Synthesis of Novel Lipophilic Nucleotide and CpG Dinucleotide Conjugates for the Stimulation of the Immune Response System

By

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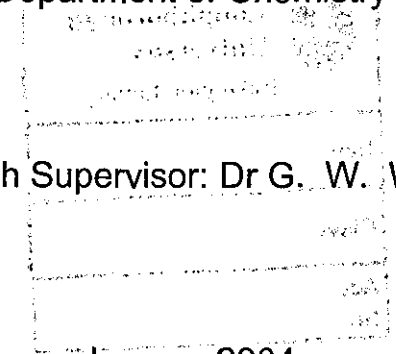

Research Supervisor: Dr G. W. Weaver

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Dedication

This research work is dedicated to my dear Mother and Father and my lovely brother.

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ABSTRACT

The research described in this thesis was aimed at synthesizing novel lipophilic nucleotide and CpG dinucleotide conjugates with the purpose of improving their immunostimulatory activity.

The first part of the discussion describes the successful synthesis of novel acetal, carbonate, carbamate and ester linkers of tocopherol and cholesterol for attaching lipophilic molecules to the nucleotide constructs. This was done in order to improve transport of the molecules across cell membranes as the lipophilic tail can assist the likelihood of oligonucleotide uptake into the cells by reducing their polarity.

Furthermore, a linker was synthesized which was used to link tumour targeting GRE1, GRE4 and G34 rabbit antibodies provided by the labs in Queens Medical Center and Aphton Corporation to different DNA constructs containing antisense insert. The resulting solutions of bound antibodies were purified to give the biologically active antibody constructs. The antibodies retained at the binding sites were eluted and showed biological activity, which unfortunately could not be maintained.

The second part reports the development of new improved methods for the global and partial protection of nucleosides employing *tert*-butyldimethylsilyl chloride (TBDMSCl) as a suitable and versatile protecting group for the hydroxyl functions of the nucleosides without the need for base protection. The conditions employed averted any silylation of the amino bases, increased the rate of reactions and afforded high yields of the protected nucleosides. Selective 5'-desilylation was also achieved and the silylated derivatives of the nucleosides were utilized in the formation of lipophilic nucleoside conjugates and subsequent synthesis of CpG dinucleotides.

The third part entails the synthesis of nucleosides covalently linked to lipophilic molecules through the exocyclic amino base or the 3'-hydroxyl group, forming novel carbonyl-type links.

This was done with the aim of improving their transport and hybridization properties and increasing their nuclease resistance. Lipophilic N-carbamate and N-acetamide conjugates of globally protected guanosine and adenosine were synthesized in very good yields. Modifications at the 3'-terminus of the nucleosides, which can potentiate their immunostimulatory activity, afforded their lipophilic O-carbonate analogues in good yields.

An alternative method for the synthesis of these conjugates was also investigated by employing the acid chloride of cholesterol, which led to low yields. Furthermore thioctic acid, which is also considered a lipophilic compound, was utilized and formation of its N-carbamate analogue of adenosine was achieved though employment of coupling reagents.

The fourth part describes the successful formation of CpG dinucleotides with or without the attachment of lipophilic molecules using both solution and solid phase synthesis. Also, the 5'-dicetyl, dicholesterol and ditocopherol phosphates of the CpG dinucleotides were synthesized in order to examine if modifications at the 5'-terminus can enhance cellular uptake and increase immunostimulatory activity. Hence, the *N,N*-diisopropyl-phosphoramidites of cetyl alcohol, cholesterol and tocopherol were prepared which were subsequently coupled to CpG through their 5'-end, in order to form the lipophilic phosphodiester CpG dinucleotides.

ABBREVIATIONS

- **Aq.** Aqueous
- **Ac** Acetyl
- **AcOH** Acetic Acid
- **Anal.** Analysis
- **Ar** Aryl
- **Bn** Benzyl
- **b.p.** boiling point
- **^{0}C** Degrees Celcius
- **Calcd** Calculated
- **$CDCl_3$** Deuterated Chloroform
- **CDI** N,N'-Carbonyl Diimidazole
- **Conc** Concentrated
- **cm^{-1}** Wavenumber
- **DCM** Dichloromethane
- **DMAP** Dimethylaminopyridine
- **DMF** Dimethylformamide
- **DMSO-d_6** Dimethylsulphoxide (deuteriated)
- **DNA** Deoxyribose Nucleic Acid
- δ Chemical Shift
- **EI** Electron Impact
- **ELISA** Enzyme-Linked ImmunoSorbent Assay
- **eq.** Equivalents
- **Et** Ethyl

- **EtOAc** Ethyl acetate
- **EtOH** Ethanol
- **h** Hour(s)
- **HRFABMS** High Resolution Fast Atom Fast Atom Bombarding Mass Spectrum
- **Hz** Hertz
- **IgG** Immunoglobin G
- **I.R** InfaRed spectroscopy
- ***J*** *coupling constant*
- **lit** Literature
- **LHMTS** Lithium bis (trimethyl silyl) amide
- **M** Molar
- **MeOH** Methanol
- **Me** Methyl
- **min** Minute(s)
- **ml** Millilitre(s)
- **mmol** Millimole(s)
- **m.p.** Melting Point
- **NMR** Nuclear Magnetic Resonance
- **Ph** Phenyl
- **ppm** Parts Per Million
- **^{i}Pr** IsoPropyl
- **R.T.** Room Temperature
- ***t*-BuLi** *t*-Butyl lithium
- **TBAF** Tetrabutylammonium fluoride

- **Tf** Trifluoromethane sulphonyl
- **TFA** Trifluorocetic Acid
- **THF** Tetrahydrofuran
- **TLC** Thin Layer Chromatography
- **TMS** Trimethylsilyl
- **w / v** Weight per Volume

INTRODUCTION

Synthetic oligonucleotides have proved to be invaluable tools of modern molecular biology[1]. The facile and efficient production of synthetic oligonucleotides has spurred interest in the application of these biomolecules to a variety of diagnostic and therapeutic indications. Oligonucleotides fuctionalized with *intercalators, cross linkers, affinity ligands and DNA/RNA* cleaving agents are examples of such applications[2]. They can also been used for the enzymatic construction of genes[3], physical studies of the stability and conformational behaviour of nucleic acids[4] and investigation of drug-nucleic acid and protein nucleic acid interactions[5].

Recent advances in chemical synthesis especially polymer supported methods[6] have markedly reduced the enormous amount of time formerly required for oligonucleotide synthesis. Compounds which stimulate the immune response have great potential[7] in the treatment of diseases such as cancer where conventional therapies using cytotoxic agents cause serious side effects due to the low discrimination between cancer cells and the cells of normal healthy tissue.

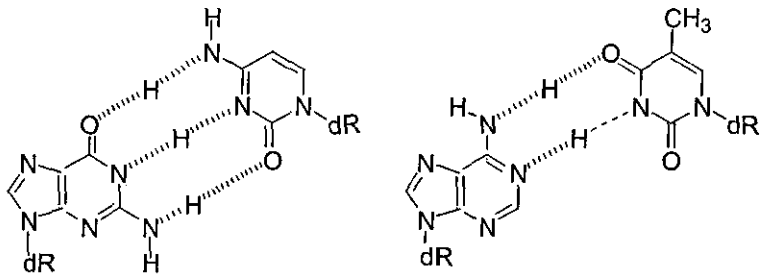
Inhibition of the gene expression, where the oligonucleotides found application, has become an attractive method for the treatment of viral disease and cancer[8].

Oligonucleotides can be used to block deleterious genes by binding to a run of complimentary bases if the gene sequence is known, thus preventing expression of the gene and production of harmful proteins (antisense oligonucleotide). Complementary oligonucleotides can be utilized either to block sequences of DNA before transcription, or to block mRNA before translation occurs. Being normal products of cell biosynthesis they are rapidly processed and degraded and have low or no toxicity, thus avoiding the side effects common with many therapeutic agents.

Antisense oligonucleotides (ODN's) have been proposed as a major class of new pharmaceuticals. In general, antisense refers to the use of small synthetic oligonucleotides; resembling single stranded DNA, to inhibit gene expression.[9-11].

Antisense binding was first described as a naturally occurring phenomenon in which cells transcribe an antisense RNA complementary to a cellular mRNA.[9,10,12] This antisense RNA was found to be a repressor of gene expression, hybridizing to a target mRNA, inhibiting its translation and decreasing the cellular levels of the protein[12].

Antisense RNA is produced from the corresponding antisense genes by transcription. In the simplest case this can take place by transcription of the complementary (antisense) DNA strand. The antisense RNA then binds to the complementary (sense) target nucleic acid via Watson-Crick base pairing[13] in which adenosine and thymidine or guanosine and cytidine interact through hydrogen bonding (**Figure 1**)



G-C Base Pair

A-T Base pair

Figure 1. Base pairing interactions for Adenosine with Thymidine (A-T) and Guanosine with Cytidine (G-C) where dR is the 2-deoxyribose sugar

The process of binding of the oligonucleotide to a complementary nucleic acid is called hybridization. These simple base pairing rules govern the interaction between the antisense ODN's and the cellular RNA, allowing the design of ODN's to target any gene of a known sequence.

A major advantage of this strategy is in the potential specificity of action. In principle, an ODN can be designed to target any single gene within the entire human genome, potentially creating specific therapeutics for any disease in which the causative gene is known. As a result there have been numerous studies of antisense ODN activity for potential antiviral[14-16] and anticancer[17,18] applications. Although the field has progressed over the past decade, recent papers indicate that the observed activity of ODN's in tissue culture can be predominantly through non-antisense mechanisms.[15,16,19]

Oligonucleotide reagents have found wide application in biotechnology and more recently in medicinal chemistry to the extent that "antisense" oligonucleotides have been shown to block the translation and transcription of genes. Inhibition of gene expression through the use of an exogenously added oligonucleotide was first reported in 1978.

Zamecnik and Stepherson[20,21] were able with a 13-mer oligonucleotide that was complementary to the RNA of Rous sarcoma virus, to inhibit the replication of this virus in cell culture. Various cellular processes can be inhibited depending on where the oligonucleotide hybridizes on single stranded regions of the DNA or mRNA. A simple model describes the inhibition of protein biosynthesis by an antisense oligonucleotide being bound to the mRNA (**Figure 2**).

Although this illustrative model does not always reflect the actual mechanisms, nevertheless it reveals the essential steps in the new principal and the problems associated with it: for the antisense oligonucleotide to be able to inhibit translation it must reach the interior of the cell unaltered. The requirements for this are stability of the oligonucleotide toward extra- and intracellular enzymes and also ability to penetrate through the cell membrane. Once the antisense oligonucleotide has reached the cytoplasm it must bind specifically and with sufficient affinity to the target mRNA to inhibit its translation into the corresponding protein. In order to meet all these requirements it is necessary for the normal oligonucleotides to be chemically modified in a suitable manner.

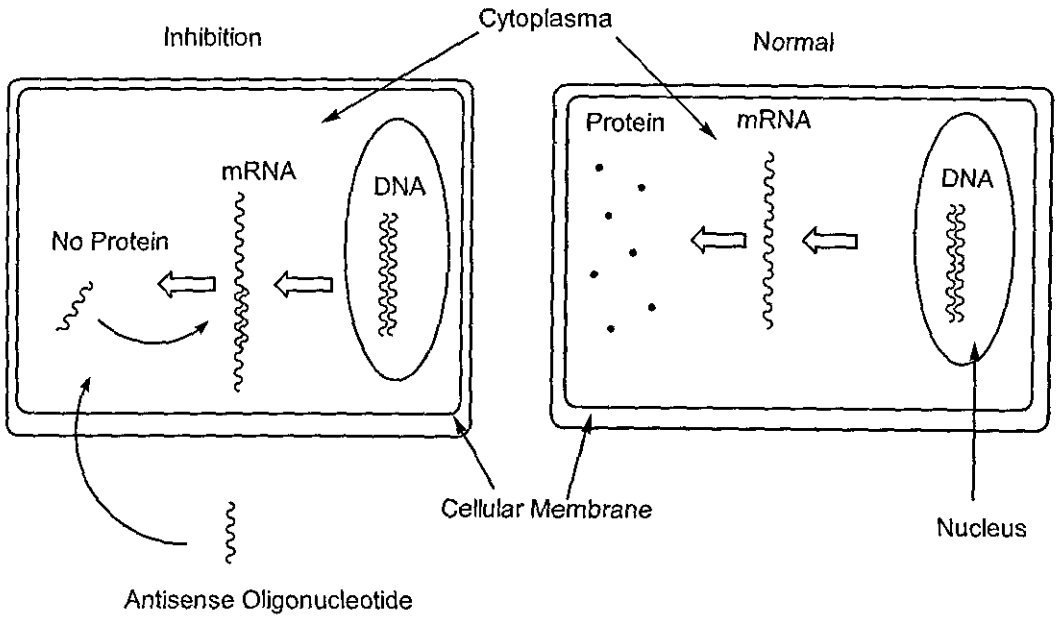


Figure 2. Principle of action of the antisense oligonucleotides

A variety of possible oligonucleotide modifications are shown in the **Figure 3** below.

It is also known[22] that the presence of certain base sequences such as GC pairs in an oligonucleotide can lead to stimulation of the immune system and in the case of therapeutic agents helps the body's own defences to fight the disease.

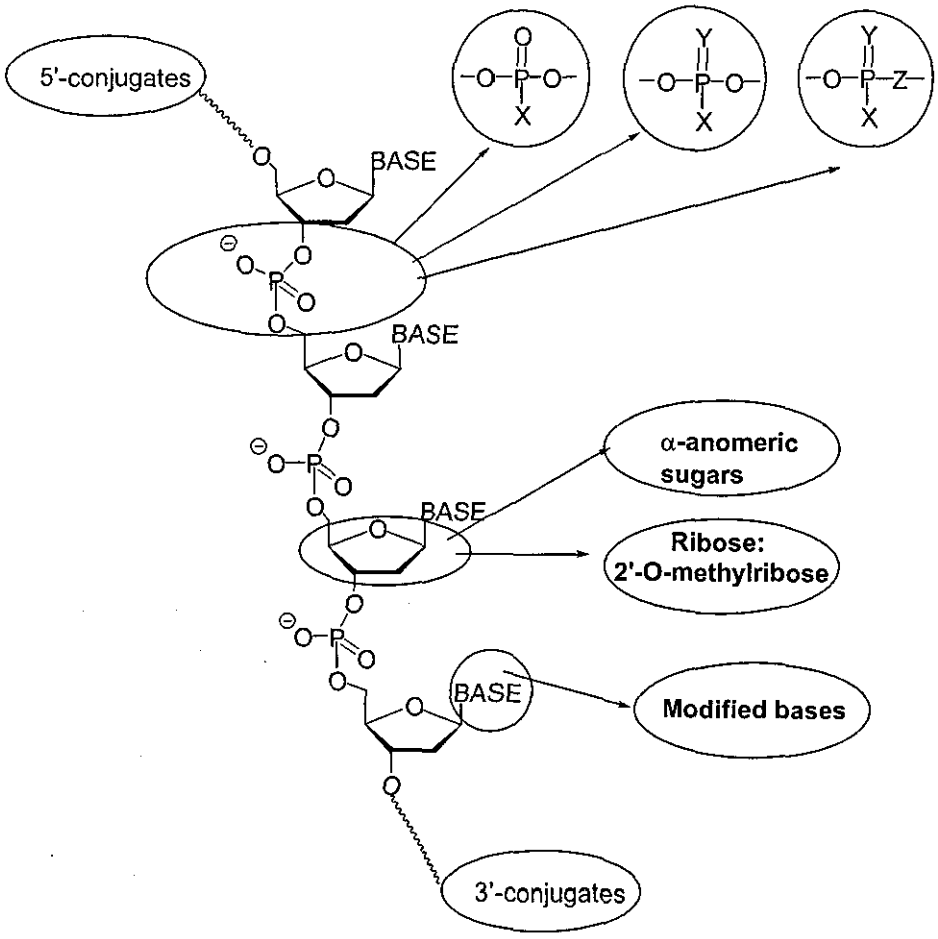

5'-conjugates
BASE
BASE
BASE
BASE
α-anomeric sugars
Ribose: 2'-O-methylribose
Modified bases
3'-conjugates

Figure 3

1. METHODS FOR THE SYNTHESIS OF OLIGONUCLEOTIDES AND THEIR ANALOGUES

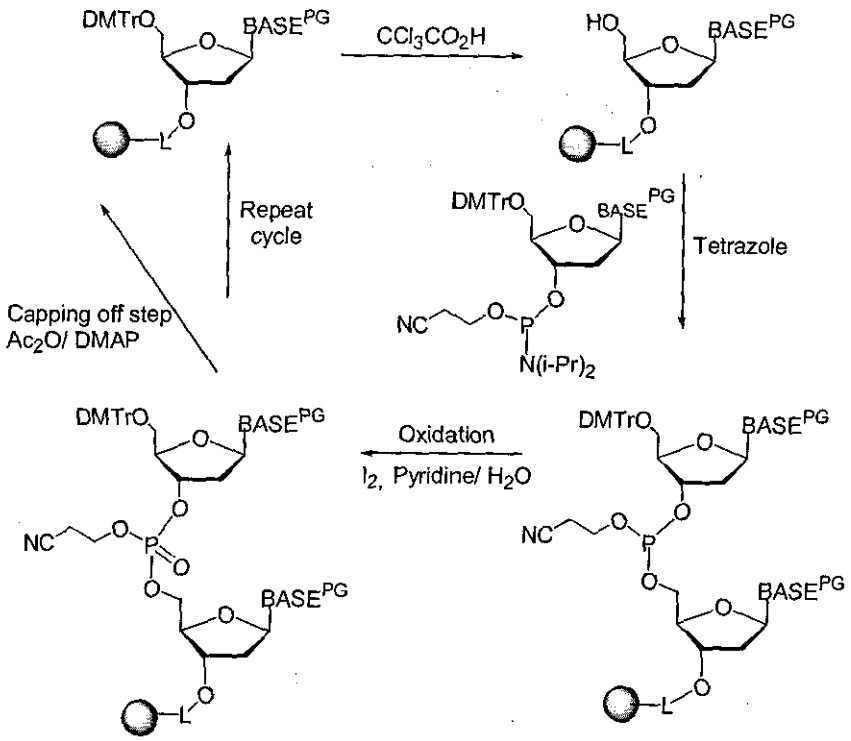
The preparation of unmodified oligodeoxynucleotides has been the center of interest of many research groups. One of the most important aspects of the synthesis is the chain-extension process in the conventional methods used up to date.

Three principal methods: the **phosphoramidite** (known as amidite method), **phosphotriester** and **H-phosphonate**, have been widely used for the synthesis of oligonucleotides. The phosphotriester approach has been used widely for solution phase synthesis, whereas the phosphoramidite and H-phosphonate methods have found application mainly in solid-phase synthesis.

(A) Phosphoramidite method

The synthesis via phosphoramidites, originally introduced by Letsinger[23] **(REF),** is currently the most efficient method[24] for preparing oligonucleotides and can be used to construct oligomers having up to 175 nucleosides using automatic DNA synthesizers. The basic steps in the phosphoramidite method are shown in **Scheme 1** below.

Basic steps in a cycle of nucleotide addition by the phosporamidite method



Scheme 1

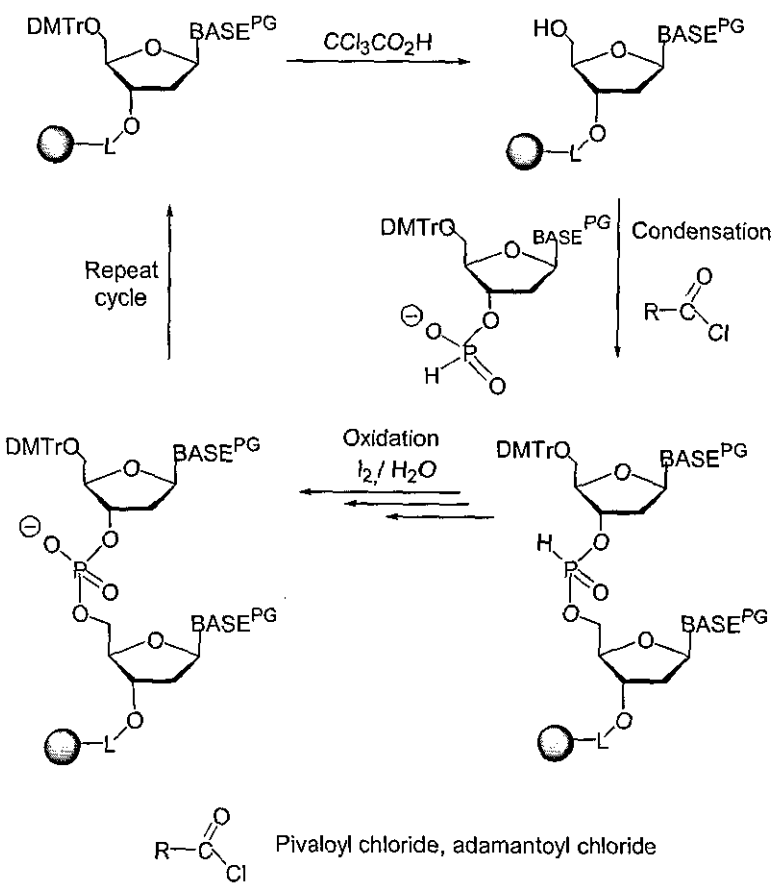
This method includes use of a highly efficient coupling reaction between a 5'-hydroxyl group of a support-bound deoxynucleoside and an alkyl 5'-Dimethoxytrityl-protected-(N-protected)-deoxynucleoside-3'-O-(N,N-diisopropyl)phosphoramidite, the alkyl group being methyl or 2-cyanoethyl. Protonation at the nitrogen converts the phosphoramidite into a highly reactive phosphitylating agent, where the tetrazole is just sufficiently acidic in order to activate the phosphitylation without causing loss of the protecting groups.

The product of the coupling is a dinucleotide phosphite, which must be oxidized with iodine and water in the presence of pyridine to the phosphotriester before proceeding with chain extension. The efficiency of the coupling is extremely high and the only side reaction is the phosphitylation of the O-6 position of guanine. Treatment though with acetic anhydride and N-methylimidazole (introduced before hand to cap off any unreacted hydroxyl groups) completely reverses this side reaction[25]

(B) H-phosphonate method

In the past two years the H-phosphonate method, which was first described by Todd[26] in 1957 has become reestablished and has in some cases replaced the amidite method, particularly because the synthons are easier to handle, and no phosphate protective group is employed.[27-29] This method entails the 5'-OH group of the growing DNA chain to react with a nucleoside 3'-H-phosphonate (**Scheme 2**)

Basic steps of the H-phosphonate method



Scheme 2

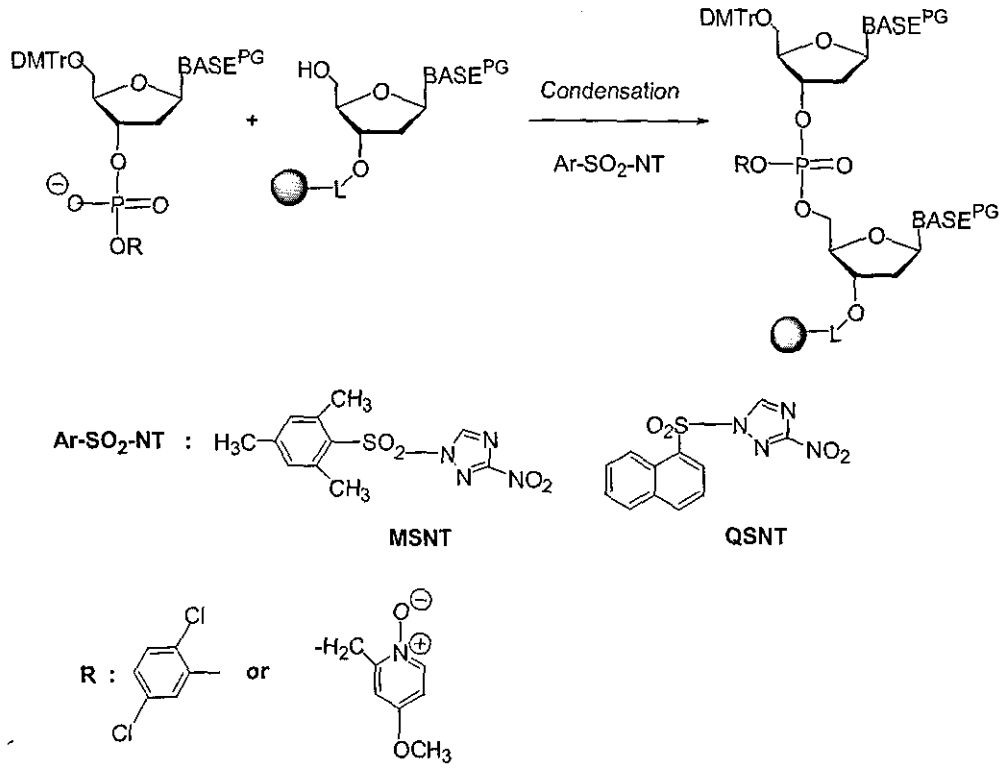
The condensing agents used are sterically hindered carbonyl chlorides such as adamantoyl or pivaloyl chloride.

The resulting phosphite diester is oxidized with *tert*-butyl hydroperoxide or iodine to the phosphotriester only after construction of the chain is complete, in contrast with the amidite method. The H-phosphonate method can also be used to prepare oligomers of considerable chain length.

(C) Phosphotriester method

The phosphotriester method[30,31], which dominated oligodeoxynucleotide preparation for a long time, is still regarded as the method of choice for large-scale synthesis[31], although the H-phosphonate approach has gained ground too.[32] In this method the required phosphotriester is obtained in one step from the 5'-OH group of the growing chain and the nucleoside 3'-phosphodiester units as shown in **Scheme 3**.

Phosphotriester method



Scheme 3

The condensation is brought about with 3-nitro-1,2,4-triazolides of an arenesulfonic acid preferably 2,4,6-triisopropylbenzene-sulfonic acid[33,34] (TIPS) or 8-quinolinesulfonic acid[35,36] (QNS).
A further improvement was achieved by using nucleophilic catalysts, e.g. 4-substited pyridine N-oxides, which intramolecularly attached to the phosphate protecting group, improve the coupling yield to 98% and reduce the time taken for a synthesis cycle to 7-8 min.[37]

The synthetic oligodeoxynucleotides can be purified by polyacrylamide gel electrophoresis followed by removal of the salts or, by HPLC.[38,39] Capillary gel electrophoresis[40,41] is proving to be extremely useful for the analysis and fractionation of small amounts of oligodeoxynucleotides and may be regarded in the future as the method of choice.
Sequence analysis of purified oligonucleotides is possible by two methods: The first method is by Maxam and Gilbert,[42] which is based on chemical cleavage and subsequent gel electrophoresis. The second one is the wandering-spot method,[43] in which the oligodeoxynucleotide is subjected to partial enzymatic degradation and fractionated by two-dimensional electrophoresis. Sequences of shorter oligodeoxynucleotides can also be analyzed by FAB or by plasma desorption mass spectrometry.[44,45] Since these rapid and accurate analytical methods can also be applied to modified oligonucleotides,[46] they could well achieve greater importance.

When normal oligonucleotides have been used as antisense oligonucleotides, problems such as instability to nucleases and insufficient membrane penetration have been encountered. That gave rise to a variety of modifications of the oligonucleotide structure in order to try to overcome those problems.

2. SYNTHESIS OF MODIFIED OLIGONUCLEOTIDES AND THEIR CONJUGATES

Modifications to the oligonucleotide have been employed most often for use in antisense inhibition where it is necessary for oligonucleotides to survive in cell cultures, or other biological environments, and also to cross the cell membrane. Modifications can be adopted to improve performance in both areas.

Nucleases are widespread and the lipophilic cell membrane is an effective barrier against passive diffusion of polyelectrolytes. Changes can be made at the bases, the sugars, the end of the chain, or at the phosphate groups of the backbone. Those at the bases and sugars are generally the most difficult chemically and require the most synthetic work. In addition they must not disrupt the ability of bases to form hydrogen bonds.

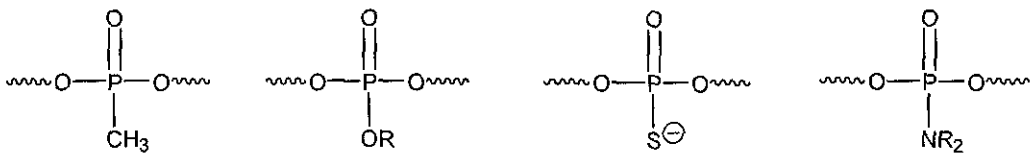
Modifications to the ends and backbone of the molecule are easier synthetically. The 5'-terminus is the most common site for conjugation as they are the site of action of nucleases and also carry the charges that inhibit cellular uptake. This represents the most direct approach for further derivatization.

A. OLIGONUCLEOTIDES WITH A MODIFIED PHOSPHATE RESIDUE

Currently there is a great interest in the chemical synthesis of oligonucleotides bearing modifications of the phosphodiester internucleotide linkages. These changes in the backbone of DNA fragments often lead to an increased stability towards enzymatic hydrolysis by nucleases and a greater overall lipophilicity without influencing too much the hybridization conditions to a complimentary single-stranded DNA or RNA sequence.

