-methanol (98:2 v/v) were combined and concentrated under reduced pressure to give the title compound (Found, in material recrystallized from dichloromethane: C, 61.3; H, 4.2; N, 6.4. } C_{22}H_{18}N_2O_7-0.5H_2O \text{ requires: C, 61.25; H, 4.44; N, 6.50%; mp 152–153°C) as a colourless solid (0.46 g, 88%).} \]
$\delta_H\{(CD_3)_2SO\}: 3.68 (2 \text{ H, m}), 4.40 (1 \text{ H, m}), 5.22 (2 \text{ H, m}), 5.48 (1 \text{ H, dd, } J 2.5 \text{ and } 6.4), 5.70 (1 \text{ H, d, } J 8.0), 6.14 (1 \text{ H, d, } J 2.5), 7.35 (4 \text{ H, m}), 7.55 (1 \text{ H, dd, } J 1.2 \text{ and } 7.8), 7.75 (1 \text{ H, dd, } J 1.2 \text{ and } 2.5), 7.92 (2 \text{ H, m}), 11.48 (1 \text{ H, br, s}).$

$\delta_C\{(CD_3)_2SO\}: 61.38 (\text{CH}_2), 82.54 (\text{CH}), 85.44 (\text{CH}), 86.39 (\text{CH}), 91.36 (\text{CH}), 101.73 (\text{CH}), 105.12 (\text{C}), 116.55 (\text{CH}), 116.83 (\text{CH}), 121.09 (\text{C}), 122.68 (\text{C}), 123.66 (\text{CH}), 123.89 (\text{CH}), 125.52 (\text{CH}), 126.43 (\text{CH}), 130.43 (\text{CH}), 130.97 (\text{CH}), 142.06 (\text{CH}), 150.28 (\text{C}), 150.48 (\text{C}), 151.71 (\text{C}), 163.30 (\text{C}).$

$R_f$ (System B): 0.54.

$2',3'-\text{O-}(2,7\text{-Dimethylxanthen}-9\text{-ylidene})\text{uridine (616b)}$

![616b](image)

Uridine (300 mg, 1.23 mmol) and 2,7-dimethyl-9,9-dimethoxyxanthene (0.54 g, 2.0 mmol) were co-evaporated with dry acetonitrile (2×10 ml). The residue was suspended in dry acetonitrile (15 ml) followed by addition of (±)-10-camphorsulphonic acid (20 mg). After 4 h, triethylamine (0.5 ml) was added. The products were then concentrated under reduced pressure. The residue was partitioned between dichloromethane (30 ml) and saturated aqueous sodium hydrogen carbonate (2×20 ml). The layers were separated. The dried (MgSO$_4$) organic layer was evaporated under reduced pressure. The residue was then fractionated by chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (98:2 v/v) were combined and concentrated under reduced pressure to give the title compound (Found, in material recrystallized from
dichloromethane: C, 63.9; H, 4.8; N, 6.1. C_{24}H_{22}N_{2}O_{7} requires: C, 64.00; H, 4.92; N, 6.22%) as a colourless solid (0.39 g, 70%), mp 153–155°C.

δ_{H}[(CD_{3})_{2}SO]: 2.39 (3 H, s), 2.40 (3 H, s), 3.63–3.70 (2 H, m), 4.36 (1 H, m), 5.14 (1 H, dd, J 2.8 and 6.3), 5.22 (1 H, t, ex, J 5.3), 5.45 (1 H, dd, J 2.9 and 6.3), 5.69 (1 H, dd, J 1.9 and 8.0), 6.15 (1 H, d, J 2.7), 7.20–7.29 (2 H, m), 7.31–7.36 (2 H, m), 7.49 (1 H, s), 7.69 (1 H, s), 7.90 (2 H, d, J 8.0), 11.47 (1 H, br, s).

δ_{c}[(CD_{3})_{2}SO]: 20.52 (CH_{3}), 61.32 (CH_{2}), 82.28 (CH), 85.26 (CH), 86.05 (CH), 90.96 (CH), 101.74 (CH), 105.38 (C), 116.26 (CH), 116.54 (CH), 120.67 (C), 122.28 (C), 125.21 (CH), 126.10 (CH), 130.97 (CH), 131.54 (CH), 132.57 (C), 132.69 (C), 141.83 (CH), 148.43 (C), 149.88 (C), 150.52 (C), 163.24 (C).

R_{f} (System B): 0.62.