Therefore such backbone-modified DNA fragments seem to be the ideal tools for being applied in the control of gene expression on the mRNA level known as the anti-sense approach. This could finally lead to agents capable of stopping the growth of viruses of malignant cells.[47]

The most extensively studied analogues have been those containing modified phosphate linkages. Since the early experiments, researchers have observed that oligonucleotides containing **methylphosphonate 1**, **phosphotriester 2**, **phosphorothioate 3** or **phosphoramidate 4** linkages (shown below) have inhibitory activity against viral replication or expression of cellular genes in a number of different tissue culture systems



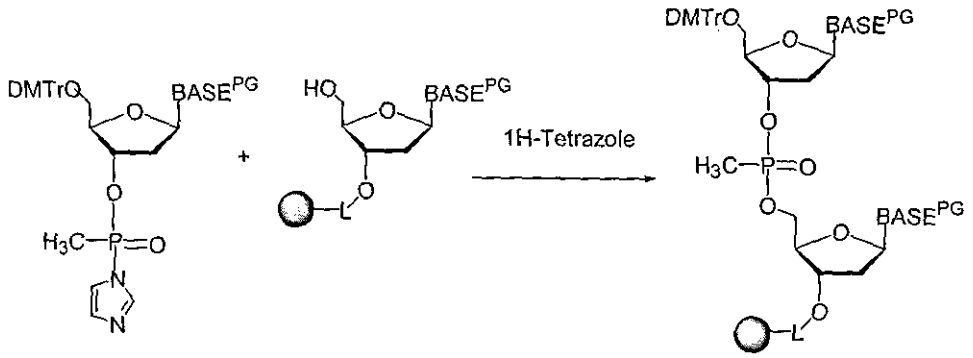
1 **2** **3** **4**

(I) Methylphosphonates (1)

The methylphosphonate linkage is uncharged and gives rise to nucleotides, which are less water-soluble than their phosphate counterpart. In order to improve uptake by the cells and extend the biological half-life, Miller and Ts'O[48,49], have concentrated on eliminating the negative charge on the internucleotide phosphate bridge.

Methylphosphonate oligodeoxynucleotides, which are called *MATAGENES* (*mas*king *ta*pe for *gene* expression)[50], are very stable with respect to cellular nucleases[51] and can enter cells although through a passive mechanism which is not related to that which appears to drive the uptake the of charged oligonucleotides.[52] A methyl group, which is neutral and sterically undemanding, replaces the negatively charged phosphate oxygen.

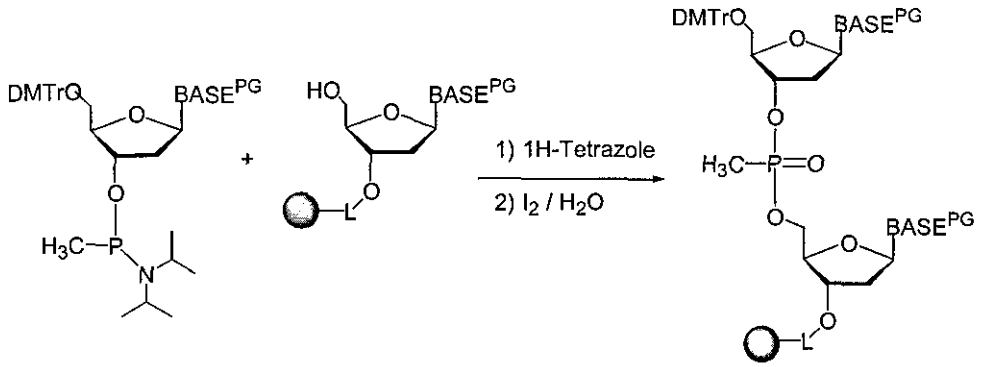
Initially the triester method found application in the synthesis of methylphosphonates which involved the condensation of 3'-methylphosphonates with 5'-unprotected nucleosides using various coupling reagents such as 1-(2-mesitylsulfonyl)-3-nitro-1,2,4-triazole (MSNT).[53,54] Miller and Ts'O[55] eventually optimized this strategy, by using 5'-protected nucleoside 3'-methylphosphonic acid imidazolides as shown in **Scheme 4**.



Scheme 4 Preparation of oligonucleotide methylphosphonates by the phosphotriester method.

The yields in each coupling step are 88-92%, which allows 15-mers to be synthesized in an isolated yield of 4%.

However the method of choice utilizes methylphosphonoamidites on a solid support[56,57] that proceeds in high yields (97%) in each coupling step (**Scheme 5**). The use of these synthons also allows unmodified oligodeoxynucleotides with one or several methylphosphonates bridges to be introduced at any desired point in the molecule, using the usual synthesis cycle.[56]



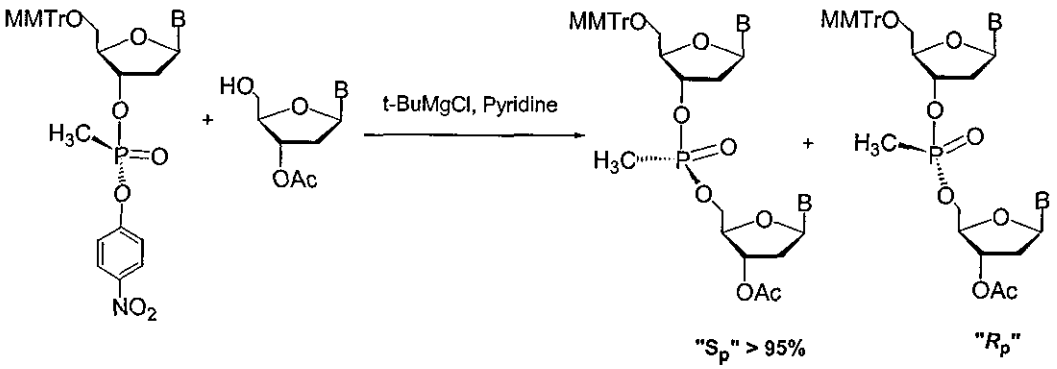
Scheme 5 Preparation of oligonucleotide methylphosphonates by the amidite method.

Since the internucleotide methylphosphonate bridge is more base labile than the natural internucleotide linkage,[57,58] milder conditions are necessary for cleavage from the support and deprotection. Whereas the latter is normally carried out with concentrated NH_4OH at 60 ^0C for 8h, in the case of methylphosphonate it is carried out again with concentrated NH_4OH at room temperature for only 2h and then with ethylenediamine /ethanol (1:1) for 7h, likewise at room temperature.[55,56]

Modifications to phosphates though pose a problem as they make the methylphosphonate bridge chiral, with an R_p or S_p configuration. Attempts were made early on to separate diastereomeric dinucleotides by chromatographic methods.[53] Stec *et al*[59,60] introduced the first promising approach to stereoselective synthesis.

This entails the R_p or S_p diastereomers of $T_{Me}T$ being obtained by stereospecific reaction of the P-chiral nucleotide components 5'-O-(monomethoxytrityl)thymidine, 3'-O-(O-(4-nitrophenylmethanephosphonate) and 3'-O-acetylthymidine as shown in **Scheme 6**.

The 5'-OH group is activated by *tert*-butylmagnesium chloride, and the reaction takes place with inversion of the stereochemistry of phosphorus. The stereochemistry is >95% but the chemical yield of ca. 70%, resulting in a little hope of successful use in solid-phase synthesis.



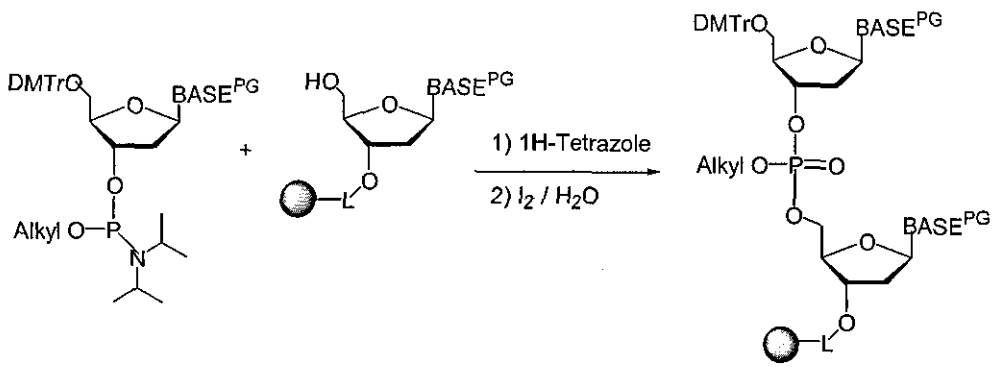
Scheme 6

Only tetramers with all R_p and S_p configuration are obtained by stepwise reaction. Predominantly S_p-configured benzylphosphonates have been reported recently[61] via the reaction of organozinc halides with PCl_3.

(II) Phosphotriesters (2)

Oligodeoxynucleotide phosphate triesters lack the usual negative charge present on the oxygen in the oligodeoxynucleotides and are accessible via several routes. They are produced as intermediates in the amidite as well as in the phosphate triester method, as well as the amidite method. In these cases, the triester serves to protect the phosphate and must accordingly, be easily cleaved. DNA phosphate esters have been of interest for a long time as products of alkylating agents, but they acquired additional significance as potential antisense oligonucleotides.[48,62]

The amidite method is widely used to synthesize phosphate triesters from nucleoside dimers and oligomers. Mainly employed for this are the 5'-protected nucleoside 3'-(O-isopropyl-N,N-diisopropyl-phosphoramidites) and their O-ethyl analogues[63-65] (**Scheme 7**)

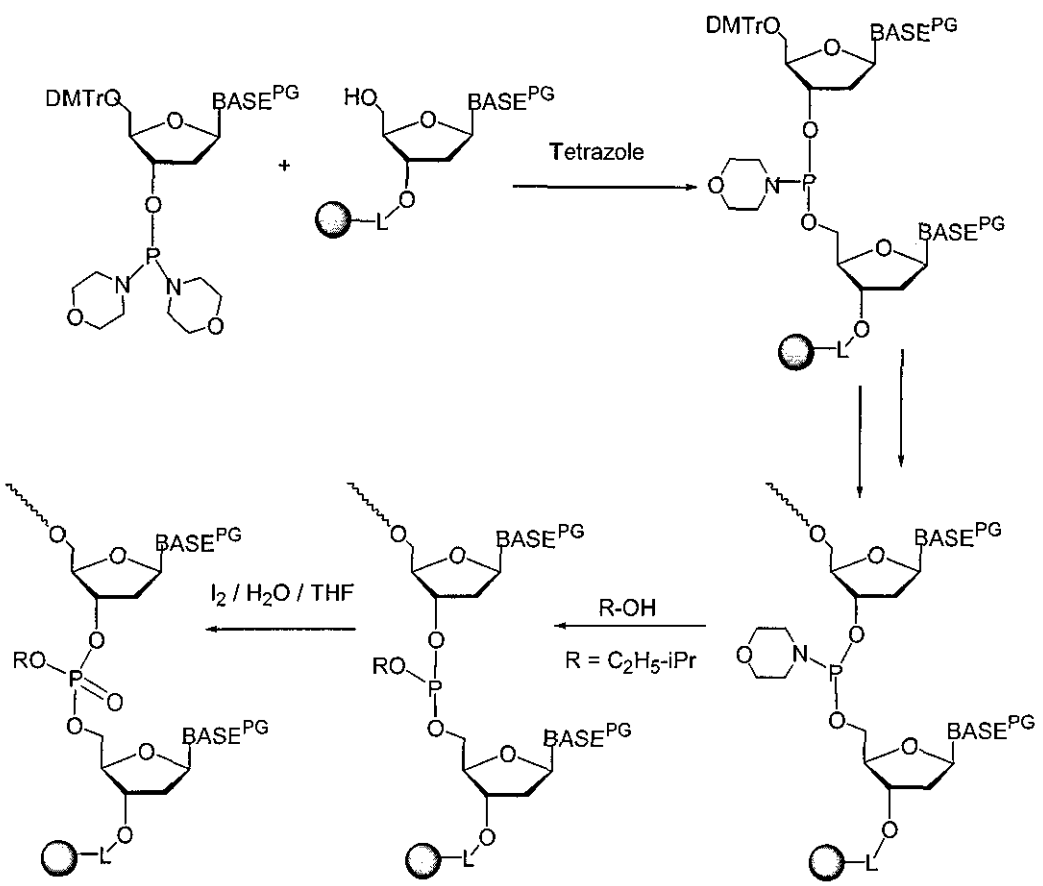


Scheme 7 Preparation of oligonucleotide phosphotriesters by the amidite method

The synthesis is efficient and moreover provides the opportunity to replace the I_2 /H_2O oxidation by a reaction with S_8 /pyridine, thus obtaining phosphorothioate triesters. It is possible to incorporate both groups at any desired point in normal oligodeoxynucleotides[64,65]. The cleavage of the support and the deprotection of the bases is carried out with 25% NH_4OH at room temperature for 48 h.

The ethyl and isopropyl phosphate triesters are stable under these conditions, whereas methyl and cyanoethyl esters are completely removed.[65]
Stec *et al.*,[66] developed in 1987 a method that combines some advantages of the classical amidite method with some of the H-phosphonate method. This employs 5'-protected nucleoside 3'-O-phosphorodimorpholidites as synthons, with hydrolysis to the H-phosphonate at each synthesis cycle (yields between 90 and 99%), and then oxidation is necessary only once at the end of the synthesis.

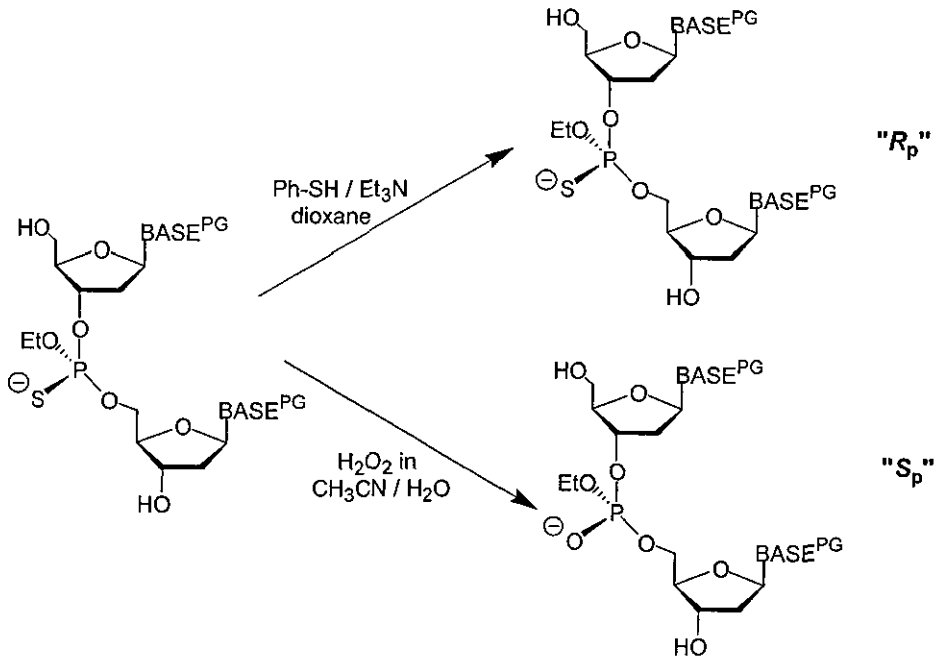
Alternatively alcohols can be used for alcoholysis, and the resulting phosphite triesters are oxidized at the end of the synthesis. This method shown below in **Scheme 8** is attractive due to its versatility.



Scheme 8 Preparation of oligonucleotide phosphotriesters by synthesis with phosphodimorpholino amidites, alcoholysis, and oxidation

The problem of chirality that has already been mentioned also occurs with the phosphate triesters. Considerable efforts have been made to separate the diastereomers arising due to this chirality center and to characterize them.[63,67] A method was employed to allow for configurational assignment at the phosphorus irrespective of the nature of triester.

Such a method is based on the stereoselective reaction of a P-chiral phosphothionotriester with H_2O_2 in acetonitrile /water to give an O-triester on one hand and with thiophenol /triethylamine in dioxane to give a phosphorothioate on the other hand.[68] This kind of reaction is shown in **Scheme 9** below.



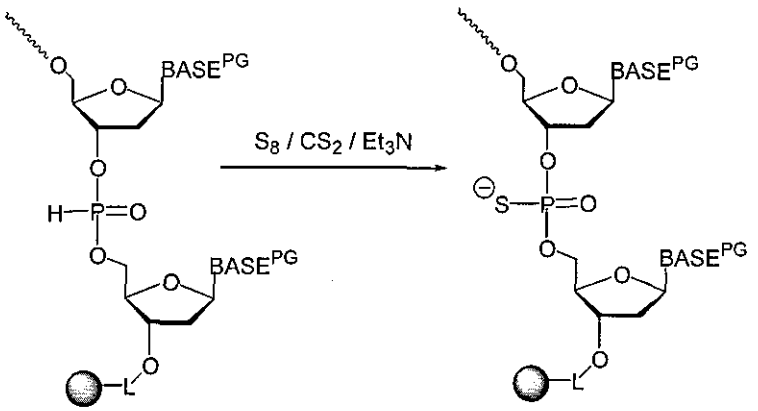
Scheme 9

(III) Phosphorothioates (3)

In phosphorothioate oligodeoxynucleotides one of the phosphate oxygen atoms not involved in the bridge, is replaced by a sulfur atom, with the negative charge being distributed asymmetrically and located mainly on sulfur. This substitution results in properties such as stability to nucleases, retention of solubility in water, and stability to base-catalyzed hydrolysis, which makes it exceptionally interesting for use in antisense technology.
The incorporation of internucleotidic phosphorothioate linkages into oligonucleotides has considerably enchanced their resistance to hydrolysis consequently increasing the stability of DNA to enzymatic hydrolysis.

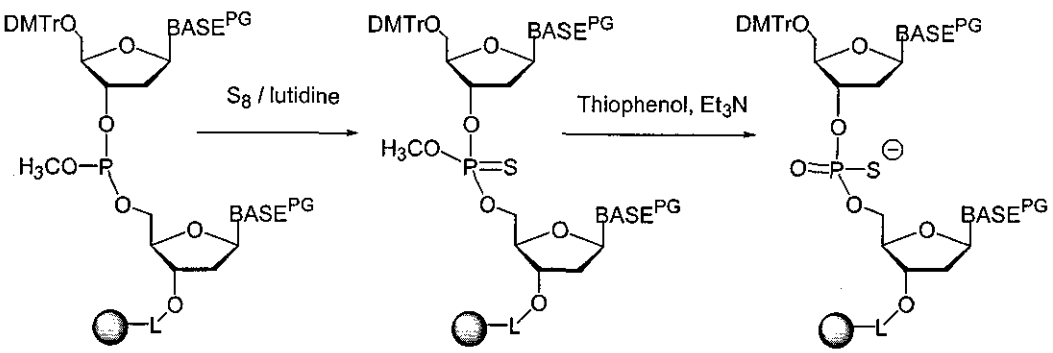
It has been demonstrated that antisense phosphorothioates complementary to the messenger RNA (the sense sequence) of the HIV-I gene inhibited the cytopathic effect of the virus in chronically infected H9 cells[69]. Just like the unmodified oligodeoxynucleotides, the phosphorothioates analogues can be prepared by the 3 main methods of synthesis, amidite, phosphotriester and H-phosphonate methods.

Synthesis of all-phosphorothioate oligodeoxynucleotides is more easily carried out as single-step sulfurization in a modification of the H-phosphonate method.[70] Fujii *et al.*,[71] was the first to describe the oxidation of the H-phosphonate with S_8 in pyridine/triethylamine, which was subsequently applied to solid-phase synthesis,[72,73] as shown in **Scheme 10**.
The advantage of this approach is that oxidation with sulfur (0.1 M S in 9:1 CS_2 / triethylamine, 2h), is necessary only once after the completion of the synthesis.



Scheme 10 Synthesis of oligonucleotide all-phosphorothioates by a modification of the H-phosphonate method

The usual amidite method is also used for the synthesis of phosphorothioates, the only change being oxidation with 0.4 M S_8 in 2,6-lutidine or 5% S_8 in pyridine at room temperature in place of the usual I_2/H_2O,[74] as shown in **Scheme 11** below.

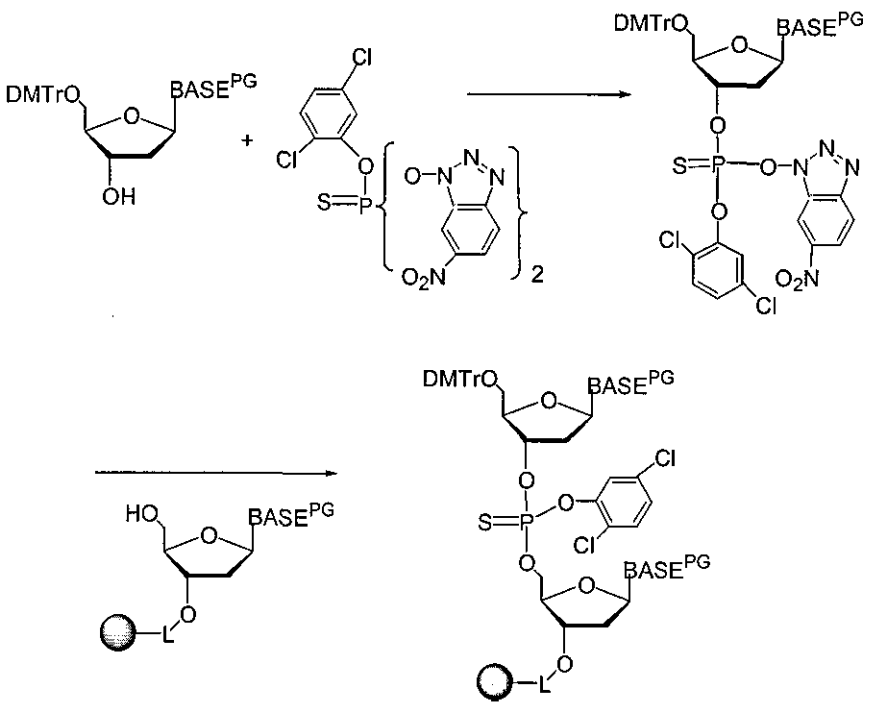


Scheme 11 Synthesis of oligonucleotide phosphorothioates by a modification of the amidite method

The yield in the subsequent coupling step remains at the same high level.[75] This method is particularly attractive because it can be carried out without extensive alteration of the normal synthesis cycle and is therefore still regarded as the method of choice.[76] Another advantage derives from the fact that phosphorothioate bridges can be introduced in this way at any desired point in the oligonucleotide synthesis.[74-76]

This is of interest particularly for studies of enzyme mechanisms[77] but also for antisense technology.[76] Subsequent oxidation with I_2 / H_2O does not seem to cause loss of sulfur. The deprotection of the thiophosphate group from the O-methyl ester is carried out at the end of the synthesis with thiophenol.

The phosphotriester method, without any modification in terms of strategy for the removal of the protective groups or the cleavage of the support, has also found applicability in the synthesis of phosphorothioate analogues.
The groups of Reese[78] and van Boom[79], have synthesized the phosphorothioate by the above method employing 2,5 dichlorophenyl phosphorodichloridothioate, as phosphorylating and coupling component. The preferred activator for the reaction is 1-hydroxy-6-benzotriazole (**Scheme 12**).

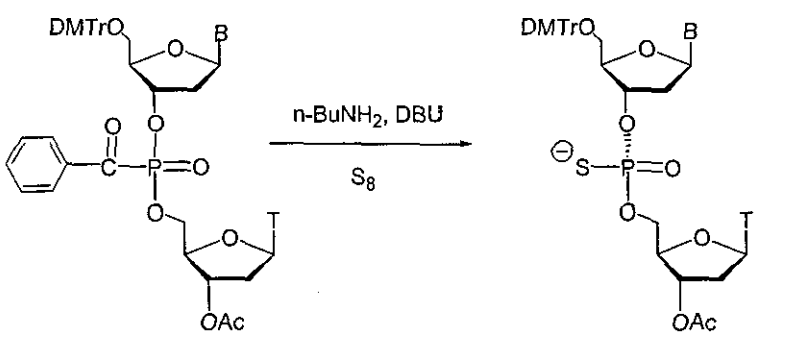


Scheme 12 Synthesis of oligonucleotide phosphorothioates by the phosphotriester method

Standard methods were used for cleavage of the support and removal of the protective groups,[79] while yields of >90% were reported for a 10 min synthesis.

The dinucleotide phosphorothioates have, as do the methylphosphonates and phosphotriesters, a chirality center at the phosphorus. There is a great interest in chromatographic separation of the diastereomeric dimers,[80,81] spectroscopic characterization[82] and the possibility of stereospecific synthesis.

Fujii *et al.*[71] published in 1986 a method (shown in **Scheme 13**) for the stereoselective preparation of the R_p diastereomeric dimmers T_ST and dA_ST. This entailed the corresponding aroylphosphonates being reacted with *tert*-butylamine in the presence of DBU and sulfur directly and exclusively to the R_p isomer with a yield of ca. 60%.



"R_p" configuration (> 99%)
yield 60%

Scheme 13

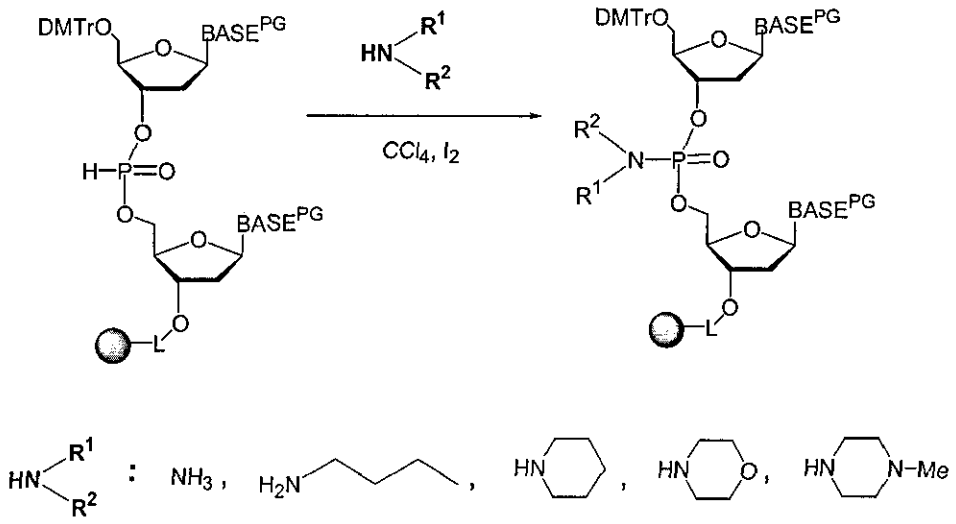
The chirality problem can be avoided if the second phosphate oxygen, which is not involved in the bridge, is also replaced by sulfur. Caruthers *et al.*[83,84] have described the synthesis of such phosphorodithioates. In this, a phosphorothioamidite is condensed with a second nucleoside with tetrazole catalysis and the resulting intermediate is oxidized with 5% sulfur to the phosphorodithioate. The synthesis can also be carried out on a solid support. To ensure high coupling yields the phosphorothioamidite must be supplied twice to the solid support in the coupling step.

(IV) Phosphoramidates (4)

The phosphoramidates are an easily obtainable group of nucleotide analogues, which can be prepared in various ways from three- and five-valent phosphorus intermediates.

The method of phosphorylating amines by condensation with phosphate diesters in the presence of triphenylphosphine and CCl_4 has often been used to prepare dinucleotide phosphoramidites.[85-87] However the yields were *moderate*[85,87] *(between 53-65%) while yields of 70%* were reached only on solid supports.[87]

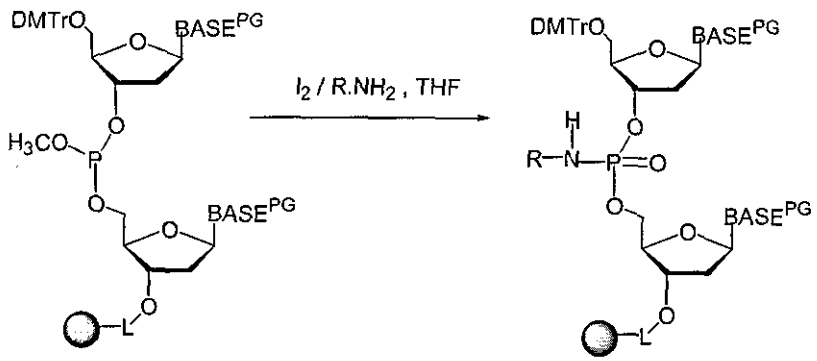
By contrast, oxidation of dinucleotide H-phosphonates in the presence of amines gives the corresponding dinucleotide phosphoramidates[88,89] in high yields.[90] The reaction can easily be extended to polymer-bound di- and oligonucleotide H-phosphonates. The oxidizing agent used for the coupling, which can be carried out with primary and secondary amines, is I_2 employing CCl_4 as the solvent[89,91] as shown in **Scheme 14** below



Scheme 14 Synthesis of phosphoroamidates by oxidation of H-phosphonates

Besides the described oxidation of H-phosphonates, which results in all phosphoramidate oligodeoxynucleotides, phosphite triesters are also oxidized with iodine in the presence of alkylamines.

This oxidation, which was first described by Nemer and Ogilvie[88] is particularly attractive[87] because it can be carried out without expensive alteration to the amidite synthesis cycle.[87] This entails oxidation with 0.1 M I_2 in 2:1 THF / alkylamine, resulting in selective elimination of the methyl protective group (**Scheme 15**).



Scheme 15 Synthesis of phosphoramidates by oxidation of dinucleotide phosphite triesters

This high yield method utilizes methanolic tert-butylamine for deprotection and cleavage of the support. An additional feature of the method is that phosphoramidite bridges can be incorporated at any desired point in the oligonucleotide. The process also allows for the generation of oligonucleotide conjugates,[87,92] e.g., with intercalating agents via alkylamino linkers that can be introduced during the oxidation step.

None of the methods used above result in the preferential formation of a diastereomer, but the mixtures in the case of the dimers can be separated by chromatography and characterized by spectroscopy.[87] *N*-Alkylphosphoramidates are suitable as functionalizing groups, via the alkyl radical and can be used to produce oligonucleotide conjugates **(REF)**.

B. OLIGONUCLEOTIDES WITH A MODIFICATION AT THE 5' OR 3'-END AND THE AMINO BASE

Oligodeoxynucleotides can be covalently linked to a wide variety of molecules and the coupling can be done through sites present naturally in nucleic acids. The naturally occurring groups that can be used are amino groups on the bases, hydroxyl groups on the sugars and phosphate groups, both terminal and internal.

In the context of antisense technology, this is usually done to improve transport and hybridization properties of the oligonucleotides. Conjugates are differentiated according to the position of their linkage to oligonucleotides and according to their function. This linkage can be introduced at one of the three stages during oligonucleotide synthesis.

- The linker can be attached to a nucleotide before incorporation into the growing chain.
- Molecules other than nucleotides can be introduced during the synthesis of the oligonucleotide.
- A linker group can be attached to a natural nucleic acid or to a synthetic oligonucleotide after deblocking.