(±)-1,2-O-(Xanthen-9-ylidene)glycerol (630a)

Glycerol (0.74 ml, 10 mmol) and 9,9-dimethoxyxanthene (1.21 g, 5 mmol) were co-evaporated with dry acetonitrile (10 ml). The residue was then taken up with dry acetonitrile (20 ml). (±)-10-Camphorsulphonic acid (10 mg) was added and the mixture was allowed to stir under argon at room temperature. After 4 h, triethylamine (0.1 ml) was added and the resulting reaction mixture was concentrated under reduced pressure. The residue was partitioned between dichloromethane (20 ml) and saturated sodium hydrogen carbonate (2×10 ml). The dried (MgSO_{4}) organic layer was concentrated and the residue was purified by chromatography on silica gel: the
appropriate fractions, which were eluted with dichloromethane–methanol (99:1 v/v) were combined and evaporated under reduced pressure to give the *title compound* (Found, in material recrystallized from ethyl acetate–petroleum spirit (bp 40–60 °C): C, 70.8; H, 5.2. C\textsubscript{16}H\textsubscript{14}O\textsubscript{4} requires: C, 71.10; H, 5.22%) as a colourless solid (1.1 g, 81.4%). mp 108–109.5 °C; R\textsubscript{f} (system B) 0.52; \(\lambda_{\text{max}}\) (EtOH)/nm 288 (c 3680).

\[\delta_{\text{H}}[(\text{CD}_{3})\text{SO}] : 3.67–3.80 \text{ (2 H, m)}, 4.05 \text{ (1 H, t, } J 7.9), 4.38 \text{ (1 H, dd, } J 6.3 \text{ and } 7.9), 4.58–4.65 \text{ (1 H, m)}, 5.14 \text{ (1 H, t, ex, } J 5.6), 7.26–7.33 \text{ (4 H, m)}, 7.46–7.51 \text{ (2 H, m)}, 7.72 \text{ (1 H, dd, } J 1.3 \text{ and } 7.8), 7.89 \text{ (1 H, dd, } J 1.3 \text{ and } 7.8).\]

\[\delta_{\text{C}}[(\text{CD}_{3})\text{SO}] : 61.07 \text{ (CH\textsubscript{2})}, 67.37 \text{ (CH\textsubscript{2})}, 78.48 \text{ (CH)}, 100.24 \text{ (C)}, 116.17 \text{ (CH)}, 116.44 \text{ (CH)}, 123.22 \text{ (C)}, 123.48 \text{ (CH)}, 123.84 \text{ (C)}, 126.46 \text{ (CH)}, 127.02 \text{ (CH)}, 130.05 \text{ (CH)}, 130.22 \text{ (CH)}, 150.65 \text{ (C)}, 151.15 \text{ (C)}.\]

\((\pm)-1,2-O-(2,7-Dimethylxanthen-9-ylidene)glycerol\) (630b)

\[\begin{align*}
\text{O} & \quad \text{OH} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O}
\end{align*}\]

2,7-Dimethyl-9,9-dimethoxyxanthene (0.81 g, 3 mmol) and glycerol (0.369 g, 4 mmol) were co-evaporated with dry acetonitrile (2×10 ml) and the residue was redissolved in anhydrous acetonitrile (20 ml). Then (±)-10-camphorsulphonic acid (10 mg) was added and the mixture was allowed to stir under argon at room temperature. After 4 h, triethylamine (0.1 ml) was added. The reaction mixture was concentrated under reduced pressure. The residue was dissolved in dichloromethane (20 ml) and washed with saturated sodium hydrogen carbonate (2×10 ml). The dried (MgSO\textsubscript{4}) dichloromethane layer was concentrated under reduced pressure. The residue was purified by chromatography on silica gel. The appropriate fractions,
which were eluted with dichloromethane – methanol (99:1 v/v) gave the *title compound* (Found, in material recrystallized from ethyl acetate – petroleum spirit (bp 40–60°C): C, 71.6; H, 6.2. C_{18}H_{18}O_4·0.2 H_2O requires: C, 71.60; H, 6.14%) as a colourless solid (0.60 g, 67%), mp 137–138°C, R_f (system B) 0.60. λ_{max} (EtOH)/nm 297 (ε 3910).

δ_{H}[(CD_3)_2SO]: 2.34 (3 H, s), 2.36 (3 H, s), 3.67–3.78 (2 H, m), 4.03 (1 H, t, J 7.9), 4.39 (1 H, dd, J 6.3 and 7.8), 4.58–4.62 (1 H, m), 5.11 (1 H, t, ex, J 5.6), 7.15 (2 H, dd, J 5.3 and 8.4), 7.24 (2 H, dd, J 1.9 and 8.3), 7.47 (1 H, d, J 1.4), 7.63 (1 H, d, J 1.6).

δ_{C}[(CD_3)_2SO]: 20.46 (CH_3), 20.50 (CH_3), 61.06 (CH_2), 67.47 (CH_2), 78.27 (CH), 100.46 (C), 115.84 (CH), 116.09 (CH), 122.72 (C), 123.37 (C), 126.18 (CH), 126.72 (CH), 130.58 (CH), 130.76 (CH), 132.13 (C), 132.23 (C), 148.69 (C), 149.21 (C).

1,2,5,6-Di-O-(xanthen-9-ylidene)-D-mannitol (631a)

D-Mannitol (1.82 g, 10 mmol) was azeotroped with dry pyridine (20 ml) and the residue was suspended in dry pyridine (30 ml) and cooled to 0°C (ice–water bath). 9,9-Dichloroxanthene (6.32 g, 50 mmol) was added and after 10 min the reaction mixture was allowed to warm up to room temperature. After 1 h, the products were poured into saturated aqueous sodium hydrogen carbonate (150 ml). After the resulting mixture had been stirred at room temperature for 1 h, the precipitate was collected by filtration and washed with water (340 ml). The air-dried solid was
suspended in ethyl acetate (25 ml) with stirring for 10 min and petroleum spirit (bp 40–60°C, 25 ml) was added. After 1 h, the mixture was filtered and the residue was washed with ethyl acetate–petroleum spirit (1:1 v/v, 40 ml) to give the title compound as an off-white solid (4.55 g, 84.5%) (Found in material recrystallized from ethyl acetate: C, 71.0; H, 4.8. C_{32}H_{26}O_{8} requires C, 70.89; H, 4.91%) mp 222.5–224°C.

δ_{H}[(CD_{3})_{2}SO]: 3.83 (2 H, t, J 7.7), 4.13 (2 H, m), 4.31 (2 H, dd, J 6.2, 8.3), 4.62 (2 H, dd, J 6.9 and 13.9), 5.25 (2 H, d, ex, J 7.7), 6.81 (2 H, m), 7.22 (6 H, m), 7.34 (2 H, m), 7.41 (2 H, m), 7.64 (4 H, dd, J 1.3 and 7.8).

δ_{C}[(CD_{3})_{2}SO]: 68.60 (CH_{2}), 70.91 (CH), 77.24 (CH), 100.46 (C), 116.56 (CH), 116.79 (CH), 123.40 (C), 123.73 (CH), 123.80 (CH), 124.02 (C), 126.79 (CH), 127.04 (CH), 130.43 (CH), 130.51 (CH), 150.97 (C), 151.52 (C).

R_{f} (System B): 0.53.

(S)-(+)-1,2-O-(Xanthen-9-ylidene)glycerol (632a)

\[
\begin{align*}
\text{632a}
\end{align*}
\]

Lead (IV) acetate (9.05 g, 20.4 mmol) was added in one portion to a stirred, cooled (ice–water bath) mixture of 1,2:5,6-di-O-(xanthen-9-ylidene)-D-mannitol (3.15 g, 5.83 mmol), sodium hydrogen carbonate (1.96 g, 23.3 mmol) and ethyl acetate (115 ml). After 1 h, the products were filtered through a bed of Celite (20 g) and the residue was washed with ethyl acetate (30 ml). The combined filtrate and washings were added dropwise over a period of 15 min to a stirred solution of sodium borohydride (1.75 g, 47.3 mmol) in ethanol (115 ml) at 0°C. After a further period of 1 h, sodium hydroxide pellets (0.8 g) and then 1.0 M aqueous sodium hydroxide (50
ml) were added with continued stirring. The products were filtered and the layers were separated. The aqueous layer was extracted with dichloromethane (3×20 ml) and the combined organic layers were concentrated to dryness. When petroleum spirit (bp 40–60°C, 100 ml) was added to a solution of the residue in dichloromethane (20 ml), the title compound (Found, in material recrystallized from ethyl acetate–petroleum spirit (bp 40–60°C): C, 70.85; H, 5.1. C₁₆H₁₄O₄ requires: C, 71.10; H, 5.22%) was obtained as a colourless solid, mp 105–107°C. Rᵣ (System B): 0.59, [α]̅D +15.7 (c 1.5, ethanol).

δ_H[(CD₃)₂SO]: 3.66–3.78 (2 H, m), 4.03 (1 H, t, J 7.9), 4.37 (1 H, dd, J 6.3 and 7.9), 4.58–4.64 (1 H, m), 5.11 (1 H, t, ex, J 5.6), 7.25–7.32 (4 H, m), 7.46–7.51 (2 H, m), 7.70 (1 H, dd, J 1.3 and 7.8), 7.87 (1 H, dd, J 1.3 and 7.8).

δ_C[(CD₃)₂SO]: 60.95 (CH₂), 67.26 (CH₂), 78.36 (CH), 100.09 (C), 116.05 (CH), 116.32 (CH), 123.09 (C), 123.37 (CH), 123.72 (C), 126.34 (CH), 126.89 (CH), 129.94 (CH), 130.11 (CH), 150.51 (C), 151.02 (C).

(R̄⁻)(−)-1,2-O-(Xanthen-9-ylidene)glycerol (634a)