The conjugates formed are designed to improve some of the existing features of the oligonucleotide, which will be endowed with some completely new property, either physical or chemical, while retaining its ability to base pair. Linker groups combined with oligonucleotides fall into two major categories:

1. **Chemically Reactive Groups.** Groups that cleave or cross-link with other nucleic acids or proteins are used to study interactions between these molecules and oligonucleotides, and to create possible therapeutic agents.
2. **Groups Promoting Intermolecular Interactions.** The best example of this category is the intercalating agents or lipophilic compounds used to strengthen the hybridization of the oligonucleotide with its complement and enhance cellular uptake.

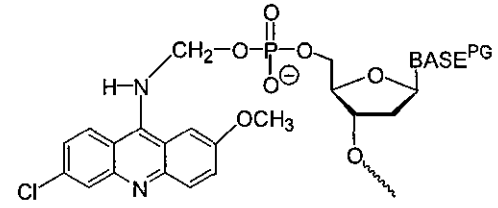
(I) Conjugation via the 5' End

Covalent linkage from the 5' end of oligonucleotides is particularly attractive because it can be carried out in the solid phase synthesis in a similar manner to the 5'-phosphorylation of oligonucleotides The group that is to be linked can be derivatized as a phosphoramidite, H-phosphonate, or phosphate and withstands the coupling and deprotection.

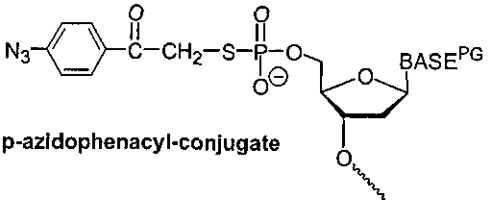
(a) Intercalating Agents

The idea of linking an intercalating compound to an oligonucleotide was first described by Letsinger.[93] There has been a number of papers in which phosphotriester, or amidite chemistry was used for covalent attachment of intercalating agents, usually acridine derivatives to the 5' end of modified and unmodified oligodeoxynucleotides.[94-96]

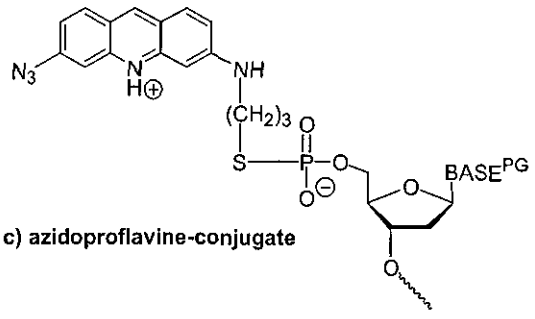
Thus, 2-methoxy-6-chloro-9-((5-hydroxypentyl)amino)acridine (shown in **Scheme 16**) has been linked via its phosphoramidite to oligodeoxynucleotide methylphosphonates and α-anomeric oligodeoxynucleotides.[97,98] The same intercalating agent has previously been incorporated using phosphotriester chemistry[99-100].



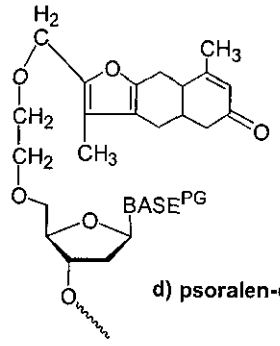
a) acridine-conjugate



b) p-azidophenacyl-conjugate



c) azidoproflavine-conjugate



d) psoralen-conjugate

Scheme 16

(b)Cross-Linkers

Cross-linkers can be linked to the 5'-position by a variety of methods.[94-96] Thus psoralen derivatives (shown above in **Scheme 16**) have been incorporated in oligonucleotides and oligonucleotide methylphosphonates via the amidite chemistry[101] and phosphotriester chemistry.[102]

Helene *et al.*, introduced azidophenacyl,[103] azidoproflavine[105] (shown above in **Scheme 16**) and proflavine[104] and to the 5'-position by first converting the oligonucleotide into the 5'-thioshosphate, which could then be alkylated via a bridge with the particular cross-linker (3-azido-6-((3-bromo-propyl)amino)acridine.[105]

(c) Lipophilic Carrier

The transport of oligodeoxynucleotides into the cells has been facilitated, by linking them to lipophilic carriers. Goodchild *et al.*[106(a)] described the incorporation of long chain alcohols as phosphate esters in the 5'-position of oligonucleotides. This can take place at the end of solid phase synthesis by use of an appropriate phosphoramidite. Letsinger[106(b)] has reported only one example of a cholesterol conjugate of oligonucleotides in the literature.

Universal methods for fuctionalizing oligodeoxynucleotides with amino or mercapto groups have been described Sinha and Connolly,[107] who used H-phosphonate and amidite chemistry to attach amino-alkyl and mercaptoalkyl linkers to the 5'end (**Scheme 17**). A large number of derivatives, such as dyes or other nonradioactive markers, can be introduced via these linkers. Simple linkage with polylysine, which is intended as a carrier for oligonucleotides[108], via a maleimide has also been described.

Y = S, NH

Synthons for the amino- and thiofunctionalization of oligonucleotides

Scheme 17

(II) Conjugation via the 3' End

Modifications at the 3'-end are less common due to the inaccessibility of this site in solid support synthesis. This problem was though solved using a support with a modification build in.

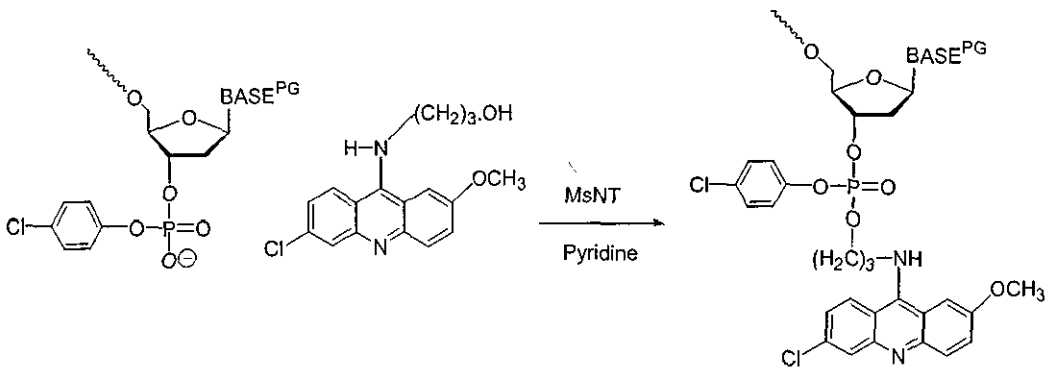
Polyamide or polypeptide chains were synthesized on a support to give conjugates with a 3'-polypeptide tail. The spacer between the nucleoside and the support was designed so that on cleavage, a reactive nucleophile is generated at the end of the oligonucleotide.

Reactions at the 3'-end can be performed more readily using solution phase synthesis. Examples are the condensation between a 3'-phosphate and the hydroxyl group of an acridine or phenanthroline derivative using triester chemistry.[109,110]

(a) Intercalating Agents

The synthesis of conjugates of intercalating agents with oligonucleotides via the 3' position has been extensively investigated; the agent mostly used was 2-methoxy-6-chloroacridine. Whereas 5'-derivatization is primarily carried out in solid-phase synthesis using amidite chemistry, the one in the 3'-position mainly employs triester chemistry[111-114], as shown below in **Scheme 18.**

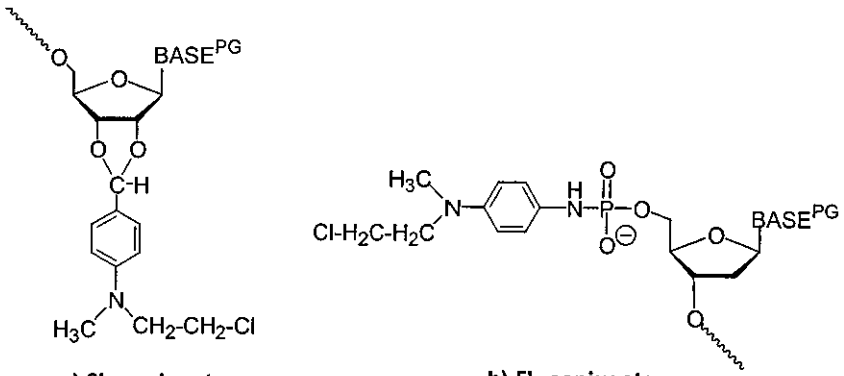
3'-Conjugation of modified oligonucleotides containing methyl esters has also been described.[115]



Scheme 18

(b) Alkylating Oligonucleotides

Grineva *et al.*[116] put into practice the idea of specific and irreversible modification of DNA by cross-linking. They used for this a dinucleotide that was fuctionalized at the 3' end via an acetal linkage with an anomeric (2-chloroethyl) amino group (**Scheme 19**). Alkylating groups have also been attached in the 5'-position via phosphoramidate linkages, as shown in **Scheme 16** in page 26.



a) 3'- conjugate **b) 5'- conjugate**

Scheme 19

The neighbouring group participation of the nitrogen means that the (chloroethyl) amino groups are particularly susceptible to alkylation and thus cross-linking. This mode of substitution was subsequently extended to oligonucleotides with a 3'-nucleoside residue and has been widely used in antisense technology.[117-119] The alkylation reaction appears to be "hybridization triggered" because no self-alkylation takes place.

(c) Oligonucleotide-Peptide Conjugates

Haralambidis *et al.*[120] described the successive solid-phase synthesis of oligodeoxynucleotides with a 3' -peptide unit. After the peptide chain has been synthesized on the support of the Fmoc method, a 4-hydroxy-butyryl linker was attached and then the oligonucleotide synthesis was carried out. The conjugate was deprotected at the end of the solid-phase synthesis Also Lebleu *et al*,[121,122] synthesized oligonucleotide-polylysine conjugates, in order to improve the transport properties of oligonucleotides.

C. INCORPORATION OF LINKER GROUPS DURING CHEMICAL SYNTHESIS OF OLIGONUCLEOTIDES

From a synthetic point of view, incorporation of the linkers during the assembly of an oligonucleotide rather than afterwards is the most rigorous approach. It gives greater control over the number and location of the modifications, the protective groups of the nucleotides minimize side reactions and advantage is taken of the benefits of solid-support synthesis for work-up and purification.

The incorporation of modified nucleotides is appealing for chemical synthesis as the nucleotide building block carrying the desired modifier can be introduced precisely at any internal or terminal position in the oligonucleotide with assurance that modification at that position is complete. The modifications though must be able to withstand the coupling reaction and the rigours of acid and basic deblocking.

Substituents may be attached to nucleotides at the base and sugar, but ideally, changes should not interfere with hybridization. Emphasis will be given on the nature of the bond-making reactions and the coupling reactions used for different linker groups.
Substituents used for the formation of conjugates could be either electrophilic linkers or phosphoramidites. They are used most commonly for coupling at the 5'-end of an oligonucleotide while it is still fixed to a solid support or the amino base of nucleotides. The linkers must be soluble in an organic solvent suitable for the coupling reaction and, again, must withstand the deblocking procedure.

(I) Electrophilic linkers

Most examples of conjugate formation use a nucleophile to react with electrophilic linkers. Oligonucleotides contain a number of reactive sites that can be used for conjugation. For the most part these are nucleophiles and include the nitrogen function on the bases, primary and secondary hydroxyl groups and phosphomono- and diesters.

Unwanted reactions may occur at other sites of the nucleotide, which are predominantly nucleophilic in nature, particularly the bases. Reactions at these sites are likely to lead to complex mixtures unless they can be controlled in some way.

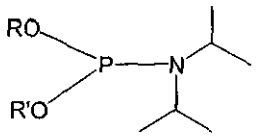
An electrophilic linker can be used providing that it does not undergo intramolecular coupling with other sites. Steric hinderance around the reacting amino group can be a troublesome. Electrophiles can be used in the form of carbonyl compounds or activated carboxylic acids and are directly coupled to the amino group of nucleosides.

Related species that have been used in literature include nitrophenyl[92,123], and pentachlorophenyl esters[124], acid anhydride[125,126] and the ester of N-hydroxysuccinimide that give an amide.[127-130] The reactions have also been extended to reactive sites other than amines and been studied in some detail to minimize base modification. The N-hydroxysuccinimide ester gave less reaction at the bases but was also less efficient overall.

Also electrophilic linkers were used to label RNA with a number of fluorescent dyes and other agents.[131,132] For the labeling of RNA with biotin, periodate was used to oxidize the 3'-terminal nucleoside to a dialdehyde that was then reacted with a primary amine and reduced with borohydride.

(II) Phosphoramidites

A site amenable to ready modification is the internucleoside phosphate, during the oxidation of intermediate phosphites. The covalent linkage is usually brought about at the 5'-end with phosphorylating agents, such as phosphoramidites that have the general structure below.



They can be employed to phosphorylate the 5'-hydroxyl group using triester chemistry and introduce one or more lipophilic groups into the backbone. The phosphate can be used for modification at a later stage or it can be selectively deblocked before cleaving from the solid support.

Letsinger and his co-workers[124] have done much of the work on conjugation here. They reported a method for linking through phosphoramidates to prepare conjugates of phenathridine and cholesterol.
Phosphoramidite derivatives of biotin[133] and anthraquinone[134] have been used similarly. Other workers have introduced derivatives of tetramethylrhodamine, and acridine using triester synthesis both by solid support and solution-phase synthesis.[109,135]

Modifications at the 3'-end are less common due to the inaccessibility of this site in solid-support synthesis. Reactions at the 3'-end may be performed more readily during solution phase synthesis.

D. INCORPORATION OF LINKER GROUPS AFTER THE SYNTHESIS OF OLIGONUCLEOTIDES

Conjugate groups can be introduced after the synthesis and deblocking of the oligonucleotide. This normally requires less effort than incorporation during synthesis but introduces other problems. Post synthetic reactions on oligonucleotides can be used to modify linkers or introduce a conjugate group. As oligonucleotides are polyionic, post synthetic reactions are usually performed in water or aqueous solvent in which the reagents will be sufficiently soluble and stable. This in itself is restricting, as many synthetic reactions are not intended to be performed under these conditions.

Since linkers are usually electrophiles, unwanted reactions may occur at many internal sites in oligonucleotides. The separation of oligonucleotides with different numbers of conjugated groups is difficult, as is their purification, which is usually not attempted.

The structure of the product is frequently assumed from the nature of the starting materials, particularly with conjugates of large molecules, where it is generally not possible to apply the more rigorous standards of organic chemistry. For example analysis by NMR spectroscopy is not possible, and identification may only be achievable by mass spectrometry for very large conjugates.

However, the reasons for making these compounds are usually for practical reasons and provided that they function as intended, then the precise number and location of the conjugate group may not be crucial.

3. PROPERTIES OF MODIFIED OLIGONUCLEOTIDES AND THEIR CONJUGATES

Oligonucleotide derivatives are considered as potential biologically active compounds capable of interfering with the functions of target nucleic acids possessing complimentary oligonucleotide sequences. The efficiency of these derivatives as reagents targeted to cellular nucleic acids can be in increased by improving their ability to penetrate into cells and by stabilizing their complexes with the target nucleic acids.

The factors that are usually assumed to limit the activity of the modified oligonucleotides are **cellular uptake, resistance to nucleases** and the **stability of the hybrid formed**.
Modifications are usually chosen to improve one or more of these properties and the conjugate itself may prove beneficial in these regards if it is lipophilic or otherwise inhibits the action of nucleases

Overall activity results from the interplay of these and other factors whose relative importance is not known. However, a steady improvement in activity has been achieved by using this rational approach that is encouraging for the designing of conjugates to meet specific requirements.

A.The effect of modification on oligonucleotide hybridization

Modifications to the internucleoside phosphates can affect hybridization in a number of different ways, but it is important that they should not prevent base pairing. Reduction in charge density lessens electrostatic repulsion between the strands and should facilitate their association.

It has been suggested that steric interactions of substituents will normally destabilize the helix, as will their electronic and other properties.[136] These might include disruption of hydration of the helix. However the grooves of the hybrid might also provide a more lipophilic environment, thereby promoting hybridization. Cases where stronger hybridization resulted on increasing the lipophilicity of the substituent can be examples of such an effect.[137,138]
The relative contributions of all these factors are governed by intrinsic factors such as the length of the oligonucleotide, the degree of modification, the localization of a given modification relative to the ends or middle of the helix, and the sequence of the bases around it.[136] Thus, the consequences of a particular modification will vary from case to case.

In their early studies of uncharged methylphosphonates and triesters, Miller and Ts'O[139,140] found that racemic di- to tetramers hybridized to unmodified strands with greater affinity than the parent phosphodiesters. While diastereoisomers of dimers differed from each other, both formed more stable hybrids than the natural charged compounds. This was attributed to the lack of charge-charge repulsion between the strands of the complex.[141,142]
An adverse effect on hybridization as the size of the substituent increased from PCH_3 to $POCH_3$ to $POCH_2CH_3$ seemed to be due to steric interactions. Similar improvements to hybridization were reported for other low molecular weight triesters and phosphoramidates.[138,143] With substituted amidites, these included a positively charged backbone.

Extensive substitution of longer oligonucleotides with various phosphoramidate linkages gave somewhat less stable hybrids than the phosphonates. Those derived from primary amines were superior to those from secondary amines.[144]

Also improvements in hybridization were reported when bulkier substituents were used in the modification of the internucleoside phosphates. In an attempt to stabilize hybridization with short oligonucleotides, Letsinger and Scott[124] attached an intercalating agent to the phosphate groups of TpT. This proved successful and permitted the use of shorter oligonucleotides that would otherwise be possible for antisense studies.
The physical chemistry of these interactions has been investigated[145,146] and it has been shown that an intercalating agent at the end of an oligonucleotide especially the 3'-end is more beneficial than on an internucleotide phosphate.[109,146] It was also shown that a second intercalating group offers no further advantage.

Modifications other than an internucleoside phosphate may also effect hybridization. α-oligonucleotides, for example, hybridize with β-RNA and DNA by forming the usual Watson-Crick base pairs. These hybrids are considerably more stable than when both strands are β.[147]

B. The effect of modification on nuclease resistance

A number of studies have demonstrated degradation of unmodified oligonucleotides at greatly varying rates in different cells. Survival times vary from minutes to day.[148-151] Consequently it has been a goal to develop derivatives that will be resistant to the enzymes that degrade nucleic acids (nucleases), on the assumption that they will be more potent.

Modifications to internucleoside phosphates that have been investigated inhibit the action of nucleases. Most reports suggest complete resistance of phosphoramidate,[137,138,152,153] phosphonate,[154,155] or phosphotriester[137,142,156] linkages towards the nucleases that have been tested. All substituted phosphoramidates were resistant. While the modified linkages themselves may be resistant, in oligonucleotides containing mixtures of modified and natural linkages, exonucleases that work progressively from one end of the chain can sometimes skip over an isolated phosphonate or triester linkage to cleave the adjacent phosphodiester at a reduced rate.[155,157]

Modifications at groups other than phosphate may also induce resistance to nucleases. α-oligonucleotides, for example, proved to be far more stable than β-oligonucleotides in a number of different biological environments.[158,159]

Increasing lipophilicity is likely to decrease the rate of degradation by nucleases that are designed to degrade polyanions. A presence of a bulky group such a lipophilic compound at an appropriate end of an oligonucleotide (either the 3' or 5' end) can increase its resistance to nucleases, thus preempting exonuclease attack.[158,160] RNA was found to be more stable than DNA in nuclear cell extracts.[161] Methylation of the 2'-hydroxyl group increases its resistance to nucleases.[162]

C. The effect of modification in the penetration of oligonucleotides through membranes (cellular uptake)

The activity of the oligonucleotides is crucially affected by how well they reach their site of action unmetabolised. The protein biosynthesis apparatus of the cell is located in the cytoplasm and comprises 55% of the cell volume. Thousands of enzymes bring about there the biosynthesis of sugars, fatty acids, nucleotides, amino acids and proteins.
The mRNA produced in the nucleus by transcription of the DNA is translated into the corresponding protein on the ribosomes in the cytoplasm. In order for the oligonucleotides to act to stop translation by hybridization, they must pass through the plasma membrane into the interior of the cell. The plasma membrane is a natural barrier and can therefore be considered as a bottleneck in the antisense oligonucleotide concept. (**Figure 2** in page 4 shows the principal of the oligonucleotide action).

A problem common to uptake studies is the difficulty in distinguishing material inside the cell from that bound to the outer membrane. Surprisingly though, despite their high charge density, oligonucleotides are taken up reasonably well by mammalian cells. This appears to be energy-requiring process and may involve receptor proteins on the cell surface. Intermolecular concentrations may rise to about 10% of those outside the cell within 15 min to 2 h.[148,151,163,164]

Shorter oligonucleotides are taken up more rapidly and phosphorothioates are taken up more slowly than unmodified oligonucleotides.[160] Uptake of uncharged methylphosphonates appears to be quite different. Intracellular levels of dimers to nonamers reached extracellular concentrations within 1.5 h.[138] This would appear to be passive diffusion across the cell membrane.

Various ways have been employed to improve the penetration of the antisense oligonucleotides through the membranes with the most important being the lipophilic modification of the oligonucleotides. Lipophilic substituents such as intercalating agents and lipophilic alcohols facilitate uptake.[165,166] Increasing lipophilicity to improve the cellular uptake is likely to decrease the rate of degradation by nucleases that are designed to degrade polyanions.

The conjugate group itself can be proved beneficial in these regards if it is lipophilic to inhibit the action of nucleases. Among those conjugates are derivatives with acridine linked at the 3'end, which has a beneficial effect on both the ability to penetrate and the stability to nuclease.[167,168]

Conjugates of poly-L-lysine were reported[169,170] to stimulate the uptake of homooligoribonucleosides in tumor cells. Poly-L-lysine can form complexes with the polynucleotides themselves, and these complexes have high affinity for the cellular membrane.[169] It is also able by interacting with the cellular receptor of HSV-1, to block the binding of the virus to the receptor.[171] In this connection, poly-L-lysine, of molecular weight of 50,000 proved to be toxic in the micromolar range.

Another group that like poly-L-lysine, is intended to increase uptake, is cholesterol. Its conjugates can penetrate through the membrane in a specific manner in order to improve the passage of oligonucleotides through membranes. This can have beneficial effects for both unmodified and modified backbones as well as for alkylating agents.[172]

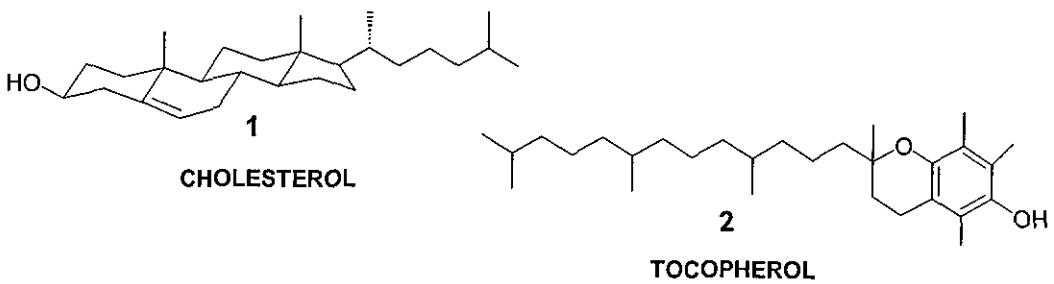
4. IMPORTANCE OF LIPOPHILIC COMPOUNDS TOWARDS OLIGONUCLEOTIDE SYNTHESIS

Major obstacles that prevent wider application of the antisense oligonucleotides are their limited oral bioavailability and permeability through cell membranes. Modification of oligonucleotides using lipophilic compounds is a possible method to improve their pharmacokinetics as they assist the likelihood of oligonucleotide uptake into the cells by reducing their polarity. These lipophilic groups fall into two groups:

(a) Those known to interact specifically with cell membranes. e.g. Cholesterol[173-175] and certain lipids[176]

(b) Those who impact general lipophilicity to the oligonucleotide e.g. Alkyl chains[177,178]

Molecules such as cholesterol **1** and tocopherol **2** (shown in **Scheme 20** below) are known to have an immune stimulating effect and can enhance the adjuvant effect of the oligonucleotide. It is unclear by what mechanism these compounds exert their ability due to their lack of sequence specificity, but the lipophilic tail has been shown to improve cellular uptake.[175,177]



Scheme 20

Vitamin E (tocopherol) and cholesterol are attractive candidates as lipophilic carriers for the following reasons:

- They are inexpensive and essentially non-toxic

- They are found mainly in association with membranes of subcellular organelles[179] such as the endoplasmic reticilum and mitochondria, rather than in the plasma membrane. This maximizes the possibility of intracellular transport

(A) TOCOPHEROL

The protection of organic materials, including living organisms, against oxidative degradation is provided by fairly small quantities of certain specific compounds called antioxidants.[180] Tocopherol (known as vitamin E) is a major lipid-soluble chain-breaking antioxidant in mammalian cells and there is evidence that it may be involved in the division of normal and cancerous cells. [181] It has already[182,183] been connected to DNA through ester linkages but little is known about the behaviour of these DNA constructs in cells.

The term vitamin E^6 refers to one or more of four structurally phenolic compounds called tocopherols. The tocopherols differ from one another only in the number and position of the methyl groups in the aromatic ring. α–Tocopherol, which is fully methylated, is the most plentiful and the most biologically active of these four compounds. It has only one functional group for derivatization; the rest of the molecule is chemically inert.

Biological membranes, which are composed largely of phospholipid molecules, cholesterol and the membrane-bound proteins,[184] must be protected against peroxidation. Vitamin E appears to play a vital role in this protection and the chemistry that can occur depends on the average position of the various reactants and their mobilities. It has been proved[181,185] that in lipid bilayers, tocopherol is oriented with the phenolic hydroxyl group "head" towards the surface while the hydrophobic phytyl "tail" is embedded in the membrane, Thus attachment of the oligonucleotide to the phenolic hydroxyl group should have a minimal effect on the interactions of the vitamin E with membranes.

(B) CHOLESTEROL

Cholesterol has a high affinity for lipid bilayers and a strengthening effect upon the vesicles (also called "liposomes"), which have delivered a widespread attention for drug delivery in cancer therapy.[186] The lipophilicity of cholesterol transiently anchors the oligonucleotide to the cell membrane and may enhance the facility of cellular uptake and stabilize complexes formed with cellular nucleic acids.[187,188]

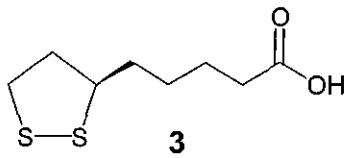
It has been shown that molecules attached to unsaturated fatty acid esters of cholesterol are incorporated most readily into the LDL core (low-density lipoprotein).[189] This protein is a selective and rapid vehicle for the delivery of lipophilic compounds to mammalian cells in tissue culture.[190] Also addition of cholesterol to the 3' or 5'-terminus of phosphorothioate oligonucleotides has been shown to improve therapeutic affect in an HIV assay.[191]

Both endogenous cholesterol, synthesized in the liver endoplasmic reticilum[192,193] and exogenous cholesterol are rapidly transported throughout the liver cell and are found in all the intracellular membranes and in small amounts in the cytosol.[194] Reports have shown[195] that exogenous cholesterol appears rapidly in a number of subcellular fractions, including the chromatin fraction of rat liver, indicating that there must be a mechanism for rapid transport of cholesterol from the liver plasma membrane to other sites of the cell.

In one cell line, cholesterol-modified oligonucleotides are localized in the nucleus, probably delivered by a cholesterol binding protein.[196] Such a protein can function as an intracellular cholesterol transport or receptor protein. It can bind to both free and esterified cholesterol in roughly equal amounts and possesses the properties expected of an intracellular cholesterol transport or receptor protein.[196]

(C) THIOCTIC ACID

Thioctic acid **3,** which is also known as lipoic acid can also be considered as a



3

THIOCTIC ACID (LIPOIC ACID)

lipophilic compound. It is widely distributed in plant and animal tissues[197] and as a lipoamide it functions as a co-factor for the multi-enzyme complex that catalyses the oxidative decarboxylation of α-keto acids and also as a growth factor in many bacteria and protozoa.[198]

Thioctic acid exhibits antioxidant functions: it scavenges hydroxyl radicals; singlet oxygen and also chelates transition metals. Thioctic acid and its reduced form (dihydrolipoic acid) appeared to be able to regenerate other antioxidants such as ascorbate and vitamin E.[199]

Due to these antioxidant properties and its high level of biological activity, it is useful for the treatment of various diseases.[200] It has beneficial effects in prevention and treatment of both type-I and type-II diabetes, and in preventing damage in myocardial and cerebral ischemia-reperfusion injury in rat. It can also act as a radioprotective agent against damages to DNA and its components produced by radiation exposure.

It has also be shown to be potentially useful for the treatment of heavy-metal poisoning, degenarative diseases of the central nervous system, and AIDS which makes lipoic acid a biologically important molecule.

5. AIMS OF THE PROJECT

The objectives of this project were:

(a) **Synthesis of novel linker groups for attaching lipophilic molecules to the oligonucleotide constructs**

This is done in order to improve transport of the molecules across cell membranes, which can be easily cleaved off once the DNA construct has entered the cell.

(b) Development of methods for the global and partial protection-deprotection of the nucleosides.

The aim is to employ suitable and versatile protecting groups for the hydroxyl functions of the nucleosides without the need for base protection.

(c) **Synthesis of conjugates of different nucleotides, where the lipophilic groups are connected, to the exocyclic amino base or the 5' primary hydroxyl group, through novel type links.**

The scope of this is to improve the transport and hybridization properties of the nucleotides and explore their stability whilst containing these novel linkages.

(d) **Formation of dinucleotides containing the desirable immunostimulating sequences, such as CpG dinucleotides.**

The aim of this is to study the chemistry of such compounds with or without the attachment of lipophilic molecules, and to investigate the stability of the linkers, towards the deprotection conditions required to complete the oligonucleotide synthesis.

A. SYNTHESIS OF LINKERS

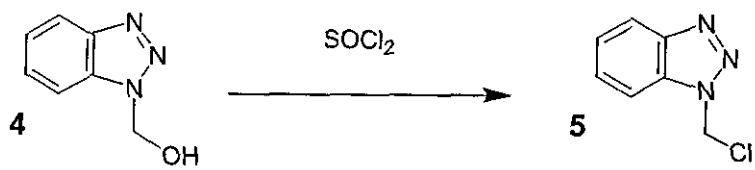
Oligonucleotides attached to lipophilic groups may become adhered to cell membranes and could therefore become unavailable for binding to mRNA.[201] This problem can be addressed by attachment of lipophilic compounds via potentially cleavable linkers, a strategy that is well known as a prodrug strategy for the delivery of therapeutic molecules.[202]

Previously, several groups have been used to tether a lipophilic group to a synthetic oligonucleotide. A tetrathymidylate sequence with unmodified phosphodiester bonds may in principle be cleaved by intracellular endonucleases considerably faster than the phosphorothioate backbone of first generation antisense oligonucleotides.[203,204]
More recently, a triglycyl spacer, the target for intracellular peptidases, has been used to conjugate cholesterol to the 3'-terminus of triple helix forming oligonucleotides.[205] A biodegradable disulfide bridge has also been employed to attach an alkyl chain to the base moiety of an oligonucleotide[206] and a phospholipid group to the 5'-terminus of an oligonucleotide.[207]

In our approach a number of reagents has been used for the synthesis of linkers of lipophilic compounds. They and are generally much simpler synthetic targets that mainly aid the incorporation of linkers during oligonucleotide synthesis. The groups introduced are achiral, thus avoiding the difficult separation of diastereisomers during the linker synthesis.
They were chosen to be stable enough to stand up to the conditions of DNA synthesis, but can also be easily hydrolyzed in the cell. They also form electrophilic linkers and act as good leaving groups for the subsequent coupling reactions. This reduces the potential for side reactions and simplifies purification. The reagents were soluble in organic solvent suitable for the coupling procedure and could withstand the deblocking procedure

(I) Acetal Linkers

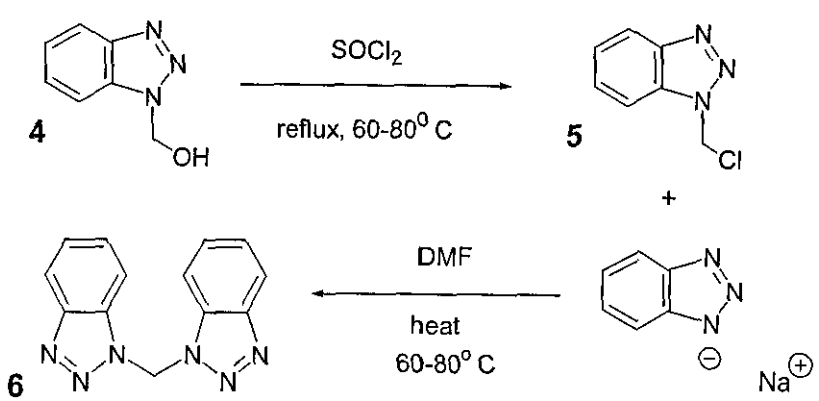
The benzotriazole methyl ethers of tocopherol cholesterol and thioctic acid **7**, and **8** and **9**, respectively (shown in **Scheme 23**), will be synthesized, by treatment of the corresponding lipophilic compounds with 1-chroromethyl benzotriazole **5**, and bis-benzotriazole methane **6. Scheme 21** shows the synthesis of 1-chloromethyl benzotriazole **5** as the acetal-forming reagent.



1-hydroxymethyl-benzotriazole **1-chloromethyl-benzotriazole**

Scheme 21

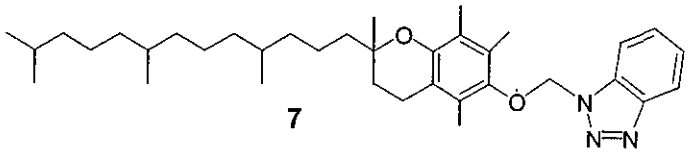
Bis-benzotriazol-1-yl methane **6**, was also synthesized in two steps[208] (starting from 1-hydroxymethyl-benzotriazole **4**), as shown in **Scheme 22** below.



***Bis*-benzotriazol-1-yl methane**

Scheme 22

Tocopherol derivative



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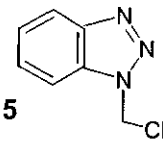

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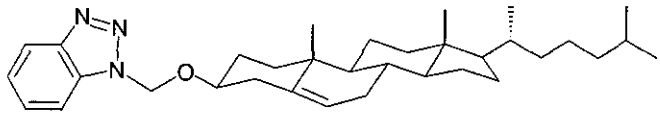
1-chloromethyl-benzotriazole

Cholesterol derivative



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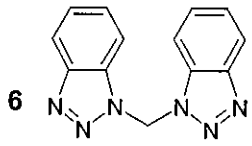
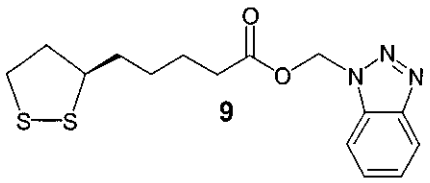


6

***Bis*-benzotriazole methane**

Thioctic acid derivative



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Scheme 23

These linkers will then be used in acetal and amide forming reactions with the 5'-terminal hydroxyl group and the NH_2 amino group of the nucleosides, respectively.

(II) Linkers containing carbonyl groups

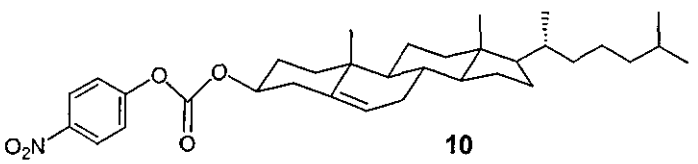
Carbamate[209,210] and carbonate[211] linkages have been shown to have biochemical properties such as template activity and resistance to enzymatic degradation. They possess linkages, which are achiral, thus avoiding the difficult separation of diastereoisomers during their synthesis and purification. The phenolic groups act as good leaving groups for the subsequent coupling reactions.

(a) Carbonate linkers

Using 4-Nitrophenyl Chloroformate

The p-nitrophenoxy group exhibits a high propensity to act as a leaving group in nucleophilic displacement reactions. This ability has been widely used in biochemistry.[212] The 4-nitrophenyl carbonate linkers **10** and **11** of the corresponding lipophilic alcohols (**1** and **2**) will be designed by reaction with the 4-nitrophenyl chloroformate **12** as shown in the **Scheme 24** below.

4-nitrophenyl carbonate linker of cholesterol



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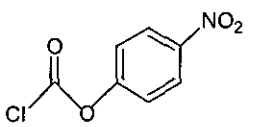
4-nitrophenyl chloroformate



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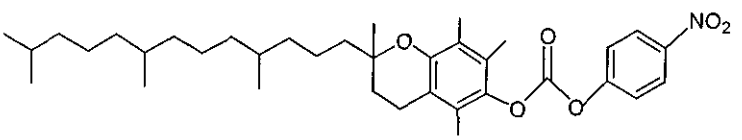
4-nitrophenyl carbonate linker of tocopherol



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Scheme 24

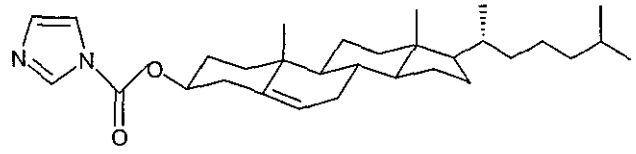
(b) Carbamate linkers

The carbamate linkage is attractive due to its stability over a wide pH range and its stability under physiological conditions. Its resistant to nuclease hydrolysis and it is also stable to hydrolysis by base, a property that favours their use in a biological context.

Using N,N'-Carbonyl-Diimidazole (CDI)

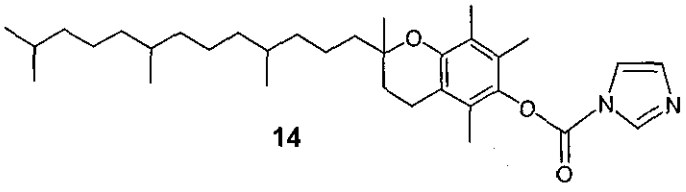
The use of CDI as a carbonyl synthon has found to afford clean, high yield reactions in the synthesis of oligonucleotide analogues.[213] Carbonylimidazolide derivatives **13** and **14** of the corresponding lipophilic alcohols (**1** and **2**), will be designed by reaction with N,N'-carbonyl-diimidazole **15** (**Scheme 25**).

carbonylimidazolide derivative of cholesterol



13

carbonylimidazolide derivative of tocopherol



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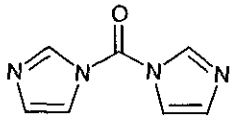
N,N'-Carbonyl-Diimidazole



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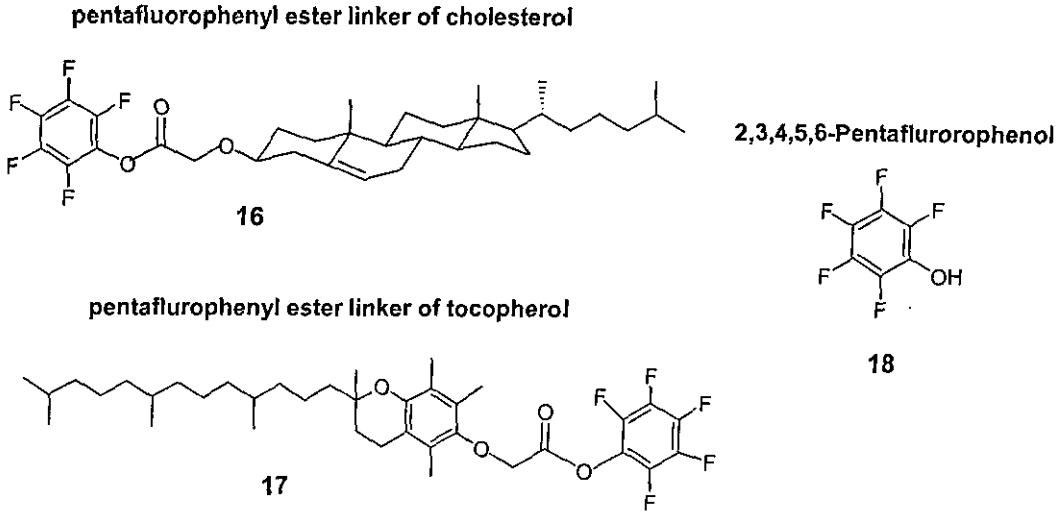
Scheme 25

(c) Ester linkers

An ester bond may be hydrolyzed by intracellular esterases.[214] Attachment of a lipophilic linker through an ester bond is hence an attractive approach to obtaining prodrug forms of oligonucleotides. The reactivity of ester function towards nucleophiles can be used for the synthesis of the desired oligonucleotide conjugates.

Using 2,3,4,5,6-Pentafluorophenol

Esters linkers will also be formed with an extra CH_2 group, which makes them more stable for further coupling reactions. **Scheme 26** below shows the pentafluorophenyl ester linkers of cholesterol **16** and tocopherol **17** formed by reaction of the corresponding lipophilic alcohols (**1** and **2**), firstly with ethyl bromoacetate and then conversion of the ethyl ester to the carboxylic acid. Subsequent reaction of the carboxylic acid with 2,3,4,5,6-pentafluorophenol **18** gave the desired ester linkers **16** and **17**.



Scheme 26

(III) Linkers for linking antibody to DNA

Linkers were also proposed for linking tumour targeting GRE1, GRE4 Rabbit Immunoglobin G antibodies (NRbt IgG), provided by the labs in Queen's Medical Center and Aphton Corporation to different DNA constructs containing antisense insert. These antibodies will be linked through a degradable linker to four different types of DNA Anticancer Agents (for detailed explanation see Discussion Part 1-E).

(a) Linearised Antisense DNA

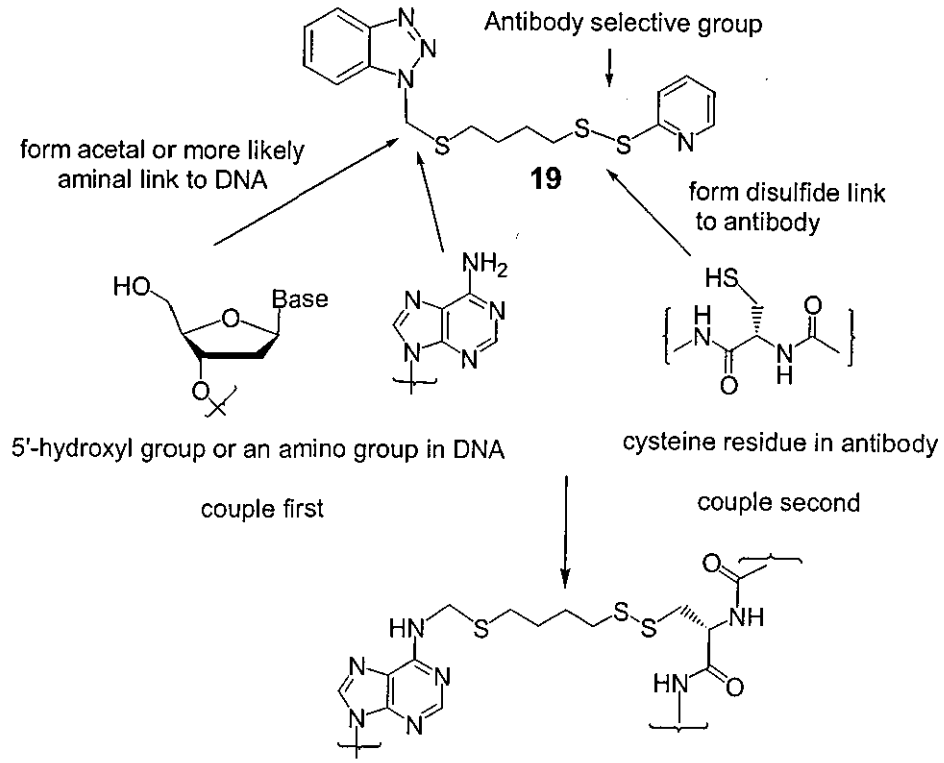
(b) Circular Plasmid DNA

(c) Apoptotic Synthetic Peptides AK79 (sequence of 9 amino acids long)

(d) Bisindolylmaleimide III HCl (selective inhibitor of protein Kinase C)

The N-S bond can form an acetal or aminal link with DNA where the disulphide bond can form a disulphide link with antibody. **Scheme 27** shows how the proposed linker **19** can connect the antibodies to DNA.

Linker for connecting gastrin antisense DNA to GRE1 and GRE4 antibodies



Scheme 27

B GLOBAL AND PARTIAL PROTECTION OF NUCLEOSIDES

There are three main points that should be accounted for in dinucleotide synthesis

(a) the selection of suitable protecting groups for the hydroxyl, amino and phosphate groups

(b) the actual synthesis of nucleosides protected on the 2'-hydroxyl and/or the 2' and 5'-hydroxyls

(c) the condensation of the protected nucleosides to dinucleotides

Progress in the chemical synthesis of oligodeoxynucleotides has climaxed with the development of automated procedures for the synthesis of gene fragments.[215] The success in the deoxy- area is due primarily to the establishment of adequate and versatile protecting groups for the amino, hydroxyl and phosphate functions.[216] Oligoribonucleotides though have posed a more difficult challenge in terms of protecting groups. The presence of the 2'-hydroxyl group not only requires protection but also places serious limitations on the nature of protecting groups used.

Methods have been explored for protection-deprotection of ribonucleosides and condensation. Alkyl silyl protecting groups have been exploited for the 2'- and 3'-position of ribonucleosides[217], monomethoxytrityl and dimethoxytrityl groups for the protection of 5'-hydroxyl groups[218] and trichloroethyl and cyanoethyl groups for the protection of phosphates.[219] Amino groups have normally been protected with benzoyl groups.

(I) Problems associated with protection of the exocyclic amino function of the nucleosides

The need to protect the amino groups of nucleoside bases has been recognized early in the development of chemical methods for synthesizing oligonucleotides.[220] The introduction and removal and the reagents required in these steps, though, limit the range of functional groups that can be tolerated in the synthesis. Also the presence of the 2'-hydroxyl group in the ribose ring requires selective protection and may sterically hinder the 3'-position during the formation of the internucleotide linkage.

In the current synthesis of DNA and RNA as well as their analogs, base-labile protecting groups generally block the nucleobases during chain elongation[221]. In the phosphoramidite method used, the NH_2 moieties of the nucleoside bases have universally been protected to prevent the N-phosphitylation in the conventionally employed synthetic methods.[222]

However highly sensitive DNA analogs could not be synthesized with such protection modes cause they readily decompose under the standard basic condition described for the removal of the protecting groups.

The 5'O-DMTr group is currently the preferred protecting group for the 5'-OH function of nucleosides, during solid phase synthesis. The introduction of this protective group, however, required the prior protection of the exocyclic amino function of nucleoside bases to prevent these from tritylating as well. Also the acid-catalyzed depurination of 6-N-benzoyldeoxyadenosine during acidic removal of the dimethoxytrityl protecting group (DMTr) can reduce the overall yield of the synthesis.[223]

Furthermore as a result of adenine depurination, a heterogeneous mixture of 5'-dimethoxytrityl deoxynucleosides is present at the conclusion of the synthesis. Thus purification, especially with chromatographic methods based on the presence of the hydrophobic DMTr group becomes more difficult due to the heterogeneity of the DMTr-containing species.

One approach to solving the problem has involved modification of the acidic detritylation conditions. Lewis acids such as $ZnBr_2$, the use of trichloroacetic acid (TCA) and trifluoroacetic acid (TFA) with variable acidity has been observed in each case to decrease depurination.[224] Several examples have been reported that use amino-protecting groups, which can be removed under neutral or basic conditions for the synthesis of phosphoramidate analogs of oligonucleotides.[225]

(II) Advantages for employing nucleosides with the exocyclic amino function unprotected (the N-unprotected method)

Because the DMTr group is ideally suited for protecting the nucleoside 5'-hydroxyl and can removed by using relatively mild acids, a more straightforward approach for synthesizing labile DNA analogs is a method utilising nucleotide building blocks having unprotected nucleobases.

As Letsinger has stated[226] ideally it would be best to avoid the N-protecting groups because they entail at least two additional steps, introduction and removal, and the reagents required for these steps limit the range of functional groups that can be tolerated in the synthesis. Furthermore, the approach without the nucleoside base protection (the N-unprotected method) may considerably diminishes the risk of depurination of deoxyadenosine, which is a serious problem in the N-protected method particularly using acyl protectors such as benzoyl.[227]

5'-O-(Dimethoxytrityl)-2-deoxyribonucleosides without the protection of the amino functions are important substances for the synthesis of various kinds of nucleic acid derivatives. These compounds are useful as building blocks for of synthesis via the hydroxyl activation method.[228] They have also served as key intermediates of N-free nucleoside-3-phosphoramidites[229], which are monomer units for the synthesis of DNA oligomers via the N-unprotected approach

Preparation of the nucleoside derivatives without the amino protecting group can be complicated though, by the fact that each of the amino groups but most specifically the guanine amino group, is subject to reaction with dimethoxytrityl chloride. For that reason a different protecting group must be utilized that does not react with the unprotected amino groups of the nucleosides but still carries all the advantages of a protecting group compatible with the conditions of the oligonucleotide synthesis

(III) Requirements for selection

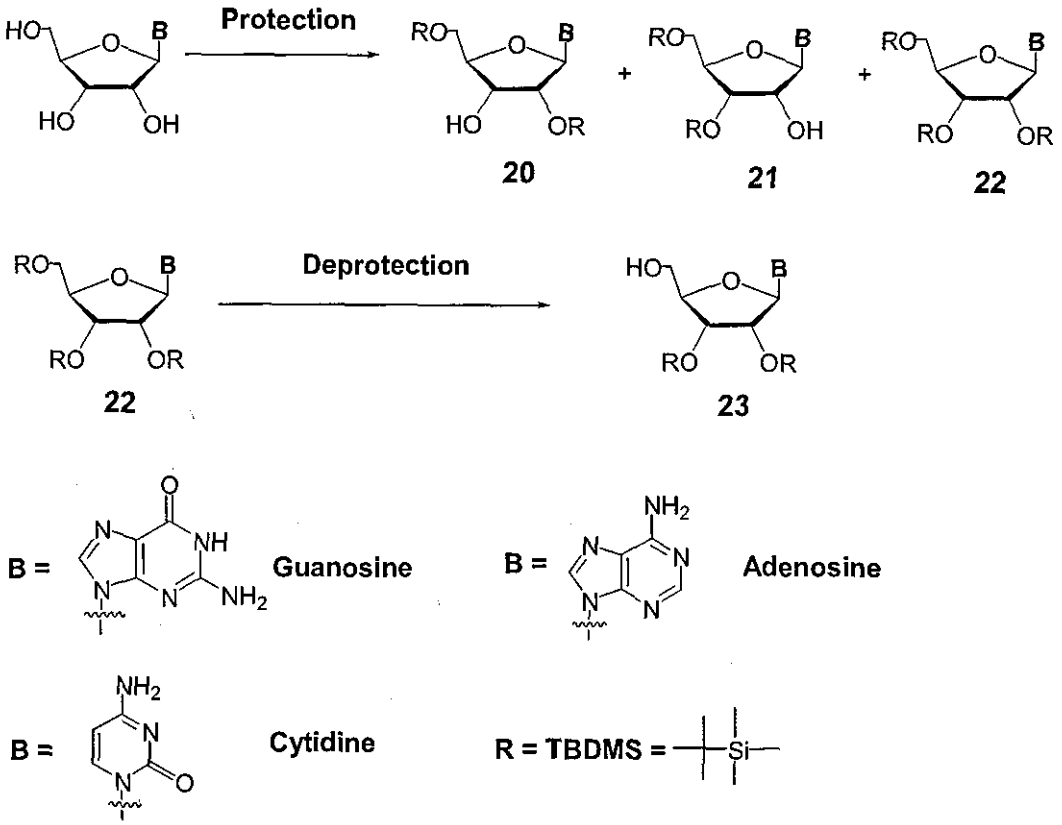
The chemical synthesis of oligonucleotides requires the availability of versatile reagents protecting the hydroxyl groups of nucleosides. A suitable protective group must be stable under the conditions of:

1. coupling, deprotection of the 5-'protecting group
2. oxidation
3. deprotection of the phosphate protecting groups and in the case of polymer-supported syntheses, cleavage from the solid support.

It must also be removed under mild conditions to prevent the attack of the released 2'-hydroxy function on the adjacent phosphodiester linkage.

Tert-butyldimethylsilyl ether (TBDMS), which was first promoted by Corey and Venkateswarlu in the prostaglandin area,[230] has proven to be an exceedingly useful group for protecting the hydroxyl moieties of the nucleosides. It is sufficiently stable, provides a rapid and sufficient synthesis of monosubstituted nucleosides. It provides a range of selectivity towards the 5'-hydroxyl group and can be stable towards acid hydrolysis.

Methods are developed for the global and partial TBDMS protection and deprotection of the OH groups of the different nucleosides, without nucleoside base protection. **Scheme 28** represents the proposed synthesis followed.



Scheme 28

C. SYNTHESIS OF LIPOPHILIC-NUCLEOTIDE CONJUGATES

A variety of nucleic acid analogues containing substantially uncharged backbones have been shown to enter the living animal cells and be resistant to nucleolytic degradation therein.[231,232] Paul Miller[233] has shown that one class of nucleic acids analogues significantly inhibits the intracellular activity of genetic sequences to which the analogues are complementary. These results suggest that uncharged nucleic acid analogues can be of value for the study of genetic mechanisms for the treatment of viral diseases, and possibly as anticancer agents.

Various nucleotide analogues containing linkages such as phosphonate,[234] thiophosphonate,[235] carboxymethyl,[236] and carbonate[237] have been reported in the literature and several of these have been found to have interesting biochemical activity. Especially analogues containing carbonyl-type bond-linkages are of interest due to their potential as anti-viral agents and repressors of gene expression at the transcriptional and translation levels. They can also be used as models for the study of nucleic acid structure and function.

Major obstacles that prevent wider application of the antisense oligonucleotides as potential therapeutics are their limited oral bioavailability and permeability through cell membranes. The permeability across the cell membrane and into the nucleus plays a critical role in determining the cellular efficacy of these oligonucleotides.

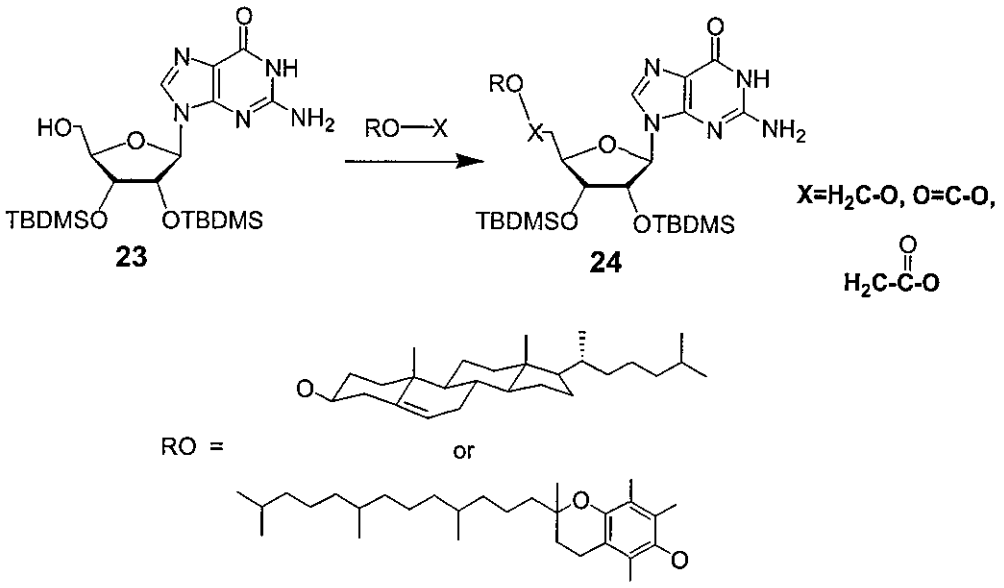
Oligonucleotides conjugated to lipophilic compounds can be taken up into the cell more efficiently,[172] exhibit better antiviral activity[178,188] and enhanced nuclease resistance.[158,160] Lipophilic-nucleotide conjugates will be synthesized as models in order to investigate the stability of modified nucleotides containing such linkages. Nucleotides can be covalently linked to the lipophilic molecules through sites present naturally in nucleic acids.

The naturally occurring sites that will be used for the coupling are amino groups on the bases, hydroxyl groups on the sugars and phosphate groups, both terminal and internal. This coupling will result in the formation of new acetal; carbamate and carbonate type links. Attachment of lipophilic groups to the amino base or the 5'-terminus of the nucleotides can improve their diagnostic utility as DNA probes and their potential therapeutic applications as antisense oligonucleotides.[238]

Initially the coupling will be conducted on a simpler system such as the single nucleoside derivatives, **22** and **23**, which are useful building blocks for investigation of the coupling chemistry of the existing linkers. The guanosine conjugate **24,** will be formed by coupling the novel linkers synthesized above, to the 5'-primary position of the 2',3'-O-TBDMS protected parent nucleoside **23** (shown in **Scheme 29**)., Adenosine conjugate **25** will also be formed by coupling to the NH_2 amino group of the 2',3',5'-O-TBDMS protected parent nucleoside **22**, as shown in **Scheme 29.**

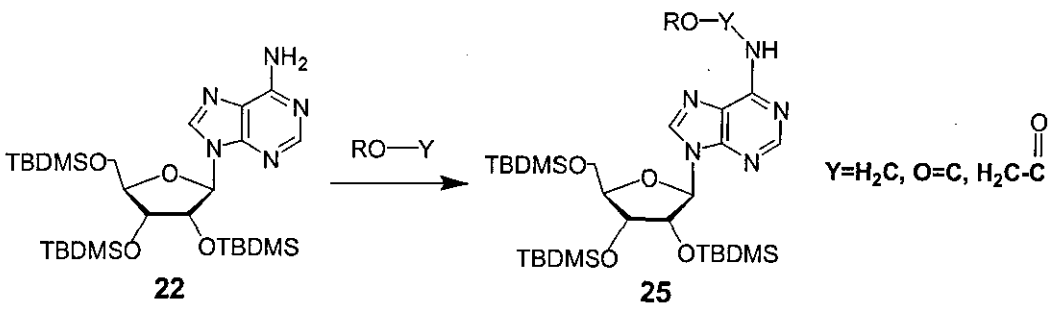
The strategy is shown below for guanosine and adenosine nucleotides.

Protected Guanosine linked to lipophilic molecules at the 5-terimus, through different linker groups



23 24

Protected Adenosine linked to lipophilic molecules at the NH_2 amino group through different linker groups



22 25

Scheme 29

Scheme 29 shows conjugates of the guanosine and adenosine nucleotides, containing novel carbonate and carbamate lipophilic linkages, respectively. This allowed for intermediates and products to be synthesized on a preparative scale. The other DNA nucleosides conjugates (using different linker groups) will be similarly investigated.

D. SYNTHESIS OF CpG DINUCLEOTIDES

Synthetic oligodeoxynucleotides containing CpG-dinucleotides (CpG-DNA) in specific sequence contexts activate the vertebrate immune system.[239] It has been reported[240,241] that bacterial DNA and phosphodiester oligonucleotides containing CpG motifs, stimulate cells of the innate immune system, induce secretion of interferons (INF's) and show antitumor activity. The mechanism of activation of immune stimulation by CpG it is believed to occur upon CpG uptake that triggers the immune cascade by binding to an intracellular receptor identified as TLR9.[242]

Growing evidence suggest that this recognition receptor exhibits considerable specificity for CpG dinucleotides and initiates immune signaling pathways. [243,244] That ultimately leads to the activation of NF-κB[245] and the subsequent up-regulation of various cytokines including IFN-γ, IL-6, IL-12 and TNF-α.[246-248] Alternately, CpG-DNA triggers DNA protein kinase activation which phosphorylates kinaseB leading to the activation of NF-κB, which further leads to the production of cytokines.[249] It is not clear if the pathways are activated sequentially or in parallel leading to a common function of activating the NF-κB pathway.