A solution of L-ascorbic acid (2.67 g, 15.2 mmol) and 9,9-dimethoxyxanthene (4.5 g, 18.6 mmol) in acetonitrile (20 ml) was evaporated under reduced pressure. The residue was suspended in dry acetonitrile (40 ml) and (±)-10-camphorsulphonic acid (50 mg, 0.22 mmol) was added. The reactants were heated, under reflux, in an atmosphere of nitrogen for 3 h. The resulting mixture was evaporated to half volume and lithium carbonate (2.29 g, 31 mmol) was added. The resulting mixture was
evaporated to dryness under reduced pressure and the residue was dissolved in water (70 ml). Aqueous hydrogen peroxide (25%, 5.5 ml, 48 mmol) was added dropwise over 5 min to the stirred, cooled (ice–water bath) solution. After a further period of 10 minutes, the reactants were allowed to warm up to room temperature. After 16 h, the products were filtered and the filtrate was concentrated to dryness under reduced pressure. After it had been co-evaporated with absolute ethanol (220 ml), the residue was extracted with boiling ethanol (270 ml). The extract was concentrated to dryness under reduced pressure. The residue was dissolved in ethyl acetate (60 ml) and sodium hydrogen carbonate (2.56 g, 30.5 mmol) was added. Lead (IV) acetate (6.8 g, 15.2 mmol) was then added in two portions to the cooled (ice–water bath), stirred mixture. After 30 min, the reactants were allowed to warm up to room temperature. After a further period of 3 h, the products were cooled (ice–water bath) and filtered through a bed of Celite. The residue was washed with ice-cold ethyl acetate (40 ml). The combined filtrate and washings were added dropwise to a cooled (ice–water bath), stirred suspension of sodium borohydride (0.756 g, 20 mmol) in absolute ethanol (80 ml). The reactants were then allowed to warm up to room temperature. After a further period of 3 h, aqueous sodium hydroxide (1.0 M, 40 ml) was added to the products and stirring was continued for an additional 30 min. The layers were separated and the aqueous layer was extracted with dichloromethane (4×60 ml). The combined organic layers were concentrated under reduced pressure and the residue was partitioned between dichloromethane (50 ml) and water (50 ml). The aqueous layer was extracted with dichloromethane (2×30 ml). The combined organic layers were dried (MgSO₄) and evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane, were combined and evaporated under reduced pressure to give the title compound as a colourless solid (1.7 g, 41.5% overall yield) (Found, in material recrystallized from ethyl acetate–petroleum spirit (bp 40–60°C): C, 70.7; H, 5.3. C₁₆H₁₄O₄·0.1 H₂O requires C, 70.63; H, 5.26%), mp 105–106°C, Rₐ 0.52 (system B), [α]₀° –15.3 (c 1.5, ethanol). The ¹H and ¹³C NMR spectra of this compound were identical to the corresponding spectra of the racemic material.
1,2:5,6-Di-O-(2,7-dimethylxanthen-9-ylidene)-D-mannitol (631b)

A suspension of D-mannitol (0.364 g, 2.0 mmol) in dry pyridine (2x5 ml) was evaporated under reduced pressure. The residue was suspended in dry pyridine (10 ml) and cooled (ice-water bath). 9,9-Dichloro-2,7-dimethylxanthene (1.34 g, ca. 4.8 mmol) was added. After 10 min, the reactants were allowed to warm up to room temperature with continued stirring. After a further period of 2 h, methanol (1 ml) was added and the products were concentrated under reduced pressure. The residue was partitioned between dichloromethane (50 ml) and saturated aqueous sodium hydrogen carbonate (50 ml). The dried (MgSO₄) organic layer was evaporated under reduced pressure. The residue was purified by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (99:1 v/v) were combined and evaporated under reduced pressure to give the title compound as a colourless froth (0.97 g, 81%) (Found: M⁺, 594.2231, ¹²C₃₆H₃₄O₈ requires m/z 594.2254), Rₚ (system B) 0.59.

δ[CD₃SO]: 2.00 (6 H, s), 2.38 (6 H, s), 3.94 (2 H, d, J 7.7), 4.19 (2 H, t, J 7.8), 4.42 (2 H, m), 4.73 (2 H, m), 5.38 (2 H, s, ex, J 7.7), 7.11–7.19 (6 H, m), 7.27 (2 H, m), 7.49 (4 H, d, J 6.9).

δ[(CD₃)₂SO]: 19.99 (CH₃), 20.44 (CH₃), 68.16 (CH₂), 70.61 (CH), 76.93 (CH), 100.51 (C), 115.80 (CH), 116.07 (CH), 122.52 (CH), 123.21 (C), 126.19 (CH), 126.43 (CH), 130.57 (CH), 130.64 (CH), 132.13 (C), 148.66 (C), 149.13 (C).
(S)-(+)-1,2-O-(2,7-Dimethylxanthen-9-ylidene)glycerol (632b)