The observation that oligodeoxynucleotides containing CpG dinucleotides (CpG-DNA) exhibit several immunological effects has led to their use as therapeutic agents and adjuvants for various diseases.[250] Several CpG-DNA drug candidates are currently being evaluated, either as monotherapies or as adjuvants (with vaccines, antibodies, antigens and allergens), in preclinical and clinical trials against cancers, viral and bacterial infections, allergies and asthma.[251,252] Knowledge gained from studies of the medicinal chemistry of CpG DNA has provided a basis for designing a second generation of CpG DNA *agents with desirable cytokine-inducing and potent immunostimulatory* activity.[253-257]

In the approach to the synthesis of macromolecular DNA, chemical synthesis of short deoxyribonucleotide segments continues to determine the progress in the synthesis of DNA molecules.

Intensive efforts have been made to date in order to enhance the rapidity and efficiency of these chemical procedures and subsequently facilitate the large-scale preparation of dinucleotide blocks.[258]

One of the main aims of this project is to develop a short, convenient, high yielding dinucleotide synthesis of phosphodiester (PO) CpG, using the phosphoramidite approach. Due to the ability of phosphodiester CpG to enhance cytotoxic T lymphocyte activity they elicit efficient antitumor activity in vivo.[259-261] Unlike the phosphorothioate analogs, PO-CpG's induce higher levels of IFN-γ in human cultures and in vivo. As a result they act as superior adjuvants by promoting immune responses when used in combination with vaccines and allergens.[262-264]

CpG dinucleotides will be synthesized manually, using solid support or solution phase synthesis. These syntheses will be performed with or without attached lipophilic groups to improve the transport properties required for the oligonucleotide uptake into the cell.

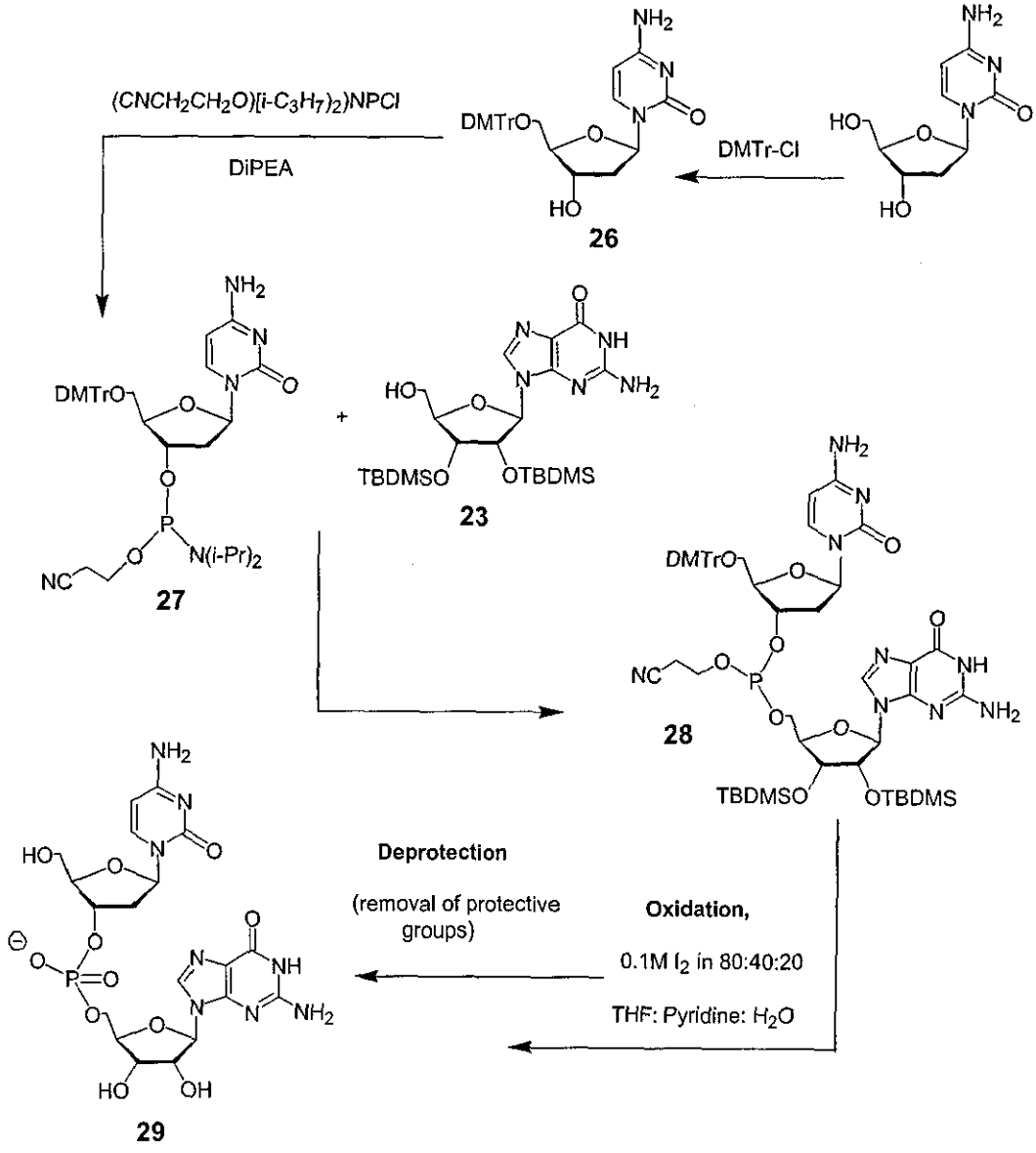
1. SOLUTION PHASE SYNTHESIS

With the recent success of various antisense oligonucleotides undergoing human clinical trials and the possibility of their commercial launch, soon very large quantities of therapeutically useful oligonucleotides may be required.[265] Thus development of methods for their large-scale synthesis has become a matter of urgency. Particularly when bigger quantities of oligonucleotides are required, solution-phase synthesis seems to be an alternative method of choice instead of the traditional solid-phase synthesis.[266,267]

Solution-phase synthesis can be applied for the formation of modified dinucleotides containing the nucleotide conjugates formed previously. In contrast with the solid phase synthesis where harsh conditions are used for the simultaneous deblocking of the N-protection and detachment from the solid support, the advantage of this method is the usage of mild deprotection conditions in order to avoid cleavage of the internucleoside linkages. Described below is the formation of CpG dinucleotides using solution phase synthesis.

(I) CpG synthesis without the attachment of lipophilic groups

This synthesis will be performed by joining the 5'-position of the N-free-2',3'-protected guanosine nucleoside **23** to the 3'-end of the N-free-5'-protected-3'-cyanoethyl-phosphoramidite of cytidine **27** (**Scheme 30**). N-free-5'-O-dimethoxytritylated derivative of cytidine **26**, will be prepared from the parent nucleoside by reacting it with dimethoxytrityl chloride (DMTr). The reaction of **26** with $(CNCH_2CH_2O)[i\text{-}C_3H_7)_2N]PCl$ in THF by the assistance of ethyldiisopropylamine, in pyridine, afforded the phosphoramidite of cytidine (**27**).



Scheme 30

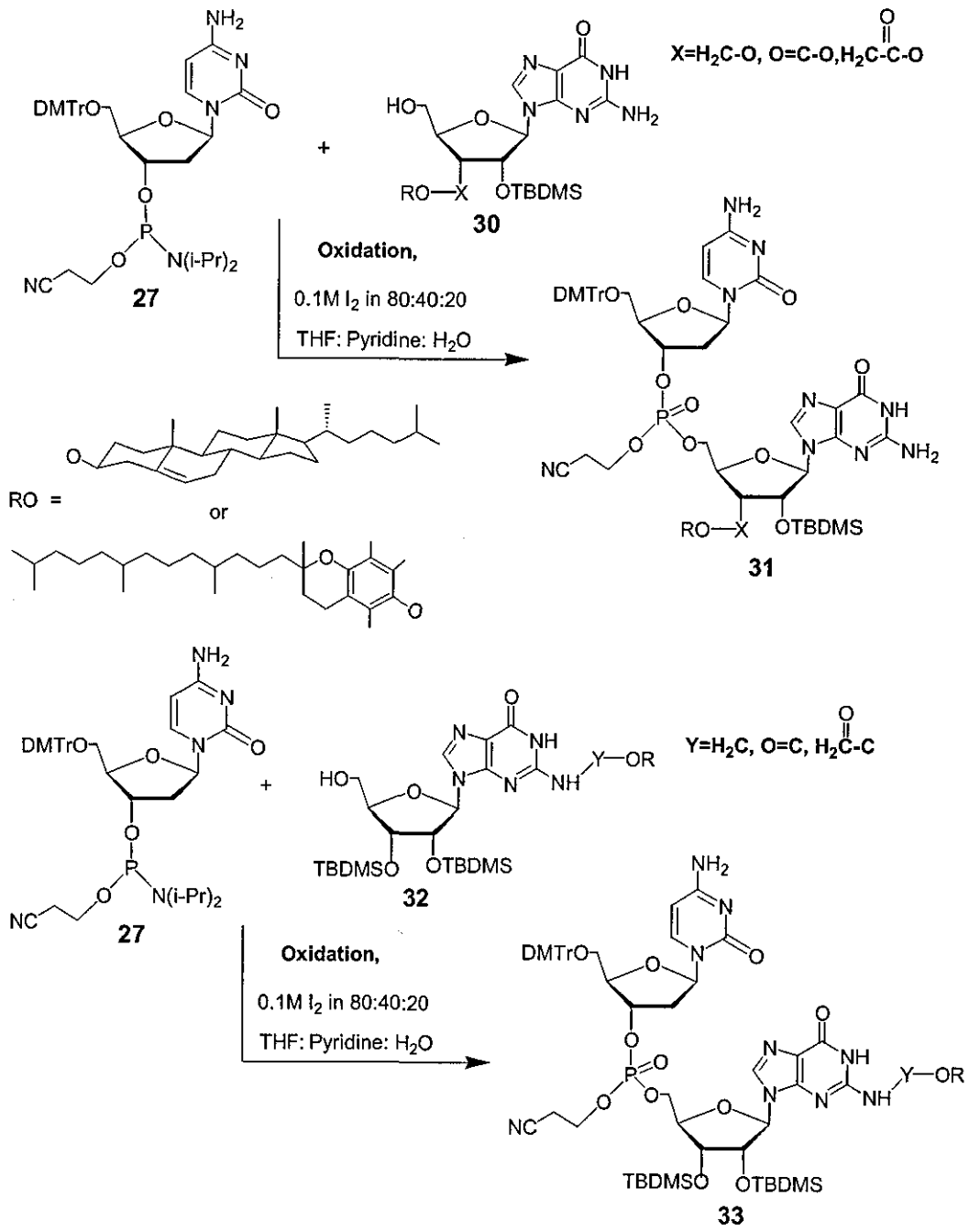
The dinucleotide **28** formed from the condensation of **27** and **23** will be subject to oxidation and subsequent deprotection to furnish the CpG dinucleotide **29**, as shown in **Scheme 30.**

(II) CpG synthesis with the attachment of lipophilic groups

The effects of structural changes in CpG-DNA have been studied that might potentiate or neutralize immunostimulatory activities in an attempt to understand the molecular recognition events between TLR9 receptor and CpG-DNA.[268] Studies have shown a number of structural and functional group requirements in the pentose sugar[269] phosphate backbone[270] and nucleobases[271] for immunostimulatory activity. These studies suggested that the receptor recognized base modifications that mimic natural C and G, while modifications at the 3'-position of the sugar are well tolerated and can have an effect on immunostimulatory activity.[272] It was also shown that an unbridged oxygen on the phosphate group between C and G of a CpG motif suppresses immunostimulatory activity suggesting that negative charge on the phosphate group is essential for protein recognition and interaction.[273]

Based on these studies it was proposed to investigate the synthesis of 3'-5'-linked CpG dinucleotides using the phosphoramidite approach but modifying the approach to allow the introduction of lipophilic groups. The previously synthesized lipophilic N-carbamate and O-carbonate conjugates of the nucleosides will be used as useful building blocks for the formation of CpG dinucleotides. Solution phase synthesis will be employed to investigate the coupling chemistry of the linkers and the stability towards the deprotection conditions.

When a guanosine nucleoside with the conjugation at the 3'-position is utilized, dinucleotide synthesis will be performed by joining the 5'-position of the N-free-2'-protected-3'-O-lipophilic conjugate of guanosine **30** to the 3'-end of the N-free-5'-protected-3'-cyanoethyl-phosphoramidite of cytidine **27**. The product furnished, will then be subject to oxidation to give the 3'-conjugated CpG dinucleotide **31** as shown in **Scheme 31** below.



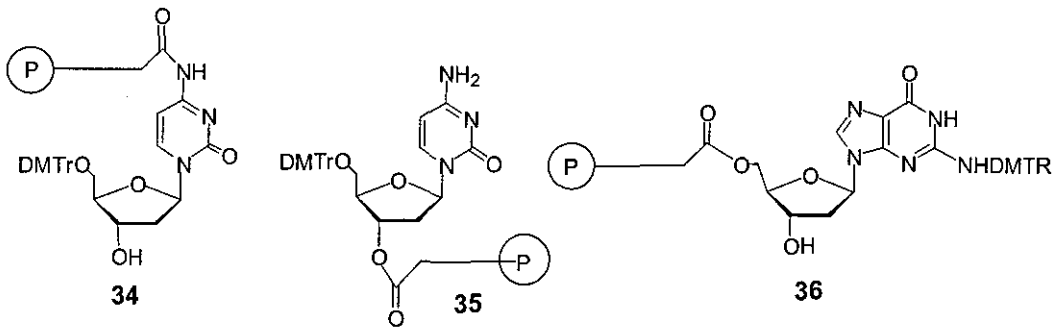
Scheme 31

When the conjugation is performed on the amino base of guanosine, CpG will be formed, by joining the 5'-position of the N-lipophilic analogue of 2',3'-protected guanosine **32** to the 3'-end of the N-free-5'-protected-3'-cyanoethyl-phosphoramidite cytidine **27**. The product furnished, will be subject to oxidation to give the N-conjugated CpG dinucleotide **33**, as shown in **Scheme 31** above.

2. SOLID PHASE SYNTHESIS

Merrifield[274] first described the solid phase synthesis of peptides in 1962. Since then considerable efforts have been invested toward the development of a suitable solid-phase strategy for the synthesis of oligonucleotides.

Letsinger and Mahadevan,[275] who described the preparation of a popcorn copolymer from styrene (88%) p-vinylbenzoic acid (12%) and p-divinylbenzene (0.2%), reported the first successful approach. This support was insoluble in water, alkaline solutions and organic solvents commonly used in oligonucleotide synthesis. The reaction of the acid chloride form of the polymer with the exocyclic amino function, 3'-OH, or the 5'-OH of properly protected nucleosides afforded the polymer bound nucleosides **34-36** respectively.[275-277] (Shown in **Scheme 32** below).



Scheme 32

Chain extensions from those polymer bound nucleosides, was achieved according to the phosphotriester approach and the resulting oligomer was subsequently cleaved from the support under basic conditions. This technique enabled the separation of the products from the solvents, excess reagents and soluble by-products by simple filtration, thereby avoiding numerous time-consuming steps.

Through the years, various polystyrene-derived supports,[278-280] in addition to polyamides,[278,280(a)] and cellulose[279(a),281] were tested in the solid-phase synthesis of oligonucleotides. The swelling of polystyrene supports with organic solvents is an advantage as it allows rapid diffusion of the reagents and solvents through the matrix.

Because of these findings, silica supports including controlled pore glass (CPG), were mainly used. During the last decade the solid phase synthesis of oligonucleotides according to the phosphoramidite approach has been mainly performed on silica-derived supports.[282] This synthesis proceeds with an average coupling of 96%, when a standard controlled pore glass support was used.

The relative importance of the type of linkage between the 3'-OH of the leader nucleoside and the spacer arm; along with the length of the spacer arm and the pore size of the solid support; with respect to the efficiency of oligonucleotide synthesis, has been investigated by Katzhendler[283] and van Aerschot.[284]

It was indeed found that the length and the distance of the spacer arm between the matrix and the leader molecule were important for optimum synthetic efficiency and product purity. A spacer length of more that 25 atoms were recommended and it was also important to avoid the possibility of folded conformations within the structure of the spacer. These may arise from internal hydrogen bonding, dipole-dipole or π interactions and could decrease the yield and purity of the final product.

The pore size of the silica supports is also of importance during the synthesis of relatively large oligonucleotides. An abrupt termination of chain propagation occurred when the synthesis of oligomers larger than 100 bases was attempted on CPG with a pore size of 500 Å.[285] This phenomenon was attributed to the increasing steric crowding around the pores and channels of the support caused by growing oligomers chains, which, presumably, reduced the diffusion of the reagents through the matrix.

Silica supports with pore size of at least 1000 Å were, satisfactory for the synthesis of large oligonucleotides.[285] For example, 98-mers were prepared with an average coupling yield of 99.3% from deoxyribonucleotide phosphoramidite monomers.[283]

(I) Synthesis of 5'-lipophilic phosphates of CpG dinucleotides, on solid support

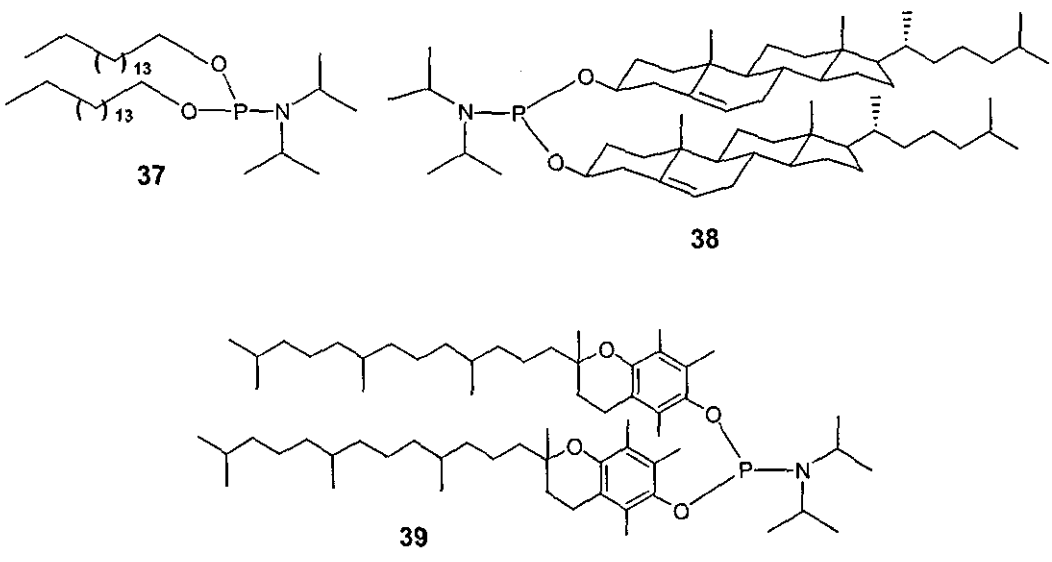
Oligonucleotides possessing 5'-phosphate group are required as valuable tools for gene construction,[286] mutagenesis,[287] and other biological applications.[288] Often, they are prepared by T4-kinase catalysed phosphorylation employing nucleoside 5'-triphosphate as the phosphate source.[289] A number of methods have been reported[290] that allow chemical 5'-phosphorylation of pre-assembled oligonucleotide precursor. Some of them include preparation of modified nucleotide-based building blocks to be attached at the last step of the oligonucleotide synthesis.[291] Another strategy based on no-nucleosidic building blocks, seems to be more universal since a single reagent can be employed.

Terminal modifications that can enhance exonuclease resistance have been studied,[260,264,292] and the modified compounds were compared with the parent (natural) deoxynucleosides. The resistance toward exonucleases was assessed, in vitro and in vivo and it was shown that stability of all the modified oligonucleotides was at least 12 times that of the parent compound.[259-261] CpG-containing phosphodiester linkages demonstrated that immunostimulatory activity is significantly reduced when the 5'-end of the oligonucleotide is not accessible, rather than the 3'-end, suggesting that the 5'-end plays a critical role in immunostimulatory activity.[253]

We were interested in the chemical solid-phase synthesis of oligonucleotides, which bear a lipophilic-phosphoramidite link at a distinct position within the CpG fragment. In this case the backbone modification is situated on the 5-phosphate group. Such modification lead to dinucleotides with a number of interesting properties. They do not form diastereoisomers and they still hybridize very specifically to a target strand.
Furthermore, they can function as templates and primers in enzymatic reactions. Restriction endonuclease recognition sites containing phosphoramidate linkages at the cleavage point also show interesting properties.

These phosphoramidate linkages should be sufficiently stable to allow the handling of such modified dinucleotides without precaution, and also be susceptible to a chemical cleavage under mild acidic conditions, resulting to the desired phosphate

Phosphoramidites coupled to lipophilic groups will be formed that can lead to preferential phosphitylation of the 5'-hydroxyl group. The N, N diisopropyl-phosphoramidites of cetyl alcohol **37** (cetyl = straight chain C_{16} group), cholesterol **38** and tocopherol **39**, will be synthesized by one-flask procedure (shown in **Scheme 33** below)



Scheme 33

The tri-coordinate phosphorus reagent enhances the attachment of lipophilic groups to the dinucleotide synthesis and improves the efficiency of uptake into the cell. Phosphitylating agents generally attack the amino as well as the hydroxyl groups of nucleosides. In conventional procedures for synthesizing oligonucleotides, selective reaction at the oxygen is achieved by using protective amino groups. For that reason, deoxynucleosides with their amino function protected, were used.

1H tetrazole can be used as a promoter in both solution-phase and solid-phase syntheses of dinucleotides via the phosphoramidite method. It is fairly reactive towards phosphoramidites, such as N,N-diisopropyl-phosphoramidites. It allows rapid and highly chemoselective condensation of the phosphoramidite and the nucleoside and it leads to a high phosphitylation yield. Excess equivalents of the phosphoramidite, the promoter and the nucleoside will be utilized in solid-phase synthesis, which can be achieved with good chemoselectivity.

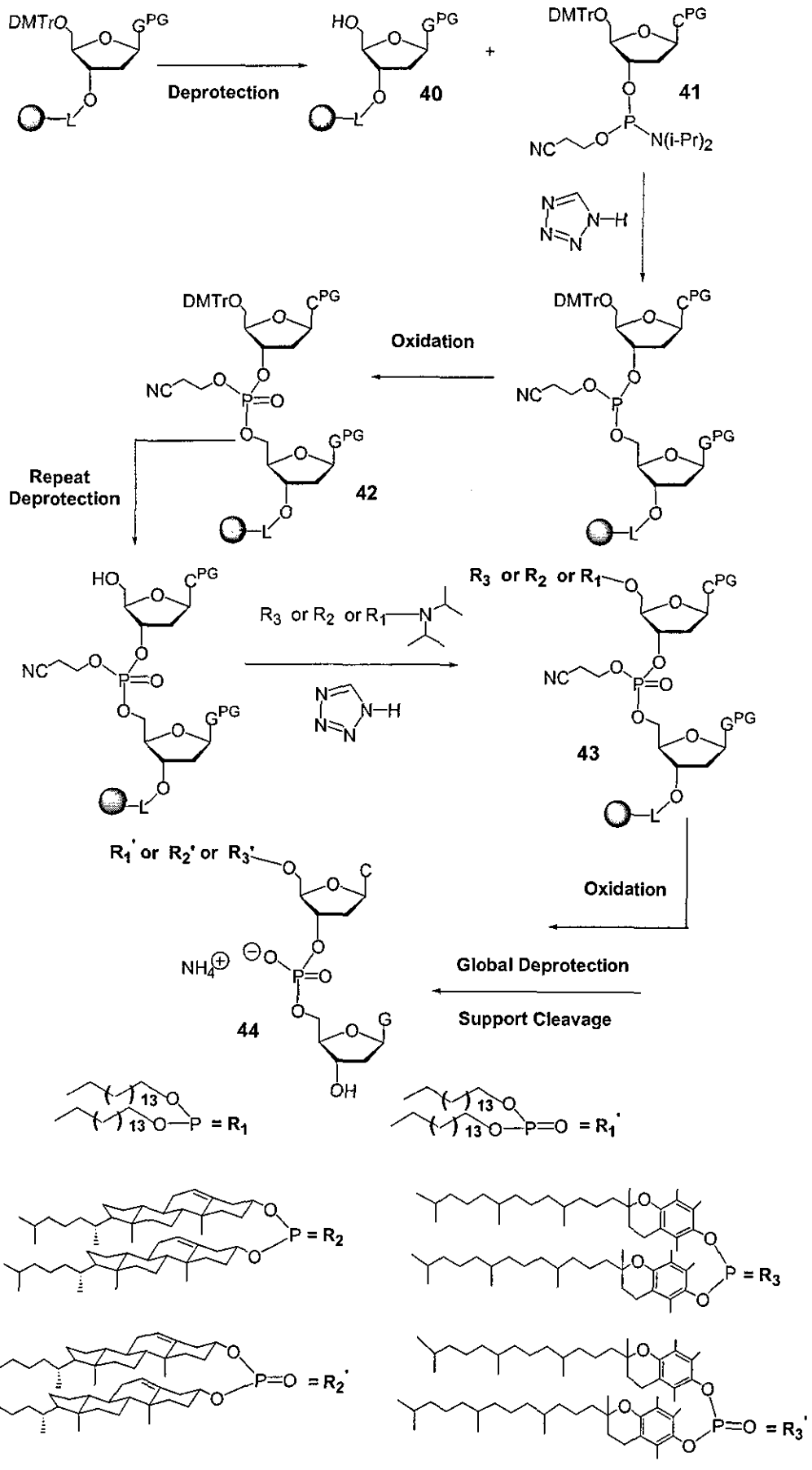
Internucleotide coupling procedure is performed with the 5'-hydroxyl group being capped with the di-lipophilic phosphate group to provide lipophilicity. In solid phase synthesis, a reaction sequence is followed, for the preparation of the 5'-dicetyl and 5'-dicholesterol and 5-ditocopherol phosphates of CpG dinucleotides, via the phosphitylation of the 5'OH group

The N-protected-5'-free guanosine nucleoside **40** (after deprotection of the 5'-OH group) anchored to a solid support (CPG) by the 3'-position, is coupled to the 3'-end of the N-protected-5'-protected-3'-cyanoethyl-phosphoramidite of cytidine **41**, by utilizing the promoter. The product formed, will then be subject to oxidation to give the 5'-DMTr-3'-cyanoethyl-phosphate solid supported dinucleotide **42**.

Deprotection is repeated in order for the 5'-OH group to become available for the leading phosphitylation reaction with the novel synthesized N',N' diisopropyl-phosphoramidites of lipophilic compounds. The formed 5'-phosphite of the 3'-cyanoethyl-phosphate solid supported dinucleotide **43,** was again oxidized followed by global deprotection. Removal of all remaining protective groups and cleavage of the solid support, gave the dinucleotide **44**, is in the form of its ammonium salt.

A schematic diagram of this synthesis is shown in the **Scheme 34** below.

Scheme 34


Deprotection
40
41
Oxidation
42
Repeat
Deprotection
43
Oxidation
Global Deprotection
Support Cleavage
44

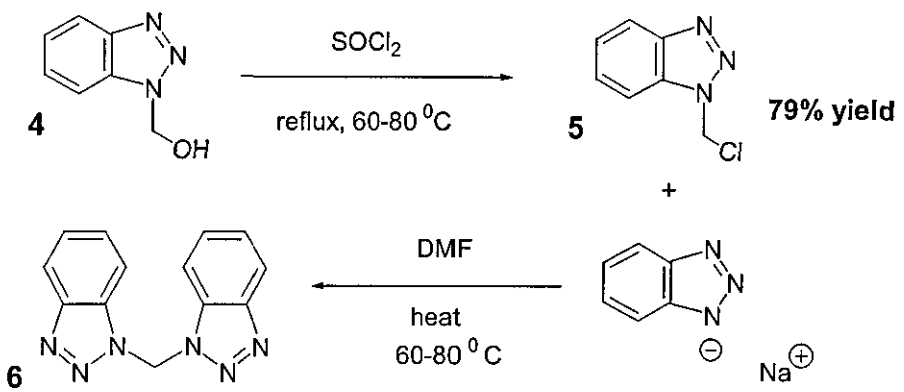
DISCUSSION

1. SYNTHESIS OF LINKERS

(A). Acetal Linkers

One of the main aims of this project was to synthesize new linker groups for the attachment of lipophilic molecules, which are designed to be easily cleaved off once the DNA construct has entered the cell. Acetal linkers were investigated firstly as they are stable enough to stand up the conditions of DNA synthesis but should also be easily hydrolyzed in the cell.

Two approaches were investigated, where *bis*-benzotriaziol-1-yl methane and 1-chloromethyl benzotriazole were employed as the acetal-forming reagents. The non-commercially available *bis*- benzotriaziol-1-yl methane **6** was prepared in two steps[208] as shown in **Scheme 35**.