Water (1.5 ml), sodium hydrogen carbonate (0.05 g, 0.6 mmol) and sodium metaperiodate (0.236 g, 1.1 mmol) were added to a stirred solution of 1,2:5,6-di-O-(2,7-dimethylxanthen-9-ylidene)-D-mannitol (0.330 g, 0.55 mmol) in THF (5 ml) at room temperature. After 3 h, the products were concentrated under reduced pressure and the residue was partitioned between dichloromethane (15 ml) and saturated aqueous sodium hydrogen carbonate (15 ml). The aqueous layer was separated and back-extracted with dichloromethane (2×5 ml). The combined organic layers were dried (MgSO₄) and added dropwise over a period of 30 min to a suspension of sodium borohydride (0.083 g, 2.2 mmol) in ethanol (5 ml). After 30 min, acetone (1 ml) was added. After a further period of 10 min, the products were evaporated under reduced pressure. The residue was partitioned between dichloromethane (15 ml) and saturated aqueous sodium hydrogen carbonate (15 ml). The layers were separated and the aqueous layer was back-extracted with dichloromethane (2×5 ml). The combined organic layers were dried (MgSO₄) and concentrated under reduced pressure. The residue was fractionated by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (99:1 v/v) were combined and evaporated under reduced pressure to give the title compound as a colourless glass (0.230 g, 69%), which later solidified (found, in material recrystallized from ethyl acetate–petroleum spirit (bp 40–60°C): C, 72.2; H, 6.0. C₁₈H₁₈O₄ requires: C, 72.47; H, 6.08%), mp 143–144°C; R₇ (system B) 0.60; [α]₂₀+18.2 (c 1.5, ethanol). The ¹H and ¹³C NMR spectra were identical to the corresponding spectra obtained from the racemic material.
L-Ascorbic acid (1.48 g, 8.3 mmol) was heated, under reflux, with 9,9-dimethoxy-2,7-dimethylxanthene (2.7 g, 10.0 mmol) in the presence of (±)-10-camphorsulphonic acid (20 mg, 0.09 mmol) in dry acetonitrile (30 ml) for 3 h as in the above preparation of \((\mathcal{R})(-)-1,2-O-(xanthene-9-ylidene)glycerol\). Subsequent reactions with aqueous hydrogen peroxide, lead (IV) acetate in ethyl acetate and sodium borohydride in ethanol–ethyl acetate, as in the above preparation of \((\mathcal{R})(-)-1,2-O-(xanthene-9-ylidene)glycerol\) and with the same stoichiometry gave, after chromatography, the title compound as a colourless solid (0.800 g, 32% overall yield) (Found, in material recrystallized from ethyl acetate – petroleum spirit (bp 40–60°C): C, 72.4; H, 6.1. \(C_{18}H_{18}O_4\) requires: C, 72.47; H, 6.08%), mp 143–144°C; \(R_f\) (System B) 0.60; \([\alpha]_D^0\) –18.2 (c 1.5, ethanol). The \(^1H\) and \(^{13}C\) NMR spectra of this compound were identical to the corresponding spectra of the racemic material.

\((\pm)-1-O\text{-Stearoylglycerol} \ (635)\)

To a stirred solution of \((\pm)-1,2-O-(xanthene-9-ylidene)glycerol\) (0.811 g, 3.0 mmol) in dichloromethane (15 ml), 1-methylimidazole (0.477 ml, 6.0 mmol) was added, followed by addition of a solution of stearoyl chloride (1.090 g, 3.6 mmol) in dichloromethane (15 ml). After 1.5 h, triethylamine (1.0 ml) and water (0.2 ml) were added and the reaction mixture was stirred for 10 min and then poured into saturated
aqueous sodium hydrogen carbonate (15 ml). The layers were separated and the organic layer was further washed with saturated aqueous sodium hydrogen carbonate (15 ml). The dried (MgSO₄) organic layer was concentrated under reduced pressure. The residue was stirred in methanol (15 ml) for 30 min. The mixture was chilled (ice-water bath) and filtered to give the putative (±)-1-O-stearoyl-2,3-O-(xanthen-9-ylidene)glycerol as a colourless solid (1.432 g, 89.7%).

This material (0.532 g, ca. 1.0 mmol) was dissolved in a solution of pyrrole in dichloromethane (3.4 ml, 1:9 v/v) followed by addition of a solution of dichloroacetic acid in dichloromethane (3.4 ml, 1:9 v/v). After 15 min, the reaction mixture was partitioned between dichloromethane (10 ml) and sodium phosphate buffer (20 ml, 1.0 M, pH 6.0). The layers were separated and the organic layer was further washed with the above phosphate buffer (15 ml). The combined aqueous layers were back-extracted with dichloromethane (20 ml). The combined, dried (MgSO₄) organic layers were concentrated under reduced pressure. The residue was taken up with diethyl ether (20 ml) followed by addition of a solution of anhydrous iron (III) chloride (0.487 g, 3.0 mmol) in diethyl ether (30 ml). After 30 min, the solid formed was removed by filtration and washed with diethyl ether (2×15 ml). The filtrate and washings were combined and washed with aqueous sodium phosphate buffer (4×25 ml, 1.0 M, pH 6.0). The aqueous layers were filtered through a bed of Celite and back-extracted with diethyl ether (2×15 ml). The combined dried (MgSO₄) ether layers were concentrated under reduced pressure to a volume of ca. 50 ml and stirred with activated charcoal (0.3 g) for 30 min. The mixture was then filtered through a bed of Celite and washed with diethyl ether (2×15 ml). The filtrate and washings were combined and concentrated under reduced pressure to give the title compound (0.320 g, 90.4%). (Found, in material recrystallized from hexane: C, 69.5; H, 11.75. C₂₁H₄₂O₄·0.25 H₂O requires C, 69.47; H, 11.80%) as a colourless solid, mp 72–73°C.

δ_H[CDCl₃] 0.81 (3 H, t, J 6.8), 1.20 (28 H, m), 1.55 (2 H, m), 2.28 (2 H, t, J 7.6), 3.53 (1 H, dd, J 5.8 and 11.5), 3.63 (1 H, dd, J 3.9, 11.5), 3.87 (1 H, m), 4.08 (1 H, dd, J 6.1 and 11.7), 4.14 (1 H, dd, J 4.7 and 11.7).
δ_{H}[CDCl_{3}] 13.88, 22.06, 24.39, 28.43, 28.68, 28.86, 29.00, 30.33, 31.26, 33.41, 62.56, 65.41, 69.21, 172.85.