***Bis*-benzotriazol-1-yl methane**

69% yield

Scheme 35

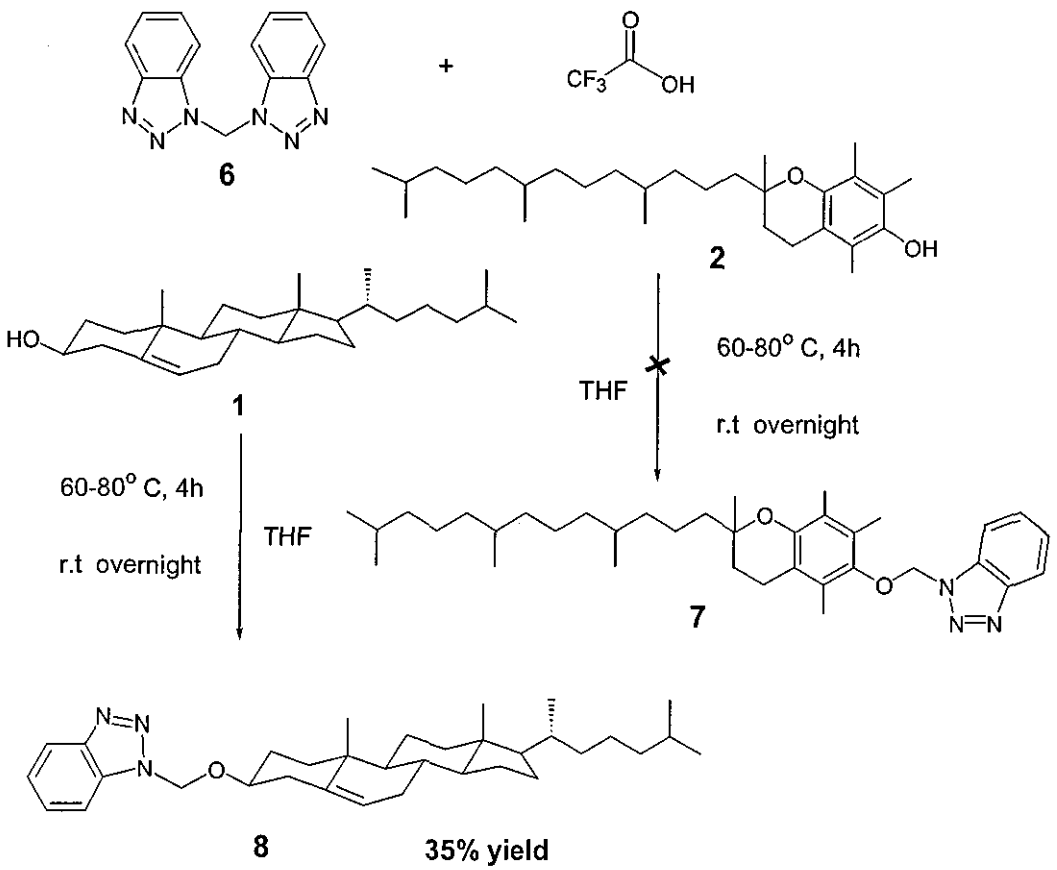
In the first step, 1-chloromethyl benzotriazole (**5**) was formed as white crystals in 79% yield, by refluxing 1-hydroxymethyl benzotriazole (**4**) with thionyl chloride.

In the second step, benzotriazole was treated with sodium hydride in DMF generating the benzotriazole anion, followed by addition to the already formed 1-chloromethyl benzotriazole. Displacement of the chlorine atom from **5**, gave the *bis*- benzotriaziol-1-yl methane **6**, as a white solid in 69% yield. Compounds **5** and **6** were identical in melting point, ^{1}H and ^{13}C-NMR and analytical analysis with those reported in the literature.[208]

(I) Linkers from cholesterol and tocopherol

1 By using bis-benzotriazole methane

For the formation of the acetal linkers, the benzotriazole methyl ethers of lipophilic alcohols, cholesterol **1** and tocopherol (vitamin E) **2**, were required.



Scheme 36

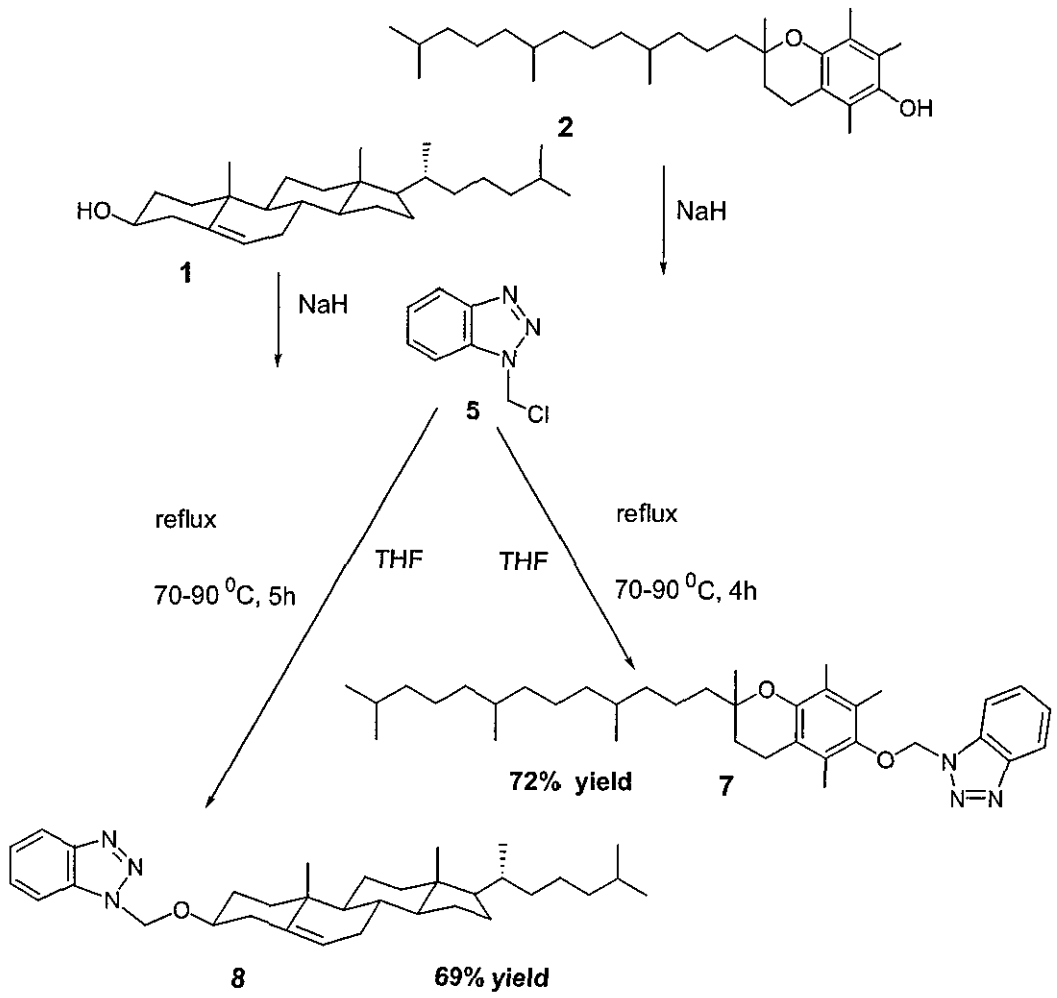
Their synthesis involved treatment of the corresponding alcohols (**1** and **2**) with *bis*-benzotriazole methane (**6**) in the presence of trifluoroacetic acid, as shown in **Scheme 36,** above. The novel benzotriazole methyl ether of cholesterol was obtained in a 46% yield as crude product. This was purified further through recrystallization, which was carried out from dichloromethane and petroleum ether and resulted in the recrystallized compound **8,** furnished as white solid in a 35% yield.

On the other hand, an attempt to synthesize the tocopherol derivative resulted in the precipitation and recovery of *bis*-benzotriazolyl methane as suggested by the spectroscopic and elemental analysis obtained. ^{1}H and ^{13}C-NMR data gave the corresponding signals for *bis*-benzotriazolyl methane while there were no aliphatic protons or carbons.

2 By using 1-chloromethyl benzotriazole

An alternative route for the synthesis of benzotriazole methyl ethers of the lipophilic alcohols was also followed. The anion of the corresponding lipophilic alcohol was generated with sodium hydride in THF and then treated with 1-chloromethyl benzotriazole **5**. Displacement of chloride in **5** by the anion of cholesterol or tocopherol, gave the formed acetal linkers **7** and **8** as shown in **Scheme 37** below.

Tetrahydrofuran (THF) was the choice of solvent for cholesterol since it is the only solvent in which cholesterol is fully soluble, even at room temperature. On the other hand, tocopherol proved to be soluble in a wide variety of solvents. As 1-chloromethyl benzotriazole was more soluble in THF compared with a less polar solvent like for e.g. acetonitrile (CH_3CN), this solvent was also employed in the case of tocopherol.



Scheme 37

The compounds obtained using the procedure shown in **Scheme 37**, were furnished as crude products in an 87 and 75% yield. Then recrystallization was carried out from ethanol and water. The novel benzotriazole methyl ethers of tocopherol **7** and cholesterol **8** were afforded as white solids in both cases, in a 72 and 69% yield, respectively.

The structures were confirmed by ^{1}H and ^{13}C-NMR spectroscopy. ^{1}H-NMR spectrum of the cholesterol derivative **8** showed a signal at 7.36 ppm corresponding to the 2 hydrogens of the CH_2 of the benzotriazole ring which

appears more down field than the CH_2 of the 1-chloromethyl benzotriazole (~6.42 ppm).

It also gave two doublets at 5.34 and 5.30 ppm and two multiplets at ~3.55 and 3.45 ppm with a ratio of 1:8 for starting material cholesterol: synthesized product, that correspond to the H_b and H_a olefinic protons of cholesterol, respectively.

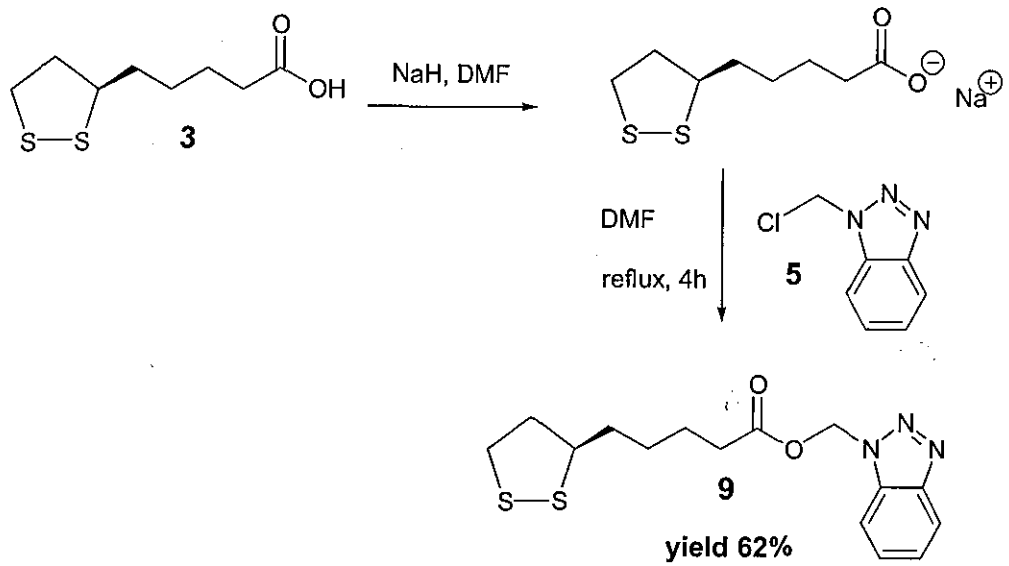
^{13}C-NMR spectrum also showed all the signals for the aliphatic and aromatic carbon atoms. This evidence suggests the conversion of cholesterol to the desired benzotriazole methyl ether of cholesterol. The benzotriazole methyl ether of cholesterol formed using 1-chloromethyl benzotriazole **5** was compared with the previously synthesized novel compound (shown in **Scheme 36**), by I.R, ^{1}H, ^{13}C-NMR and melting point and was found to be identical, while the yield obtained was improved (69% yield).

As for the tocopherol derivative **7**, the spectral and elemental analysis suggests its formation. ^{1}H and ^{13}C-NMR spectroscopic analysis showed all the signals for the aliphatic and aromatic protons, while the OH broad band of the tocopherol was absent in the I.R spectrum.

In the ^{1}H-NMR spectrum the signals of the aromatic protons have moved up-field while in both ^{1}H and ^{13}C spectrum the signals, which correspond to the carbon and the 2 hydrogens of the CH_2 of the benzotriazole ring, appear more downfield than the CH_2 of the 1-chloromethyl benzotriazole. These evidence support the formation of the tocopherol ether.

(II) Linkers from thioctic acid

Thioctic acid **3**, as it is known to have a biological importance, was employed in order to examine how it can be used for the formation of a sulfur linkage, which can then be introduced into the synthesized oligonucleotide constructs. For the synthesis of this linker the known route, used before in the synthesis of the benzotriazole methyl ethers of cholesterol and tocopherol performed under basic conditions, was followed.



Scheme 38

As shown in **Scheme 38**, the chlorine substituent of the synthesized 1-chloromethyl benzotriazole (**5**), undergoes nucleophilic substitution by the nucleophilic anion of thioctic acid, which is generated by treatment with sodium hydride in DMF. This reaction furnished the crude product as dark yellow solid in a 77% yield.

DMF was a more effective solvent for this reaction as it dissolves thioctic acid, better than THF. Further purification of the crude was performed through recrystallization from ethyl acetate and petroleum ether. The pure benzotriazole methyl ester of thioctic acid **9** was obtained as a pale yellow solid, in a 62% yield.

The spectral and elemental analysis obtained for compound **9**, supported the proposed structure with ^{1}H-NMR ^{13}C-NMR spectroscopic analysis showed a singlet at 6.03 ppm and a peak at 67.2 ppm, respectively. These signals correspond to the 2H and the aliphatic carbon of the methylene group, respectively, that connects the benzotriazole ring to the anion of the thioctic acid (O-C**H$_2$**-N).

The ^{1}H-NMR spectrum also gave all the signals for the aromatic protons and carbons, as well as, for the protons and carbons corresponding to the thioctic ester.

(B). Carbonate Linkers

Carbonate linkages have shown to be resistant to enzymatic degradation and possess linkages, which are achiral.

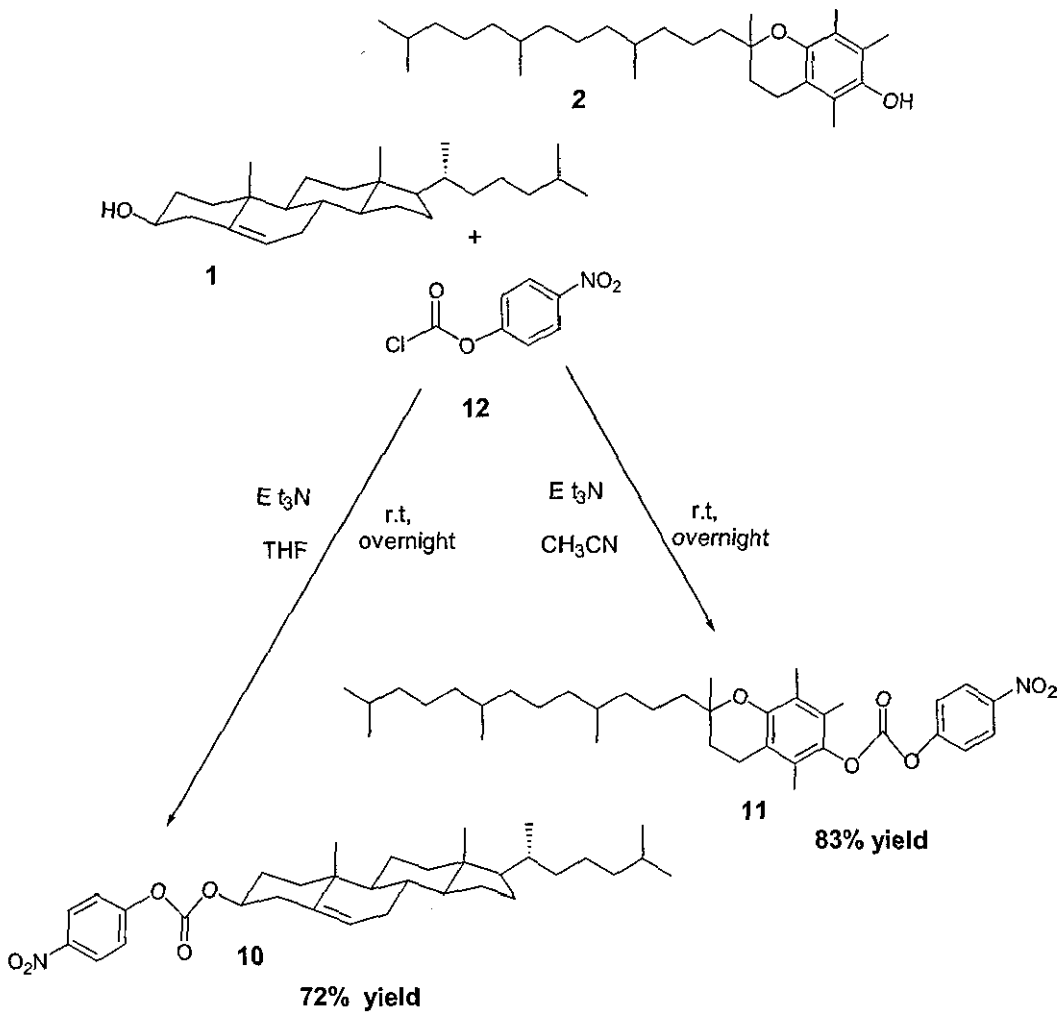
By using 4-Nitrophenyl Chloroformate

Commercially available 4-nitrophenyl chloroformate **12** was investigated as the linker-forming reagent. The *p*-nitrophenoxy group exhibits a high propensity to act as a leaving group in nucleophilic displacement reactions, and can be introduced as a better leaving group for the future formation of nucleotide conjugates.

Cholesterol **1** and tocopherol **2**, were reacted with 4-nitrophenyl chloroformate (**12**), using triethylamine as the base as shown in **Scheme 39** below. While N-methylimidazole and 4-(dimethylamino)pyridine can also serve as efficient catalysts, triethylamine was employed due to its ease of removal by evaporation.

The acid chloride **12** was used as the precursor and in the presence of triethylamine the α proton adjacent to the hydroxyl group of tocopherol is removed, thus forming the nucleophile that will attack and displace the electrophile (chloride). The pKa of tocopherol is approximately 10 making triethylamine strong enough to deprotonate the phenolic hydroxyl of tocopherol whereas the pKa of cholesterol will be about 16 and cannot be deprotonated by triethylamine. In that case the nucleophilic hydroxyl group of cholesterol will attack the electrophile and the base will remove the proton to generate the carbonate group.

1 Equivalent of the electrophilic reagent and 1.1 equivalent of the base were used, whilst release of the chlorine atom in acid chloride furnished the desired carbonate linkers. Again THF and CH_3CN were found to be effective solvents for the cholesterol and tocopherol reaction, respectively.



Scheme 39

The novel 4-nitrophenyl carbonate derivatives of cholesterol and tocopherol were obtained in 87 and 92% yield, respectively, as crude products.

The cholesterol carbonate linker was recrystallized from dichloromethane and petrol to furnish the title compound **10**, as yellow solid in 72% yield. Trituration of the tocopherol derivative from petroleum ether, afforded the desired compound **11** as yellow oil in 83% yield. As these carbonate linkers are moderately stable, care was taken in storage and they were kept under anhydrous conditions.

(C) Carbamate Linkers

The carbamate linkage is attractive due to its stability over a wide pH range and its stability under hydrolysis by base.

By using N,N'-Carbonyl-Diimidazole (CDI)

The use of CDI as a carbonyl synthon can afford clean, high yielding reactions in the synthesis of oligonucleotide analogues.

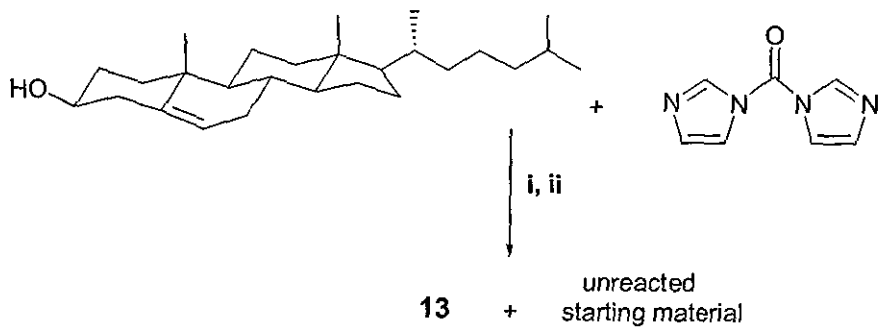
Initially, the formation of the carbamate linker of cholesterol was investigated, in order to establish the reaction conditions and the number of equivalents of the reagents needed for the reaction to reach completion. It was thought that the desired linkage could be formed by suitable basic activation of the hydroxyl function of cholesterol.

Various experiments were performed using different bases and different equivalents of N', N'-carbonyl-diimidazole as shown in **Table 1** below.

Table 1

Base	CDI	Yield (%)
Lithium bis (trimethyl silyl) amide (1.1 eq)	1.1 eq	34% + 45% unreacted cholesterol
Lithium bis (trimethyl silyl) amide (1.2 eq)	1.1 eq	42% + 36% unreacted cholesterol
NaH (1.5 eq)	1.1 eq	43% + 38% unreacted cholesterol
NaH (1.5 eq)	1.5 eq	42% + 28% unreacted cholesterol+ 12% recovered imidazole
NaH (2 eq)	1.1eq	48% + 30% unreacted cholesterol

All reactions were performed at room temperature using THF as solvent as shown in **Scheme 40**, below.



Reagents and conditions **i)** : Lithium bis (trimethyl silyl) amide **45** , CDI, THF,
ii) : NaH, CDI, THF

Scheme 40

Firstly the organolithium compound lithium bis (trimethyl-silyl) amide **45**, which is known to have strong basicity, was employed.

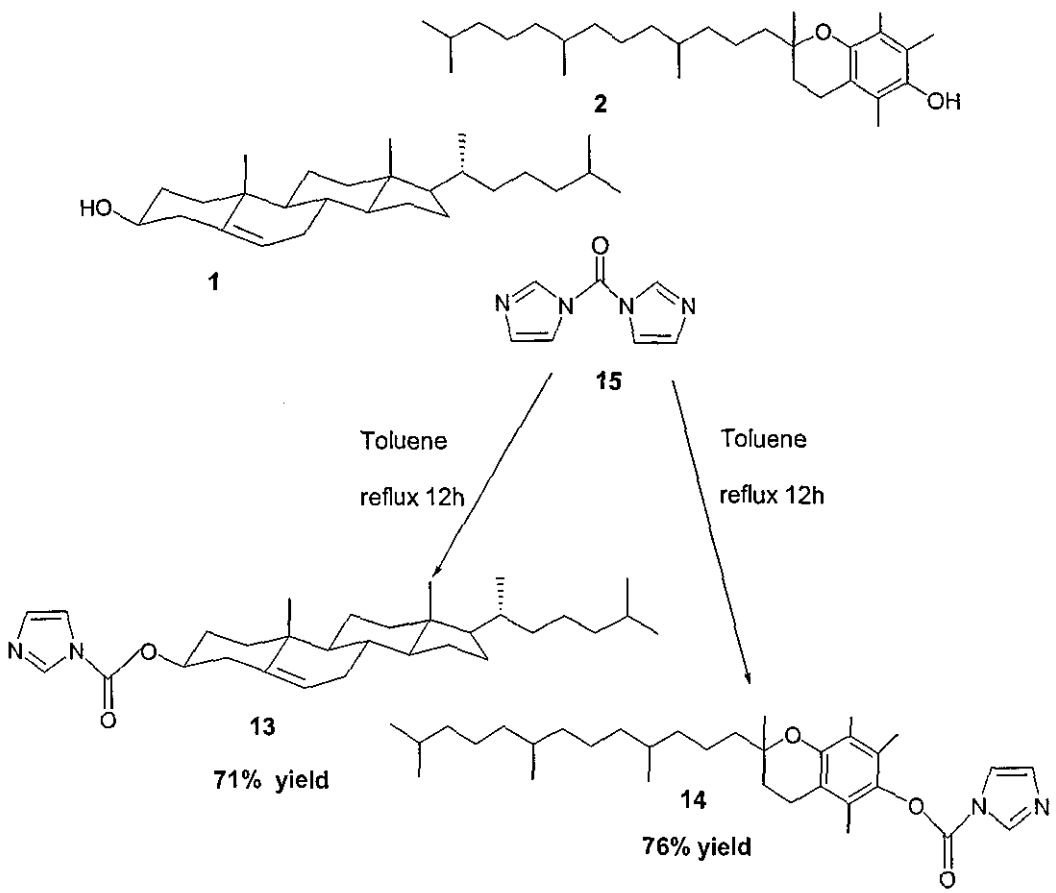
The use of 1.1 equivalent of **45** and carbonyl reagent, in THF at -78 ^{0}C, gave the required product only in 34% yield and 45% of the starting material was recovered. Increase of the base equivalent's to 1.2 led to a slight increase of the yield, along with recovery of the starting material. This suggests that the metal alkoxide formed may be too stabilized for the reaction to reach completion.

Another method using sodium hydride in THF was also investigated. When 1.5 equivalent of base and 1.1 equivalent of CDI were employed, 43% of the product was afforded along with 38% of unreacted cholesterol. Increase of CDI's equivalent to 1.5 only led to an additional recovery of imidazole. A final attempt was made, using 2 equivalent of NaH but again reaction did not reach completion.

Since the above reactions did not afford a higher yield of the desired product, an alternative procedure was followed based on a reported procedure[293] for the conversion of the hydroxyl function of cholesterol to its carbonylimidazolide derivative. This was based on using toluene, both as the solvent and activator. Reaction of cholesterol with 1.1 equivalents of CDI **15,** in refluxing toluene for 12h followed by aqueous extraction, furnished the cholesterol imidazole carbamate linker in a 78% yield as the crude product.

Recrystallization was carried out from dichloromethane and petrol, which gave the novel carbamate linker of cholesterol **13** as white solid in 71% yield. (Shown in **Scheme 41**).

The successful development of the above procedure prompted an investigation into extending the reaction to tocopherol. The same method was followed with a decrease of the reaction time to 10h. An attempt was made to purify the crude product by chromatography but it was unsuccessful due to the instability of the active carbamate. Trituration from hot hexane afforded the carbamate linker **14** as pale-yellow oil in an increased 76% yield. This linker was used without further purification.



Scheme 41

The products are stable and can be stored indefinitely. The absolute structure of compound **13** was determined by X-ray crystallography. The structure is shown in the Appendices.

(D) Ester linkers

Ester functions are known to have reactivity towards nucleophiles. Attachment of a lipophilic moiety through an ester bond is an attractive approach to obtaining oligonucleotide conjugates. However under conditions of oligonucleotide deprotection, esters are usually cleaved more rapidly than base protecting groups.

For this reason, ester lipophilic conjugates of nucleotides can be prepared by performing the conjugation step post synthetically in solution or when the base residue requires no protection the ester conjugates can be assembled successfully using solid phase chemistry.

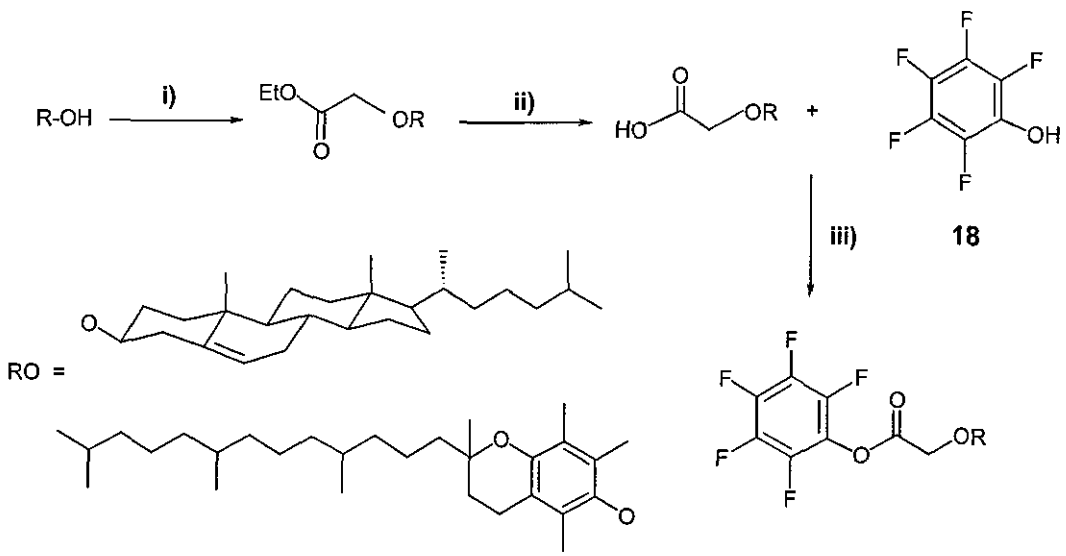
Using 2,3,4,5,6-Pentafluorophenol

As part of the investigation into preparing linkers as useful intermediates for further formation of nucleotide constructs containing lipophilic groups, the formation of ester linkers was proposed.

The esters linkers proposed contain an extra CH_2 group which improves stability needed for further conjugate-forming reactions. 2,3,4,5,6-pentaflurorophenol **18**, was employed to form the active ester which is a good electrophile and can undergo nucleophilic displacement.

In order to synthesize the pentaflurorophenyl ester linker of cholesterol or tocopherol, a sequential procedure that consisted of three steps (starting from the lipophilic compound) was followed as shown in **Scheme 42**.

Formation of the ethyl ester of the lipophilic compound by alkylation with bromoacetate, followed by hydrolysis to the carboxylic acid and subsequent reaction with 2,3,4,5,6-pentaflurorophenol **18** and either EDCI or DCC as coupling agent, leads to the desired pentaflurorophenyl ester linkers **16** and **17**.



Reagents: i) NaH, Ethyl bromoacetate, **ii)** Aq NaOH, EtOH **iii)** EDCI or DCC, DMF

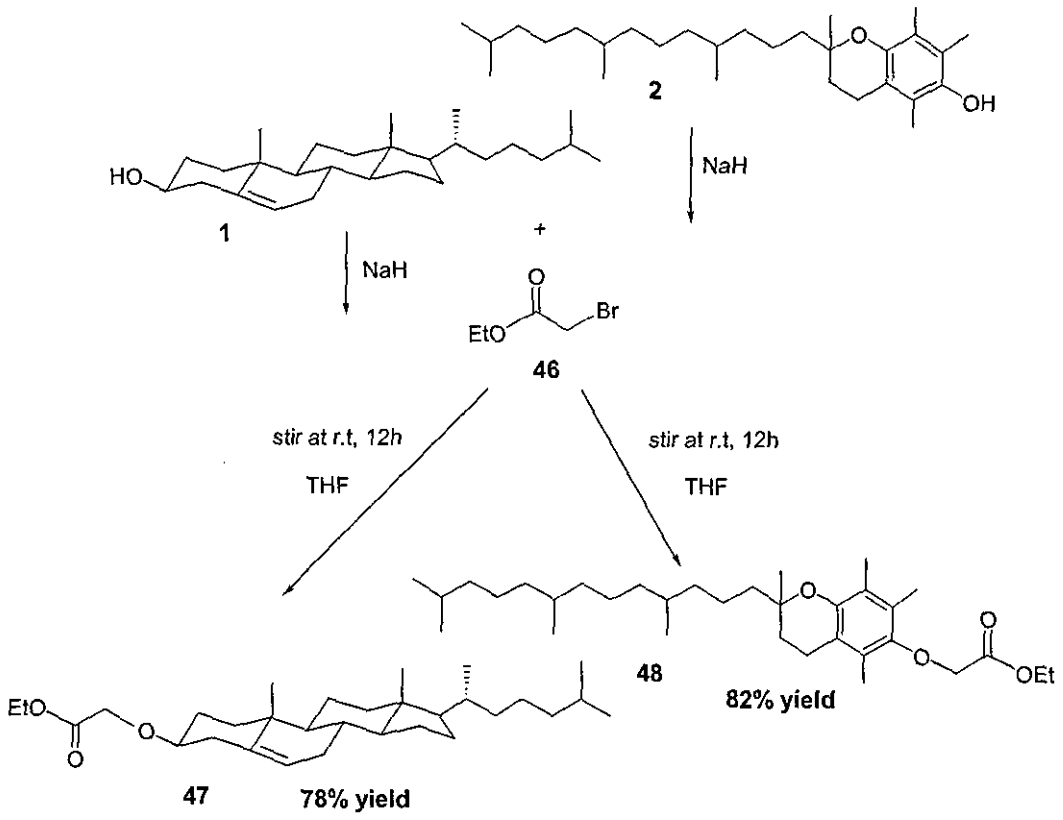
Scheme 42

This stepwise synthesis was firstly tested on cholesterol. Cholesterol was treated with ethyl bromoacetate in toluene under reflux but this reaction failed to give the product and led to the recovery of starting material.

It was then decided to investigate the displacement of bromo substituent by a nucleophile, formed by generating the anion of cholesterol, using a suitable base. The reaction involved deprotonation of the hydroxyl group to generate the nucleophile that displaces the alkyl bromide to give an O-alkylated product. The use of sodium hydride in THF was found to be an effective base in the preparation of the benzotriazole methyl ether linker of cholesterol (see **Scheme 37**).

When 1.5 equivalent of base and 1.1 equivalent of ethyl bromoacetate were employed, 61% of the product was afforded along 18% of unreacted cholesterol. Increase of the equivalent of the coupling reagent to 1.5 gave the ethyl ester of cholesterol **47** as off-white solid in 78% yield, after recrystallization from dichloromethane and petroleum ether.

The same conditions were applied for tocopherol and crude ethyl ester of tocopherol was furnished in 91% yield, as shown in **Scheme 43** below. The crude acetate derivative of tocopherol was triturated using hot hexane to afford the desired product **48** as pale yellow oil in 82% yield.

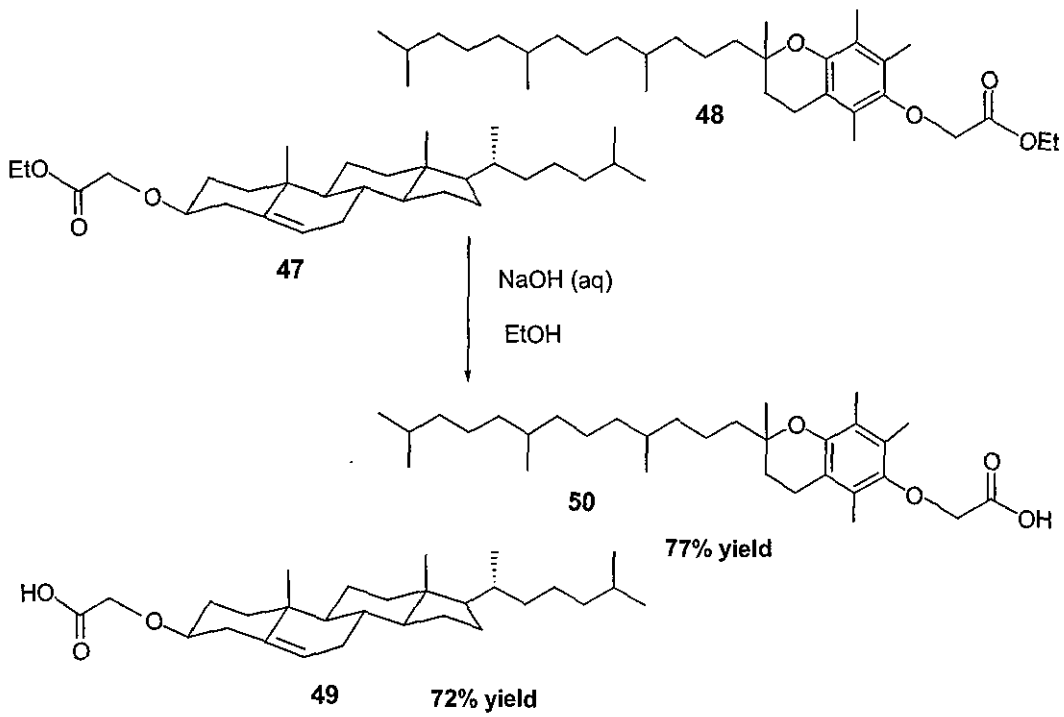


Scheme 43

Displacement of the bromo substituent by the anion of the lipophilic alcohol formed by sodium hydride in tetrahydrofuran, gave the ethyl ester derivatives of cholesterol **47** and tocopherol **48** in very good yields.

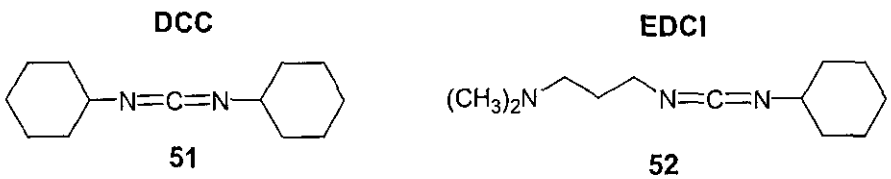
Hydrolysis of the cholesterol and tocopherol esters with aqueous sodium hydroxide in ethanol afforded the corresponding carboxylic acids **49** and **50** in a 72 and 77% yield, respectively (Shown in **Scheme 44**).

The purified form of **49** was furnished as an off-white solid after recrystallization from ethyl acetate and dichloromethane, while trituration with hot hexane gave **50** as an off yellow solid.



Scheme 44

For the third and final step of the synthesis, the previously synthesized carboxylic acids **49** and **50** were reacted with 2,3,4,5,6-pentafluorophenol **12** in the presence of a coupling reagent. The coupling reagents used were the **1**, 3-dicyclohexylcarbodiimide (DCC)[294] **51** and the water-soluble 1-ethyl-3- (-3'-dimethylaminopropyl) carbodiimide hydrochloride (EDCI)[295] **52**, as shown in **Scheme 45.**



Scheme 45

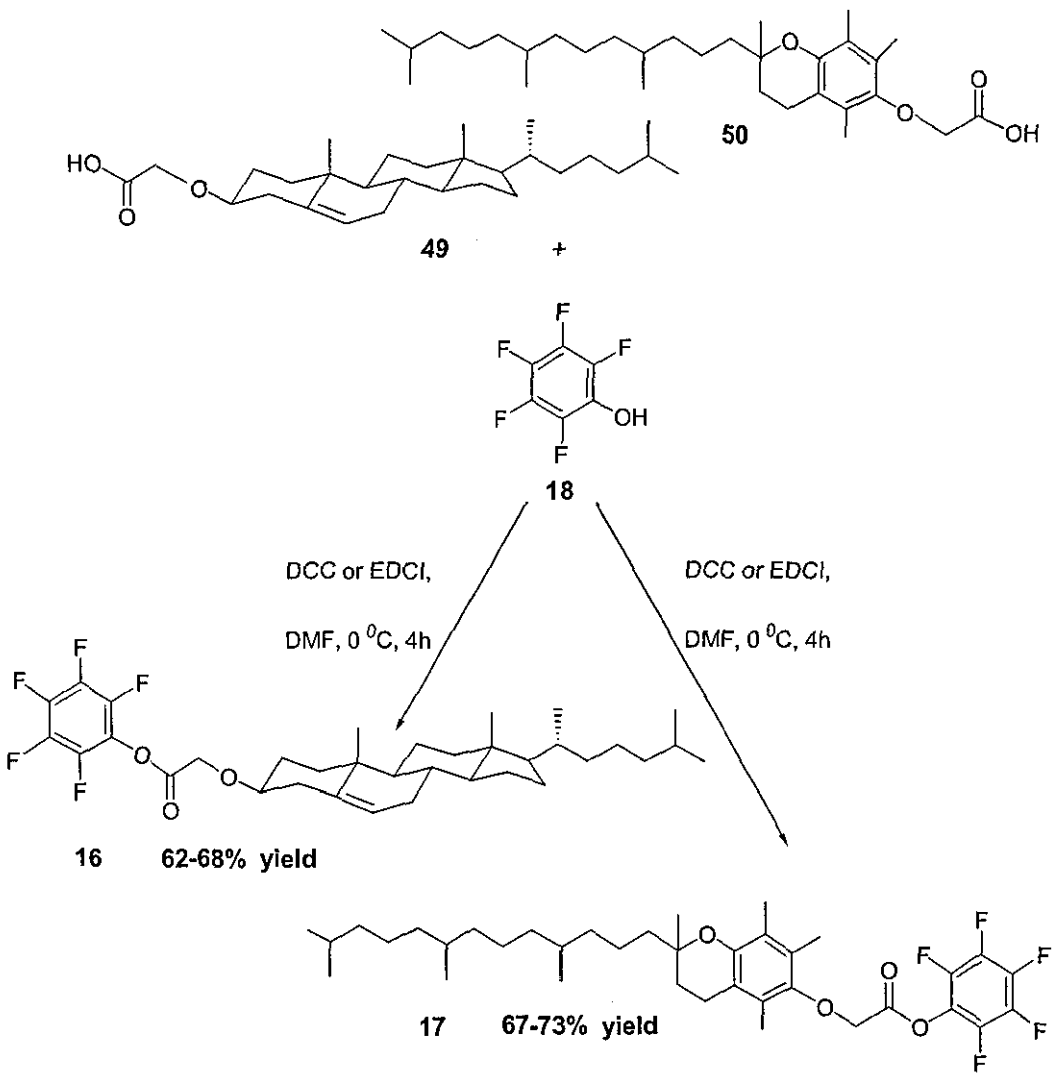
As THF is reported[296] to reduce reaction rates and encourage the formation of the N-acylurea side product and in this case, DMF was used as a more polar solvent. The coupling was carried out using the carboxylic acid of the corresponding lipophilic alcohol (1eq), 2,3,4,5,6-pentaflurorophenol (1eq) and coupling reagent (1.1eq), which was added portion wise to the solution of acid and alcohol in anhydrous DMF, cooled to 0 ^{0}C (**Scheme 46**).

The reactions were performed in DMF over 4h at room temperature to afford the desired pentaflurorophenyl ester linkers of cholesterol **16** and tocopherol **17** after recrystallization from diethyl ether and hexane, as white solid and off-yellow solid respectively.

Table 2 below shows that EDCI proved to be more effective over DCC as the yields obtained when the first was used were improved over the latter, even though the same conditions were used.

Table 2

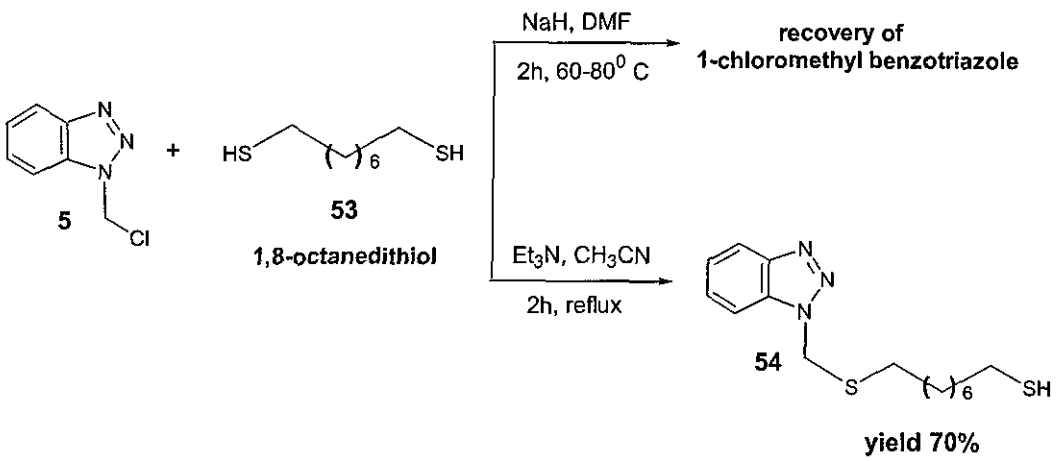
Lipophilic Alcohol	Coupling Reagent	Yield (%)
Cholesterol	DCC	62
"	EDCI	68
Tocopherol	DCC	67
"	EDCI	73


50
49
+
18
DCC or EDCI,
DMF, 0 °C, 4h
DCC or EDCI,
DMF, 0 °C, 4h
16
62-68% yield
17
67-73% yield

Scheme 46

(E) Linker for linking antibody to DNA

Another proposal was made for the synthesis of a linker in order to connect the gastrin antisense DNA to GRE1 antibody. The linker **19** (shown in Scheme 48) was synthesized in two steps. The first step involved the reaction of 1-chloromethyl benzotriazole (**5**) with 1,8-octanedithiol (**53**) in the presence of triethylamine as shown in the **Scheme 47** below.



Scheme 47

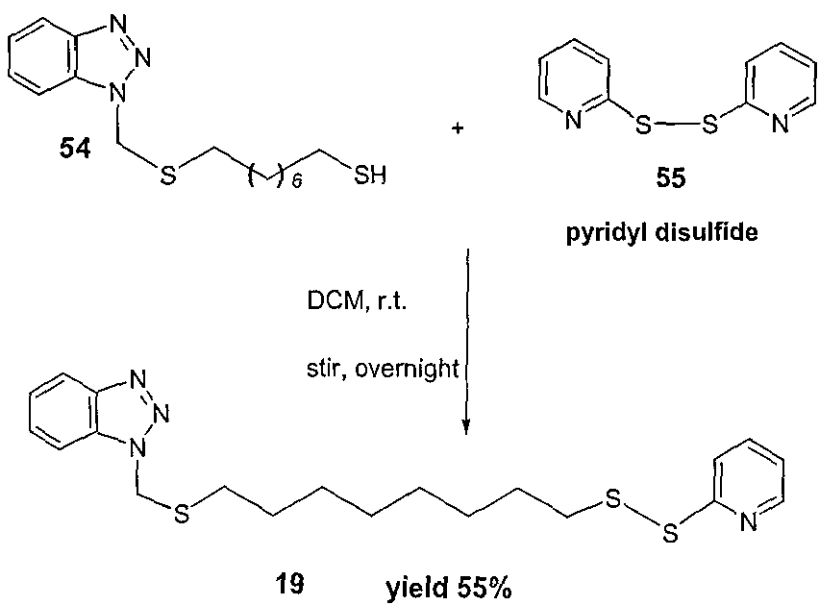
Several attempts were made to synthesize the thioaminal compound (**54**). Originally sodium hydride was employed as the base, DMF as the solvent and a 1:1 ratio of **5** and **53** was used but unreacted starting material (1-chloromethyl benzotriazole) was recovered (15%) after 2h of reaction time. An increase of the reaction time did not change the rate of reaction and unreacted **5** was again recovered.

It was then decided to use triethylamine as a base and acetonitrile as a solvent while the above ratio was changed to 1:1.25. After refluxing for 2h the reaction mixture was left to stand for 30 min, which forced the unreacted 1-chloromethyl benzotriazole to precipitate. The white crystals were removed and the work up proceeded in the usual manner to afford **54** as yellow oil in a 70% yield. This compound was in pure form and was used without further purification.

The ^{1}H, ^{13}C-NMR and elemental analysis data obtained supported the proposed structure where the ^{1}H-NMR spectrum showed a singlet at δ 5.69 ppm which corresponds to the 2H of the CH_2 bonded both to the nitrogen of the benzotriazole ring and the sulfur of the octanedithiol. This signal suggests the formation of compound **54** since the CH_2 of 1-chroromethyl benzotriazole appears at 6.40 ppm.

The second step of the proposed strategy was carried out, by stirring overnight in dichloromethane a mixture of the synthesized **54** and pyridyl disulfide **55**, as shown in **Scheme 48**. The crude product was purified on silica eluting with petroleum ether and ethyl acetate (3:1) to afford the desired linker **19** as yellow oil in a 55% yield.

The spectral and elemental analysis data was consistent with the proposed structure of the linker. The ^{1}H-NMR spectrum gave all the signals of the aromatic protons (of the benzotriazole and the mercaptopyridine ring) whereas the ^{13}C-NMR showed all the signals for the eleven aromatic and the 9 aliphatic carbons.



Scheme 48

The synthesized linker **19** was used to link tumour targeting GRE1, GRE4 and G34 rabbit antibodies (NRbt IgG), to different DNA constructs containing an antisense insert. Four different types of DNA anticancer agents were used:

(a) Linearised Antisense DNA

(b) Circular Plasmid DNA

(c) Apoptotic Synthetic Peptides AK79 (sequence of 9 amino acids long)

(d) Bisindolylmaleimide III HCl (selective inhibitor of protein kinase C)

Immunoglobin G (IgG) is the major class of antibody molecules in blood plasma. NRbt IgG stands for Rabbit Immunoglobin G (antibody).

GRE1 and GRE4 rabbit antibodies (NRbt IgG) were in a solution form immobilized in Protein A columns, where G34 was in the solid state with a molecular weight of 2676. All of the antibodies were supplied by the labs in Queen's Medical Centre in Nottingham.

The synthetic peptides used were each consisted of 9 amino acids. First consisted of LRQAGDDFC with a molecular weight of 986.97 and the second of LRRMSDEFC with a molecular weight of 1135.11

A stands for Alanine (Ala), C for Cysteine (Cys), D for Aspartic acid (Asp), E for Glutamic acid (Glu), F for Phenylalanine (Phe), G for Glycine (Gly), L for Leucine (Leu), M for Methionine (Met), Q for Glutamine (Gln), R for Arginine (Arg), S for Serine (Ser).

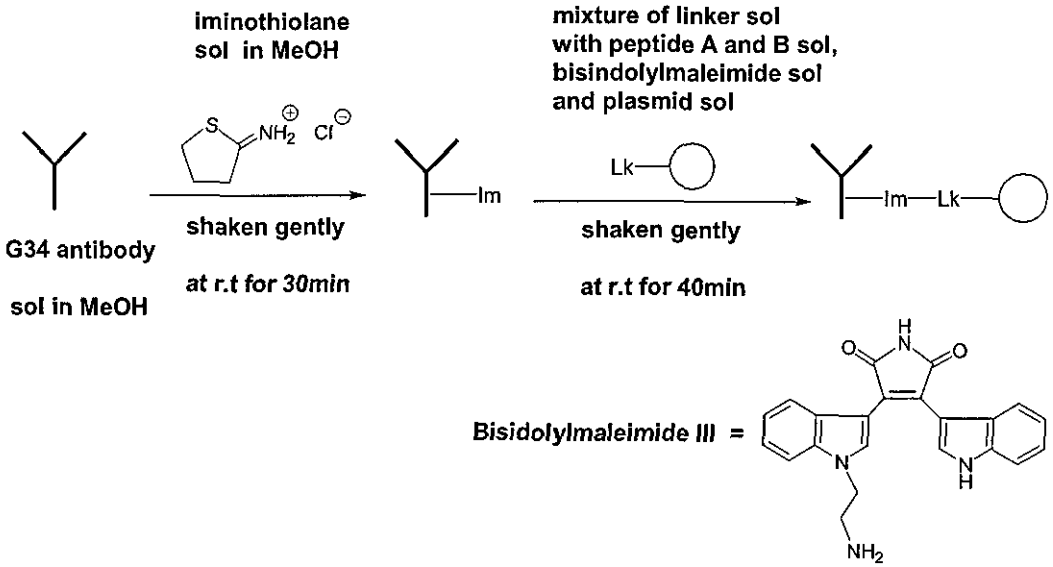
The Circular Plasmid DNA used had a molecular weight of 4352640, while Bisindolylmaleimide III HCl had a molecular weight of 420.9. The linearised antisense DNA provided was in 1 μmol solution.

Iminothiolane with a molecular weight of 137.6, was used to form available thiol groups, which can then be coupled to the disulfide linker **19** (MW 418.72)

(I) For G34 antibody (solid state)

Iminothiolane was attached to the free lysine residues of the affinity-purified antibody G34, by mixing a solution of G34 in MeOH with a solution of iminothiolane also in MeOH (**Scheme 49**). The resulting reaction mixtures were shaken gently at room temperature for 30min.

Solutions of the synthesized linker **19** in CH_3CN were respectively added to solutions of peptide A and B in H_2O and MeOH (3:1), Bisindolylmaleimide III HCl in H_2O and plasmid in MeOH. After the resulting reaction mixtures were shaken gently at room temperature for 30min, they were added to the previous bound G34 reaction mixtures as shown in **Scheme 49** below. The reaction mixtures obtained were shaken gently at room temperature for 40 min and then stored at 4 ^{0}C.



Scheme 49

(II) For GRE1 and GRE4 antibodies (solution state)

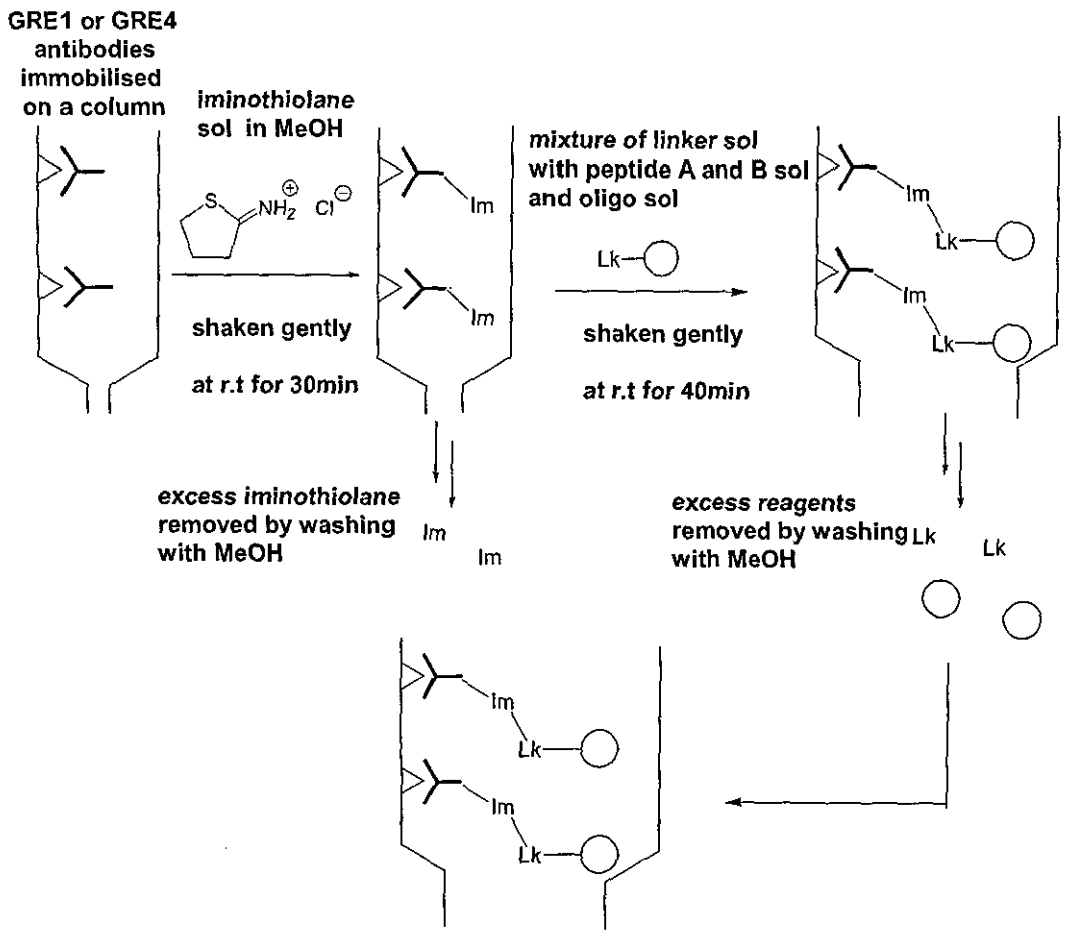
Iminothiolane was attached to the free lysine residues of GRE1 and GRE4 antibodies, which were immobilized on affinity columns, by adding a solution of iminothiolane in MeOH onto the Protein A columns (**Scheme 50**). The bound antibody columns were shaken gently at room temperature for 30min. Excess of iminothiolane was removed by washing with methanol as shown in Scheme 43.

Peptides A and B, and the Linearised Antisense DNA (oligo) were coupled with the existing linker, by mixing a solution of linker **19** in CH_3CN with a solution of peptide A and B in H_2O and MeOH (3:1) and oligo in MeOH, respectively.

After that, peptides A and B and oligo mixtures were added to the antibody columns. The aim was to use 10 fold of oligo and linker reaction mixture for the GRE1 column and 100 fold of the same reaction mixture for the GRE4 column. 10 fold of peptide A and B and linker reaction mixtures were used for the remaining two GRE1 and GRE4 columns.

Peptides A and B and the oligo were successively bound to the antibodies GRE1 and GRE4, after gently shaking the columns at room temperature for 40min as shown in Scheme 50 below. Excess of reagents was removed by washing with methanol and the columns were then stored at 4 ^{0}C.

The above experiment using the protein A columns with immobilized IgG was repeated. In this case though after the reaction to attach Peptides A, B and oligo to the antibodies, the washing of the excess of reagents did not take place. This was done in order to evaluate whether the bound materials were removed previously with washing since the detection system showed no biologically active antibody constructs (see results below)



Scheme 50

The resulting solutions of bound G34 antibody and bound GRE1 and GRE4 Protein A columns were sent out for purification of the biologically active antibody constructs. The antibodies retained at the binding sites were eluted using 0.1 M glycine buffer. The elutions, which were assayed, using ELISA as detection system, were performed in the labs in the Queen Medical Center. The steps taken in ELISA (Enzyme-Linked ImmunoSorbent Assay), shown in **Scheme 51** below, are as follows:

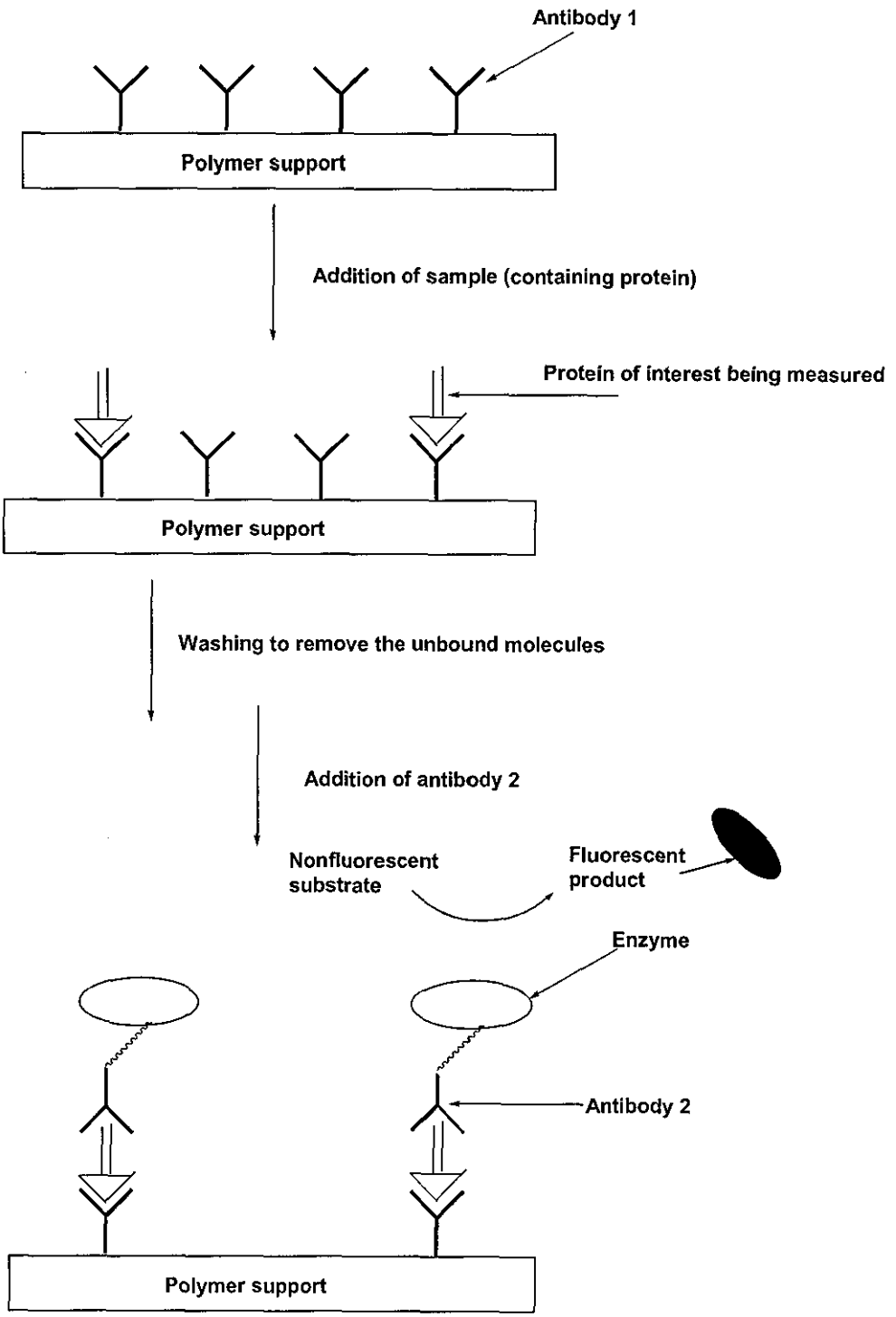
(i) Attachment of the specific antibody to a polymer support

(ii) Addition of the sample (containing the protein of interest)

(iii) Washing to remove the unbound material

(iv) Addition of the second antibody attached to an enzyme. This antibody is specific for a different site on the protein being detected.