The above experiment was repeated on the same scale and with the same stoichiometry, starting from (±)-1,2-\(O\)-(2,7-dimethylxanthen-9-ylidene)glycerol (0.895 g, 3.0 mmol) instead of (±)-1,2-\(O\)-(xanthen-9-ylidene)glycerol. The intermediate putative 1-\(O\)-stearoyl-2,3-\(O\)-(2,7-dimethylxanthen-9-ylidene)glycerol (1.61 g) was obtained as a colourless solid. This material (0.565 g) was converted into (±)-1-\(O\)-stearoylglycerol (0.322 g, 85% overall yield) under precisely the same conditions described above. The physical properties (mp, \(^1\)H and \(^{13}\)C NMR spectra) of this material were identical to those obtained above, starting from (±)-1,2-\(O\)-(xanthen-9-ylidene)glycerol.

\(\text{(S)}\)(\(+\))-1-\(O\)-Stearoylglycerol (604)

To a stirred solution of \((\text{S})\)(\(-\))-1,2-\(O\)-(xanthen-9-ylidene)glycerol (0.811 g, 3.0 mmol) in dichloromethane (15 ml), 1-methylimidazole (0.477 ml, 6.0 mmol) was added, followed by addition of a solution of stearoyl chloride (1.090 g, 3.6 mmol) in dichloromethane (15 ml). After 1.5 h, triethylamine (1.0 ml) and water (0.2 ml) were added and the reaction mixture was stirred for 10 min and then poured into saturated aqueous sodium hydrogen carbonate (15 ml). The layers were separated and the organic layer was further washed with saturated aqueous sodium hydrogen carbonate (15 ml). The dried (MgSO\(_4\)) organic layer was concentrated under reduced pressure. The residue was stirred in methanol (15 ml) for 30 min. The mixture was chilled (ice – water bath) and filtered to give the putative (\(\text{S}\))1-\(O\)-stearoyl-2,3-\(O\)-(xanthen-9-ylidene)glycerol as a colourless solid (1.484 g).
This material (0.565 g, ca. 1.0 mmol) was dissolved in a solution of pyrrole in dichloromethane (3.4 ml, 1:9 v/v) followed by addition of a solution of dichloroacetic acid in dichloromethane (3.4 ml, 1:9 v/v). After 15 min, the reaction mixture was partitioned between dichloromethane (10 ml) and sodium phosphate buffer (20 ml, 1.0 M, pH 6.0). The layers were separated and the organic layer was further washed with the above phosphate buffer (15 ml). The combined aqueous layers were back-extracted with dichloromethane (20 ml). The combined, dried (MgSO₄) organic layers were concentrated under reduced pressure. The residue was taken up with diethyl ether (20 ml) followed by addition of a solution of anhydrous iron (III) chloride (0.487 g, 3.0 mmol) in diethyl ether (30 ml). After 30 min, the solid formed was removed by filtration and washed with diethyl ether (2×15 ml). The filtrate and washings were combined and washed with aqueous sodium phosphate buffer (4×25 ml, 1.0 M, pH 6.0). The aqueous layers were filtered through a bed of Celite and back-extracted with diethyl ether (2×15 ml). The combined dried (MgSO₄) ether layers were concentrated under reduced pressure to a volume of ca. 50 ml and stirred with activated charcoal (0.3 g) for 30 min. The mixture was then filtered through a bed of Celite and washed with diethyl ether (2×15 ml). The filtrate and washings were combined and concentrated under reduced pressure to give the title compound (0.325 g, 79% overall yield). (Found, in material recrystallized from hexane: C, 69.9; H, 11.9. C₂₁H₄₂O₄·0.1 H₂O requires C, 69.99; H, 11.8%) as a colourless solid, mp 68.5–69.5°C; [a]₂₀ +3.64 (c 4.1; C₅H₅N).

δ_H[CDCl₃] 0.81 (3 H, t, J 6.8), 1.20 (28 H, m), 1.55 (2 H, m), 2.28 (2 H, t, J 7.6), 3.53 (1 H, dd, J 5.8 and 11.5), 3.63 (1 H, dd, J 3.9, 11.5), 3.87 (1 H, m), 4.08 (1 H, dd, J 6.1 and 11.7), 4.14 (1 H, dd, J 4.7 and 11.7).

(R)-(-)-1-O-Stearoylglycerol (639)

To a stirred solution of (S)(+)-1,2-O-(xanthen-9-ylidene)glycerol (0.811 g, 3.0 mmol) in dichloromethane (15 ml), 1-methylimidazole (0.477 ml, 6.0 mmol) was added, followed by addition of a solution of stearoyl chloride (1.090 g, 3.6 mmol) in dichloromethane (15 ml). After 1.5 h, triethylamine (1.0 ml) and water (0.2 ml) were added and the reaction mixture was stirred for 10 min and then poured into saturated aqueous sodium hydrogen carbonate (15 ml). The layers were separated and the organic layer was further washed with saturated aqueous sodium hydrogen carbonate (15 ml). The dried (MgSO₄) organic layer was concentrated under reduced pressure. The residue was stirred in methanol (15 ml) for 30 min. The mixture was chilled (ice - water bath) and filtered to give the putative (R)-1-O-stearoyl-2,3-O-(xanthen-9-ylidene)glycerol as a colourless solid (1.406 g).

This material (0.565 g, ca. 1.0 mmol) was dissolved in a solution of pyrrole in dichloromethane (3.4 ml, 1:9 v/v) followed by addition of a solution of dichloroacetic acid in dichloromethane (3.4 ml, 1:9 v/v). After 15 min, the reaction mixture was partitioned between dichloromethane (10 ml) and sodium phosphate buffer (20 ml, 1.0 M, pH 6.0). The layers were separated and the organic layer was further washed with the above phosphate buffer (15 ml). The combined aqueous layers were back-extracted with dichloromethane (20 ml). The combined, dried (MgSO₄) organic layers were concentrated under reduced pressure. The residue was taken up with diethyl ether (20 ml) followed by addition of a solution of anhydrous iron (III) chloride (0.487 g, 3.0 mmol) in diethyl ether (30 ml). After 30 min, the solid formed was removed by filtration and washed with diethyl ether (2×15 ml). The filtrate and washings were combined and washed with aqueous sodium phosphate buffer (4×25 ml, 1.0 M, pH 6.0). The aqueous layers were filtered through a bed of Celite and back-extracted with diethyl ether (2×15 ml). The combined dried (MgSO₄) ether
layers were concentrated under reduced pressure to a volume of ca. 50 ml and stirred with activated charcoal (0.3 g) for 30 min. The mixture was then filtered through a bed of Celite and washed with diethyl ether (2×15 ml). The filtrate and washings were combined and concentrated under reduced pressure to give the title compound (0.333 g, 77% overall yield). (Found, in material recrystallized from hexane: C, 69.8; H, 11.9. C_{21}H_{42}O_{4}·0.2 H_{2}O requires C, 69.61; H, 11.8%) as a colourless solid, mp 69–70°C; [α]_{D}^{20} -3.68 (c 4.1; C_{3}H_{7}N).

δ_{H}[CDCl_{3}] 0.81 (3 H, t, J 6.8), 1.20 (28 H, m), 1.55 (2 H, m), 2.28 (2 H, t, J 7.6), 3.53 (1 H, dd, J 5.8 and 11.5), 3.63 (1 H, dd, J 3.9, 11.5), 3.87 (1 H, m), 4.08 (1 H, dd, J 6.1 and 11.7), 4.14 (1 H, dd, J 4.7 and 11.7).

δ_{H}[CDCl_{3}] 13.88, 22.06, 24.39, 28.43, 28.68, 28.86, 29.00, 30.33, 31.26, 33.41, 62.56, 65.41, 69.21, 172.85.

9,9-Di-(pyrrol-2-yl)xanthene (647)

(a) (±)-1-O-Stearoyl-2,3-O-(xanthen-9-ylidene)glycerol (0.532 g, 1.0 mmol), the putative intermediate in one of the above preparations of (±)-1-O-stearoylglycerol, was dissolved in pyrrole – dichloromethane (1:9 v/v; 3.4 ml; ca. 4.9 mmol of pyrrole) and dichloroacetic acid – dichloromethane (1:9 v/v; 3.4 ml; ca. 4.1 mmol of dichloroacetic acid) was added to the stirred solution at room temperature. After 15 min, the products were partitioned between dichloromethane (15 ml) and saturated aqueous sodium hydrogen carbonate (15 ml). The layers were separated. The organic layer was washed with saturated aqueous sodium hydrogen carbonate (15 ml), dried (MgSO_{4}) and evaporated under reduced pressure. The residue was fractionated by
short column chromatography on silica gel. The appropriate fractions, which were eluted with petroleum ether (60–80°C)–ethyl acetate (95:5 v/v), were combined and evaporated under reduced pressure to give the *title compound* as a colourless solid (0.285 g, 92%). (Found in material recrystallized from aqueous methanol: C, 80.3; H, 5.0; N, 8.9. C_{21}H_{16}N_{2}O \cdot 0.1 \text{H}_{2}\text{O} requires: C, 80.28; H, 5.20; N, 8.92%), mp 189–190°C; R_{f} (system C) 0.44.

δ_{H}[(\text{CD}_{3})_{2}\text{SO}] 5.37 (2 H, m), 5.86 (2 H, dd, J 2.6 and 5.6), 6.63 (2 H, dd, J 2.6 and 4.4), 7.04 (4 H, m), 7.14 (2 H, m), 7.28 (2 H, m), 10.35 (2 H, br, s).

δ_{C}[(\text{CD}_{3})_{2}\text{SO}] 44.79, 106.36, 108.82, 116.11, 118.99, 123.33, 127.76, 128.31, 129.70, 135.65, 150.84.

(b) 9,9-Dimethoxyxanthene (0.484 g, 2.0 mmol) was dissolved in pyrrole–dichloromethane (1:9 v/v; 6.7 ml, ca. 9.7 mmol of pyrrole) and dichloroacetic acid–dichloromethane (1:9 v/v; 6.7 ml; ca. 8.1 mmol of dichloroacetic acid) was added to the stirred solution at room temperature. After 20 min, the products were poured into saturated aqueous sodium hydrogen carbonate (15 ml). The layers were separated, and the organic layer was washed with saturated aqueous sodium hydrogen carbonate (15 ml), dried (MgSO_{4}) and then evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel to give a colourless solid (0.462 g, 74%), identical in all respects (mp, R_{f}, ^{1}\text{H} \text{and} ^{13}\text{C} \text{NMR spectra}) to the product obtained above.
2,7-Dimethyl-9,9-di-(pyrrol-2-yl)xanthene (648)

(a) (±)-1-O-Stearoyl-2,3-O-(2,7-dimethylxanthen-9-ylidene)glycerol (0.565 g, 1.0 mmol), the putative intermediate in one of the above preparations of (±)-1-O-stearoylglycerol, was dissolved in pyrrole–dichloromethane (1:9 v/v; 3.4 ml; ca. 4.9 mmol of pyrrole) and dichloroacetic acid–dichloromethane (1:9 v/v; 3.4 ml; ca. 4.1 mmol of dichloroacetic acid) was added to the stirred solution at room temperature. After 15 min, the products were partitioned between dichloromethane (15 ml) and saturated aqueous sodium hydrogen carbonate (15 ml). The layers were separated. The organic layer was washed with saturated aqueous sodium hydrogen carbonate (15 ml), dried (MgSO₄) and evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel. The appropriate fractions, which were eluted with petroleum ether (60–80°C)–ethyl acetate (95:5 v/v), were combined and evaporated under reduced pressure to give the title compound as a colourless solid (0.280 g, 82%). (Found in material recrystallized from aqueous methanol: C, 80.86; H, 5.65; N, 8.12. C₂₃H₂₀N₂O requires: C, 81.15; H, 5.92; N, 8.23%), mp 215–216°C; Rᵣ (System C) 0.50.

δ_H[(CD₃)₂SO] 2.19 (6 H, s), 5.36 (2 H, dd, J 2.9 and 4.5), 5.85 (2 H, dd, J 2.6 and 5.5), 6.63 (2 H, dd, J 2.5 and 4.3), 6.80 (2 H, d, J 1.7), 6.99 (2 H, d, J 8.2), 7.06 (2 H, dd, J 1.9 and 8.3), 10.35 (2 H, br, s).

δ_C[(CD₃)₂SO] 20.46, 44.35, 107.87, 108.32, 115.34, 118.42, 126.85, 128.35, 129.27, 131.27, 135.27, 148.51.
(b) 9,9-Dimethoxyxanthene (0.540 g, 2.0 mmol) was dissolved in pyrrole-
dichloromethane (1:9 v/v; 6.7 ml, ca. 9.7 mmol of pyrrole) and dichloroacetic acid-
dichloromethane (1:9 v/v; 6.7 ml; ca. 8.1 mmol of dichloroacetic acid) was added to
the stirred solution at room temperature. After 20 min, the products were poured into
saturated aqueous sodium hydrogen carbonate (15 ml). The layers were separated,
and the organic layer was washed with saturated aqueous sodium hydrogen carbonate
(15 ml), dried (MgSO₄) and then evaporated under reduced pressure. The residue was
fractionated by short column chromatography on silica gel to give a colourless solid
(0.442 g, 64%), identical in all respects (mp, Rf, ¹H and ¹³C NMR spectra) to the
product obtained above.

Hydrolysis studies of 2',3'-O-protected uridine derivatives (612, 613, and 616a,b)
in methanol–water–trifluoroacetic acid (7:2:1) at 30°C

A stock solution of an internal standard (3'-O-benzoylthymidine, 0.050 g) in methanol
(35 ml), water (10 ml) and trifluoroacetic acid (5 ml) was prepared. Substrates (ca.
0.001 g) were dissolved in preheated (to 30°C) stock solution (1.0 ml) and the
resulting solutions were maintained at 30 (±0.1)°C in a Digi-block heating apparatus.
After appropriate intervals of time, aliquots (20 μl) were removed, quenched with 0.1
M triethylammonium acetate buffer (pH 7.0, 40 μl) and methanol (40 μl), and
analysed by HPLC (25 cm×4.6 mm Hypersil ODS 5 μ column). The HPLC column
was eluted with acetonitrile–0.1 M triethylammonium acetate buffer (pH 7.0) mixtures.
References


References


References


References

505–506.
181. Regberg, T.; Stawiński, J.; Stromberg, R. *Nucleosides Nucleotides*, **1988**, 7,
23–35.
2291–2294.
257–269.
133–134.
1809–1810.
6, 271–276.
802.
195. Jones, S. S.; Rayner, B.; Reese, C. B.; Ubasawa, A.; Ubasawa, M.
Heathcliffe, G. R.; Atkinson, T. C.; Newton, C. R.; Markham, A. F. *Nucleic
References


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