(v) Measurement of the fluorescent compound that is being formed by the catalytic action of this enzyme.



Scheme 51

In the experiment to validate ELISA, avidin was used to coat the plate of the polymer support. It appeared that the directly bound Rbt IgG (the ones retained on the binding sites) induced a specific signal, which was dependant on the concentration of avidin used. The NRbt control produced a much lower signal. An avidin coating concentration of 1: 500 was used for all the following assays and the results are shown below

Assay 1

The GRE1 and GRE4 Protein A columns were eluted with 0.1 M glycine buffer initially (pH 2.8) and no protein was detectable. The pH was increased to alkaline but again no protein was detectable in the elution.

Assay 2

Four samples were assayed. The samples consisted of the elutions from the columns assessed in assay 1 and the reaction mixtures in which the G34 NRbt IgG was linked to peptides A and B, bisindolylmaleimide III HCl and plasmid. The reaction mixtures were in the form of a suspension that may have non-specifically affected the absorbance and so they were assessed with and without centrifugation.

The results shown in **Figure 5** below show very low levels of specific immuno-reactivity (relating to the conjugate of NRbt IgG and biotin) in the reaction mix suspension and the supernatant following centrifugation.

Assay 2: Column elutions from immobilized reaction mix and suspension reaction mix

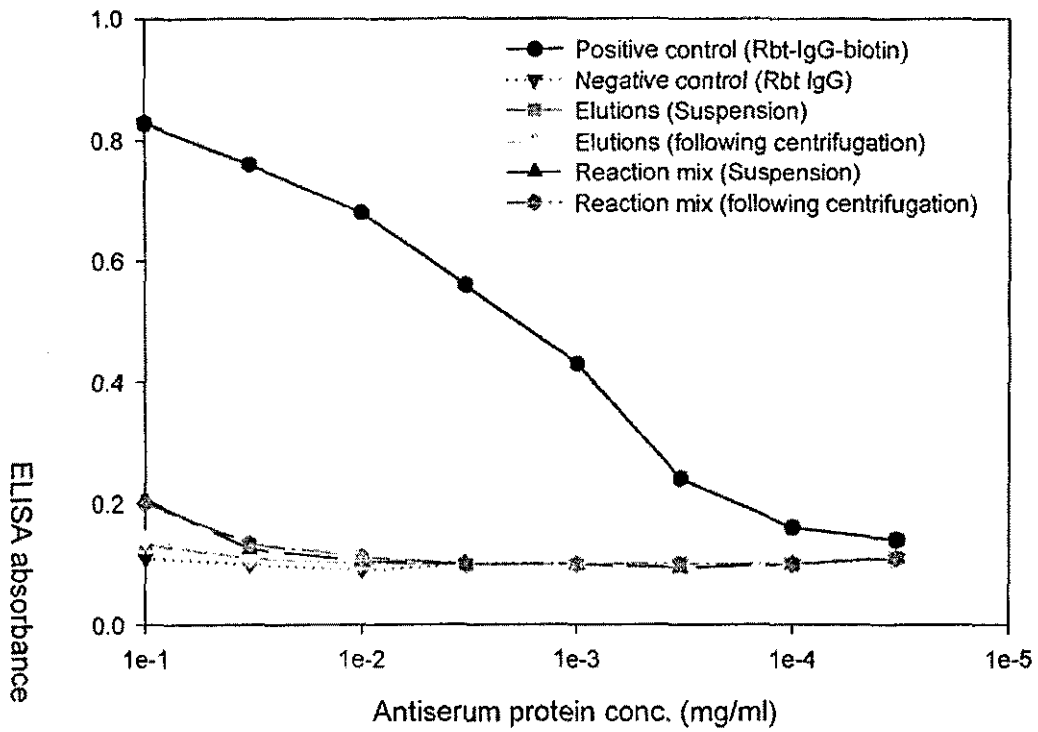


Figure 5

Assay 3

Four samples were assayed. The samples consisted of four consecutive elutions from the protein A columns with immobilized IgG which had not been washed after the reaction to attach the oligo, protein A and B to the Rbt IgG. The antibodies were eluted with 0.1 M glycine buffer (pH 2.8) and the results are shown in **Figure 6** below. The second elution showed an increased signal.

The protein concentration of the elutions (1mg of NRbt protein was coupled to the Protein A column) was also determined by the use of absorbance at 280nm and they are shown in **Table 3** below.

Assay 3: Immunoreactivity of elutions from assay of immobilized NRbt

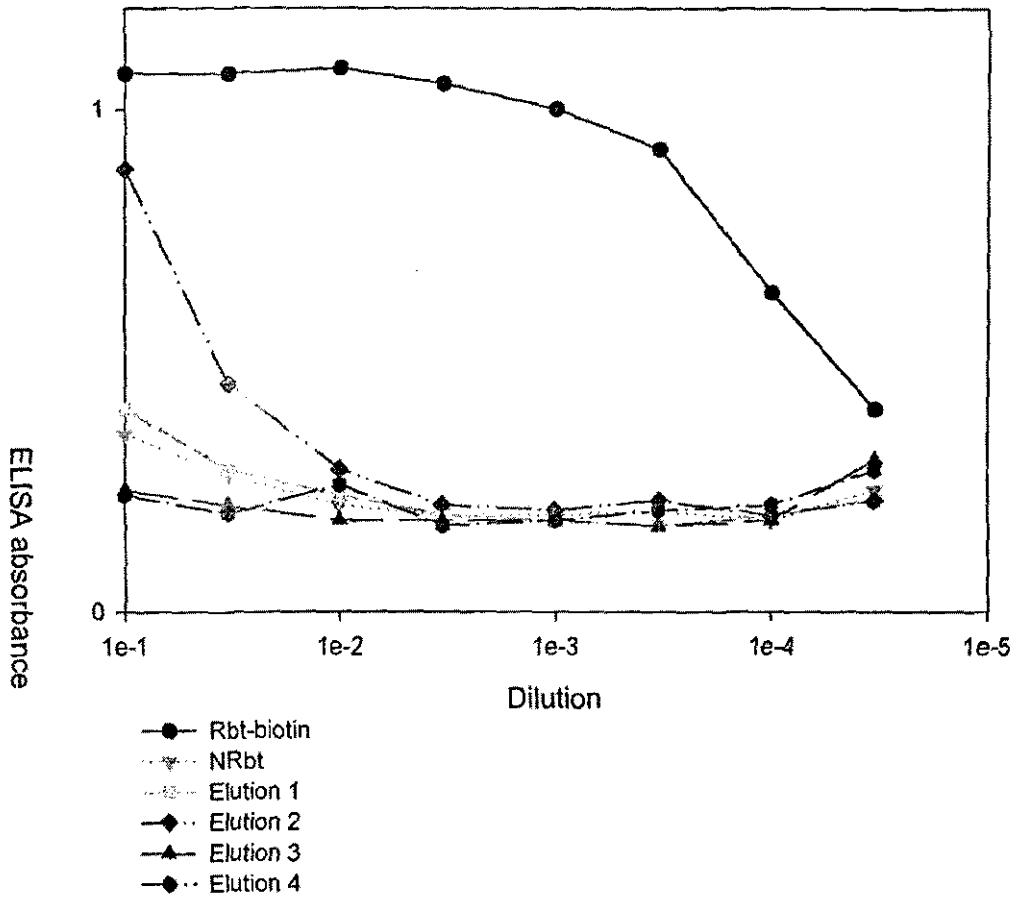


Figure 6

Table 3

Elution	Protein conc. (mg/ml)
1	0.17
2	0.65
3	0.03
4	0.00

Employment of the synthesized linker **19** is still under investigation to establish whether the tumour targeting rabbit antibodies (NRbt IgG) can be linked to a biotinylated oligonucleotide through **19** in a manner that appears to maintain increased biological activity. Plans were made to repeat experiments with GRE1 and GRE4 specific antibodies. This would be done to determine whether specific ELISA immuno-reactivity could be gained with further purification of the eluted antibodies retained at the binding sites. A study will also be done to assess the background signal gained with the biotinylated oligo.

2. GLOBAL AND PARTIAL PROTECTION OF NUCLEOSIDES

(A). Protection of the 5'-position of nucleosides

Nucleosides are conventionally attached to a solid support by the 3'-hydroxyl position, thus the 5'-position needs to be protected. The protection reaction is regioselective for the primary 5'-hydroxyl compared to the 3'-hydroxyl partly because of the bulk of the protective groups.

Thymidine was used firstly as the sample nucleoside, as its nucleoside base does not need protection, in order to investigate hydroxyl protection using different protecting groups. Following adenosine and guanosine nucleosides were also protected. The protective groups triphenylmethyl chloride (TrCl), 4, 4'-dimethoxytrityl chloride (DMTr) and *tert*-butyldimethylchlorosilane (TBDMSCl) were introduced at the 5'-position using different sets of conditions

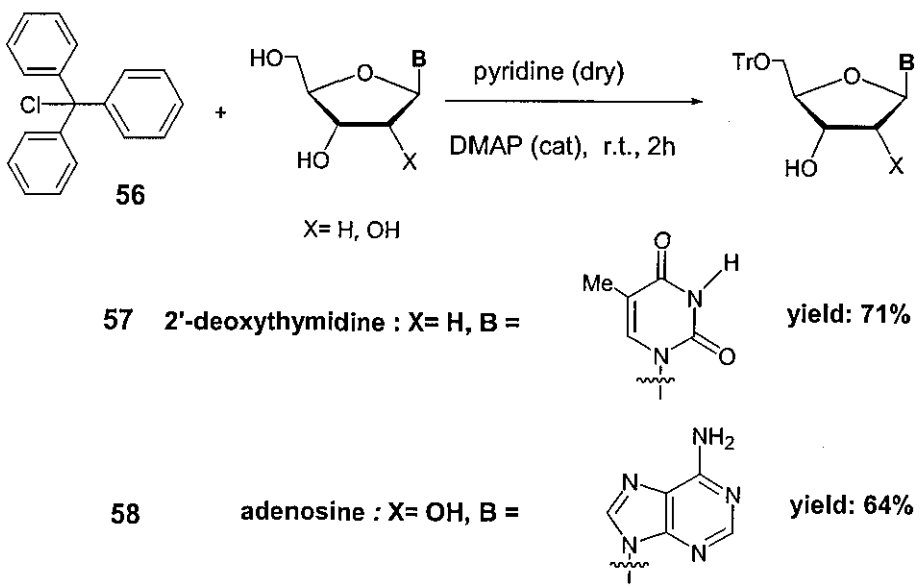
(I) Trityl Protection

Trityl (triphenylmethyl) chloride **56** (**Scheme 52**) is conventionally a protecting group for primary hydroxyl functions but it has also been used to protect the 3' and 5' positions of 2'-deoxythymidine. The 5'-O-tritylthymidine is a key intermediate in the synthesis of thymidine derivatives and favours the introduction of 3'-substituents, which are susceptible to mild reducing or basic conditions.

Tritylation of 2'-deoxythymidine and adenosine in pyridine with the presence of a catalytic amount of 4,4'-dimethylaminopyridine afforded **57** and **58** after recrystallization from ethanol and petrol in a high 71% yield and 64% yield as white crystalline solids, respectively.[297-300] (Shown in **Scheme 52**).

The ^{1}H-NMR spectrum of compound **57** showed a multiplet at δ 6.94-7.22 ppm and another multiplet at δ 7.30-7.55 which correspond to the 9 ortho and para hydrogens and the 6 meta hydrogens respectively, of the trityl groups of the protective group. ^{1}H-NMR spectroscopic analysis of compound **58** showed the same multiplets at δ 6.76-7.05 ppm and δ 7.12-7.44 ppm.

Trityl chloride (TrCl)



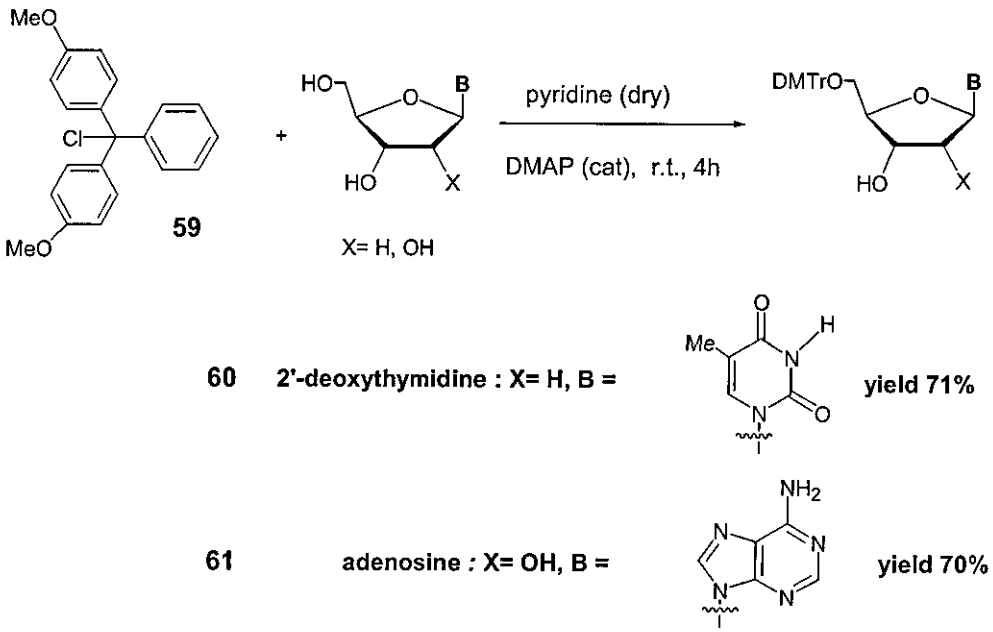
Scheme 52

(II) Dimethoxytrityl Protection

The most useful protective group for the 5'-position is the 4',4'-dimethoxytriphenyl-methyl group **59** (**Scheme 53**), which is the most easily cleaved of a family of acid- labile protective groups, the labilities of which increase as the number of methoxy groups increases.

Among the N-free 5'-O-dimethoxytritylated nucleosides, the adenosine and thymidine derivatives were prepared from the parent nucleosides in one step synthesis. The 5'-O-selective dimethoxytritylation of adenosine and 2'-deoxythymidine was conducted in pyridine, using 4, 4'-dimethoxytrityl chloride in the presence of a catalytic amount of 4-dimethylaminopyridine.

4,4'-dimethoxytrityl chloride (DMTrCl)



Scheme 53

In the case of 2'-deoxythymidine the crude product was recrystallized from ethanol and petrol to give the 5'-O-protected derivative **60** as a white microcrystalline solid in 71% yield.

For adenosine dichloromethane and petrol were used to afford **61** in 70% yield again as a white microcrystalline solid (**Scheme 53**). Both of the compounds showed spectral data consistent with the reported data for **60** and **61.**[301,302]

The selective incorporation of the protecting groups **56** and **59** at primary hydroxyl is undoubtedly one of the features accounting for the usage of these groups in the protection of the 5'-hydroxyl function of nucleosides. The sensitivity of a specific trityl ether linkage to acidic conditions is dependant on the electron-donating properties of the substituents carried by the trityl group.

Each *p*-methoxy substituent enhances the rate of acid cleavage, consequently the di-*p*-methoxytrityl group is most commonly used. In the synthesis of oligonucleotides on polymer support, the removal of the 5'-O-DMTr group under acidic conditions can be particularly useful in evaluating the efficiency of the chain elongation step. The release of the di-p-methoxytrityl carbocation gives a bright orange colour, which confirms the deprotection of the 5'-hydroxyl group.

(III) *tert*-Butyldimethylsilyl Protection

tert-Butyldimethylsilyl chloride (TBDMSCl) **62** shown in **Scheme 54**, reacts selectively with hydroxyl groups in nucleosides and even in the presence of excess TBDMSCl the reaction occurs preferentially with the 5'-hydroxyl and not with amino groups.

The TBDMS group is especially advantageous as it does not possess a chiral center and its derivatives are frequently nicely crystalline and suitable for analysis. It is stable to phosphorylation conditions and can be removed under conditions that do not affect other acid (e.g. trityl) or base labile protecting groups.[230]

The ratio of nucleoside: TBDMSCl: imidazole is important in terms of yield and rate of reaction. Two solvents systems have been employed for the silylation of the four nucleosides: (i) the DMF-imidazole and (ii) pyridine. The presence of imidazole as catalyst is essential to the reaction with DMF since its absence gives around 5% yield. However if pyridine is used as solvent in place of DMF, it effectively replaces imidazole as catalyst.

1. Protection of the 5'-hydroxyl

The DMF-imidazole system usually led to faster rates of reaction while pyridine, which works as both solvent and base, provided higher yields of the desired products. Many different approaches were attempted using different equivalents and reactions times but the reaction conditions shown in the **Table 4** below list the conditions that led to optimum yields of **63**, **64**, **65** and **66** (Shown in **Scheme 54**).

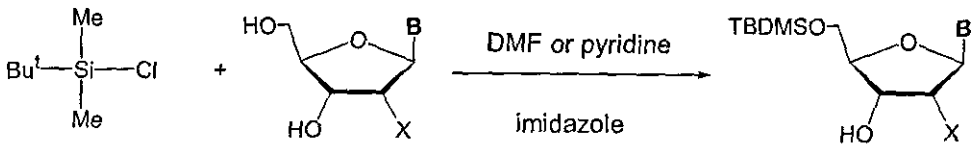
Table 4

Nucleoside	TBDMSCl (eq)	Imidazole (eq)	Solvent[a]	Time (h)	Yield (%)
2'-deoxythymidine	1.1	2.2	DMF	1	67
2'-deoxythymidine	1.5	3	DMF	8	68
2'-deoxythymidine	1.2	0	Pyridine	8	77
Adenosine	1.1	2.2	DMF	3	74
Adenosine	1.3	2.6	DMF	2	77
Adenosine	1.2	0	Pyridine	8	86
Guanosine	1.2	2.4	DMF	3	72
Guanosine	1.5	3	DMF	2	75
Guanosine	1.2	0	Pyridine	6	81
Cytidine	1.2	2.4	DMF	4	73
Cytidine	1.3	2.6	DMF	2	78
Cytidine	1.2	0	Pyridine	6	88

[a] *Conditions were* 4ml of DMF per 1mmol of nucleoside and 2ml of pyridine per 1mmol of nucleoside

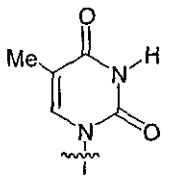
Originally the reactions were tried, as a suspension of the nucleosides in DMF, pyridine and THF. *tert*-Butyldimethylsilyl chloride was found to react very slowly with the hydroxyl groups and in unsatisfactorily yields even when excess of silylating agent in excess solvent was left to react for many hours. The lengthy reactions times and the poor yields obtained was due to the poor solubility of the nucleosides in the variety of solvents utilized, even though these are the most polar solvents available.

tert-Butyl dimethyl silyl chloride
(TBDMSCl)



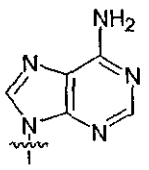
62

63 **2'-deoxythymidine : X= H, B =**



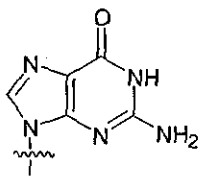
yield 67-77%

64 **adenosine : X= OH, B =**



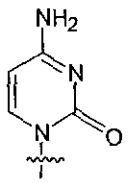
yield 74-86%

65 **guanosine : X= OH, B =**



yield 72-81%

66 **cytidine : X= OH, B =**



yield 73-88%

Scheme 54

It was observed that a simple change in the procedure led to a much cleaner reaction mixture. The suspension of the nucleoside in either concentrated DMF or pyridine was first heated up at around 70-90 ^{0}C for approximately 20min till complete dissolution of the nucleoside. Then the silylating reagent was added neat followed by imidazole in the case of DMF.

The major advantage of the method developed is that the reactions were performed in a concentrated solution instead of suspension as previously used, which accelerated the rate of reaction. This method is based on direct addition of the silylating agent to the dissolved nucleoside. Many ways of mixing the reagents were tried but the procedure developed (see Experimental) gave maximum results.

Good yields of the 5'-protected nucleosides were obtained in all four cases. The 5'-TBDMS-2'-deoxythymidine **63** was furnished in 66% yield after 1h reaction in DMF-imidazole or 77% yield after 8h reaction in pyridine. Again in DMF but increasing the ratio to 1: 1.5: 3 and the reaction time to 8h had a negligible increase in the yield.
With adenosine the DMF-imidazole system gave 5'-TBDMS-adenosine **64** in 74% yield after 3h and 77% after 2h but by increasing the ratio to 1:1.3:2.6. Pyridine again gave the best result with 86% yield after 8h of reaction.

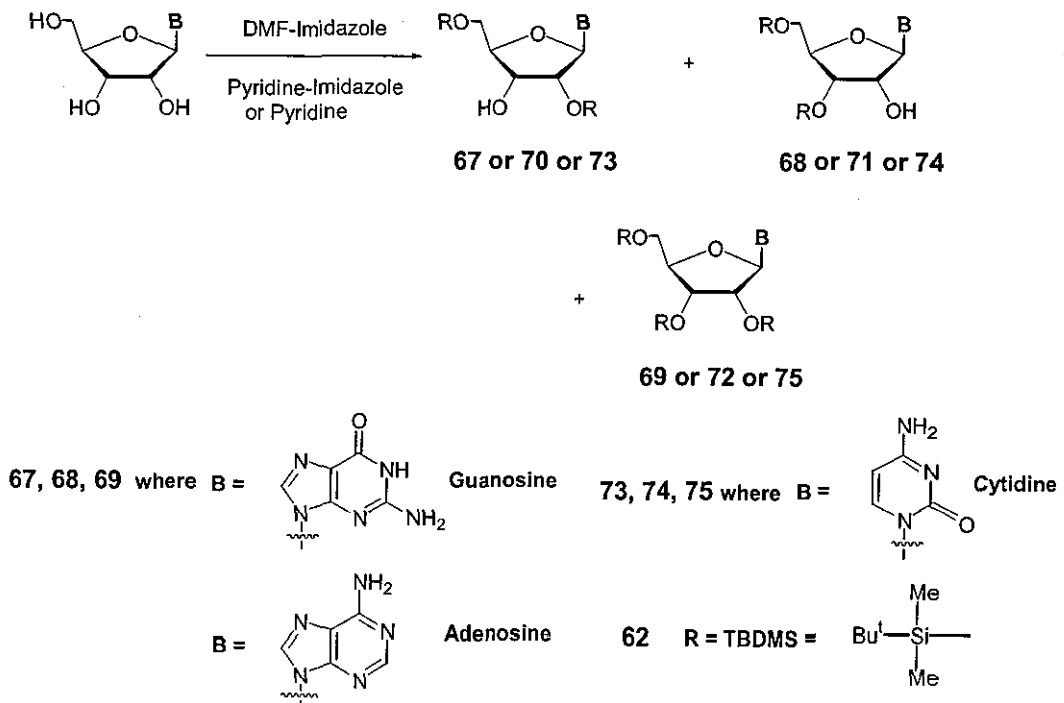
Similar yields were obtained for 5'-TBDMS-cytidine **65** (78% in DMF-imidazole and 88% in pyridine). In this case the 1:1.1:2.2 ratio gave poor results and for that reason was increased to 1:1.2:2.4 and the reaction time to 4h.
With guanosine yields of the 5'-TBDMS-guanosine **66,** were generally lower compared to the previous nucleosides (75% in DMF-imidazole and 81% in pyridine). Even when the ratio was increased to 1:1.5:3 **66** was furnished in 72% yield after 2h of reaction. The melting point, I.R, and ^{1}H NMR spectra of all four 5'-O-protected derivatives product were identical with those prepared according to the literature.[303]

Using the conditions described in **Table 4** averted any silylation of the amino bases of the nucleosides, while the method developed allowed for reactions to be performed in homogeneous solutions, increased the rate of reactions. Using larger excesses of the silylating agent in some cases led to silylation of the base especially in the case of guanosine. It was also noted that TBDMSCl deteriorates with time and repeated exposure to air such that higher ratios of the respected nucleosides must be used to obtain the maximum yields.

The preference of TBDMSCl for reaction with hydroxyl groups adds considerable versatility to nucleoside protecting groups The major advantage of the TBDMSCl reagent is that it rapidly converts the nucleosides into silylated derivatives and is stable to acidic and basic conditions, dry solvents and nonpotic bases such as pyridine. When pyridine was used as the solvent in place of DMF for adenosine, the pyridine effectively replaces imidazole.

2. Protection of the 2' and 3'-hydroxyls and Global Protection

Adenosine, guanosine and cytidine were used for protection in this case as shown in **Scheme 55**



Scheme 55

Again the two known systems (DMF-imidazole and pyridine) were used along with the new pyridine-imidazole conditions. Various reaction conditions were applied using different equivalents of TBDMSCl and imidazole and different reaction times in order to access the reaction rate and selectivity. The method developed for the 5'-OH protection (heating of the nucleoside suspension till dissolution before addition of the silylating reagent) was also used in this case.

It was found that DMF-Imidazole system worked exceptionally well in the case of the global protection of guanosine and adenosine with 100% selectivity in derivatization of the 2',3',5'-hydroxyl versus the 2',5' and 3',5'-hydroxyls. It gave the most rapid rates with completion of the reactions within 30min of stirring at room temperature and excellent yields.

Pyridine reactions were slower but gave more selectivity in favour of the formation of the 2'-hydroxyl and higher yields, especially when imidazole was also added as in the case of guanosine and adenosine. These results are summarized in **Table 5** below.

Table 5

Nucleoside	TBDMSCl (eq)	Imidazole (eq)	Solvent[a]	Time (min)	Yields (%) 2',5'	3',5'	2',3',5'
Cytidine	2.2	4.4	DMF	60	50	34	10
	3.6	6.6	DMF	90	54	25	8
Cytidine	3	0	Pyridine	90	64	22	2
Cytidine	3	7	Pyridine	60	16	21	65
	7	14	Pyridine	60	----	----	89
Guanosine	2.2	4.4	DMF	60	41	32	10
	3.3	7	Pyridine	90	51	19	8
Guanosine	5	0	Pyridine	90	67	16	4
Guanosine	7	14	Pyridine	60	4	8	76
	4	10	DMF	30	----	----	85
Adenosine	2.2	4.4	DMF	60	45	32	8
Adenosine	3	0	Pyridine	60	50	26	4
	5	0	Pyridine	60	62	19	2
	3.3	7	Pyridine	90	52	16	9
Adenosine	4	10	DMF	90	4	4	84
	4.4	8.8	DMF	30	----	----	92

[a] Conditions were 4ml of DMF per 1mmol of nucleoside and 2ml of pyridine per 1mmol of nucleoside

Good yields of the desired 2',5'-protected nucleosides were obtained when pyridine was employed in all three cases. For example the 2',5'-disilylguanosine **67** was obtained after 90 min reaction in 67% and 51% yield when pyridine and pyridine-imidazole systems were used respectively. 2',5'-Disilyladenosine **70** was formed after 60 min in pyridine in 62% and 50% yield when 3 and 5 equivalents of silylating reagent were used respectively.

Addition of imidazole and increase of the time to 90 min only resulted to a 52% yield. 2',5'-disilylcytidine **73** was obtained in 64% yield after a 60 min reaction in pyridine while addition of imidazole only gave 16% yield of the desired derivative.

On the other hand employment of the DMF-imidazole system led to the decrease of selectivity in favour of the 2',5'-protected nucleosides. With guanosine the 2',5' and 3',5'-disylated **67** and **68** derivatives were furnished in 41% and 32% yield respectively, while the 2',5' and 3',5'-disylated adenosine **70** and **71** were obtained in 45 and 32% yield respectively.

With cytidine the yield of the 2',5'-disilylcytidine **73** was slightly higher (50%) but again the selectivity suffered with the 3',5'-derivative **74** obtained in 34% yield.

On the contrary in the case of the 2',3',5' TBDMS protection employment of the DMF-imidazole system led to excellent selectivity in derivatization of the global protected derivatives of adenosine and guanosine. They were furnished in satisfactory yields within 30 min of reaction, 92% for 2',3',5'-silyl adenosine **72** and 85% for 2',3',5'-silyl guanosine **69**.

With cytidine though, the pyridine-imidazole system proved to be more effective than DMF-imidazole, resulting in the selective production of the 2',3',5'-silyl cytidine **75** in 89% yield after a 60 min reaction time.

One excellent synthetic feature of the silylated nucleosides is that isomeric compounds separate very cleanly on silica gel chromatography and can be obtained by short column chromatography often in solvents such as ether and hexane. Products containing silyl groups on the exocyclic amino groups were not obtained in any quantity during these reactions. It appears that N-silylated nucleosides may be intermediates in the reactions but they either act as silylated agents themselves (i.e., transferring the N-silyl to a hydroxyl) or are hydrolyzed during work-up.

The usage of TBDMSCl protecting group leads to the conclusion that for the nucleosides possessing a free amino group, the above ratio of the reagents used increases for optimum yields of adenosine cytidine and guanosine derivatives.

3. Selective 5'-desilylation of 2',5'-and 2',3',5'-silylated nucleosides

The 2',3',5'-O-TBDMS and the 2'-5'-O-TBDMS nucleosides are useful building blocks for investigation of the coupling chemistry of the existing linkers. One of the main aims of this project was the employment of the linked nucleoside conjugates for the formation of CpG dinucleotides. In order to utilize them the 5'-end needs to be free for coupling and subsequently removal of the 5'-TBDMS groups is essential. It was proposed to investigate the 5'-selective desilylation method first on the non-coupled nucleosides in order to study the effects and then apply the optimized conditions on the linked nucleosides.

One of the greatest challenges in the field of the TBDMS protection is the selective deprotection of the primary TBDMS groups in the presence of their secondary counterparts, a procedure with considerable utility in nucleotide synthesis.[304] In the past, removal of silyl groups was done either by acid hydrolysis, base, Lewis acids or in the presence of tetrabutylammonium fluoride (TBAF).

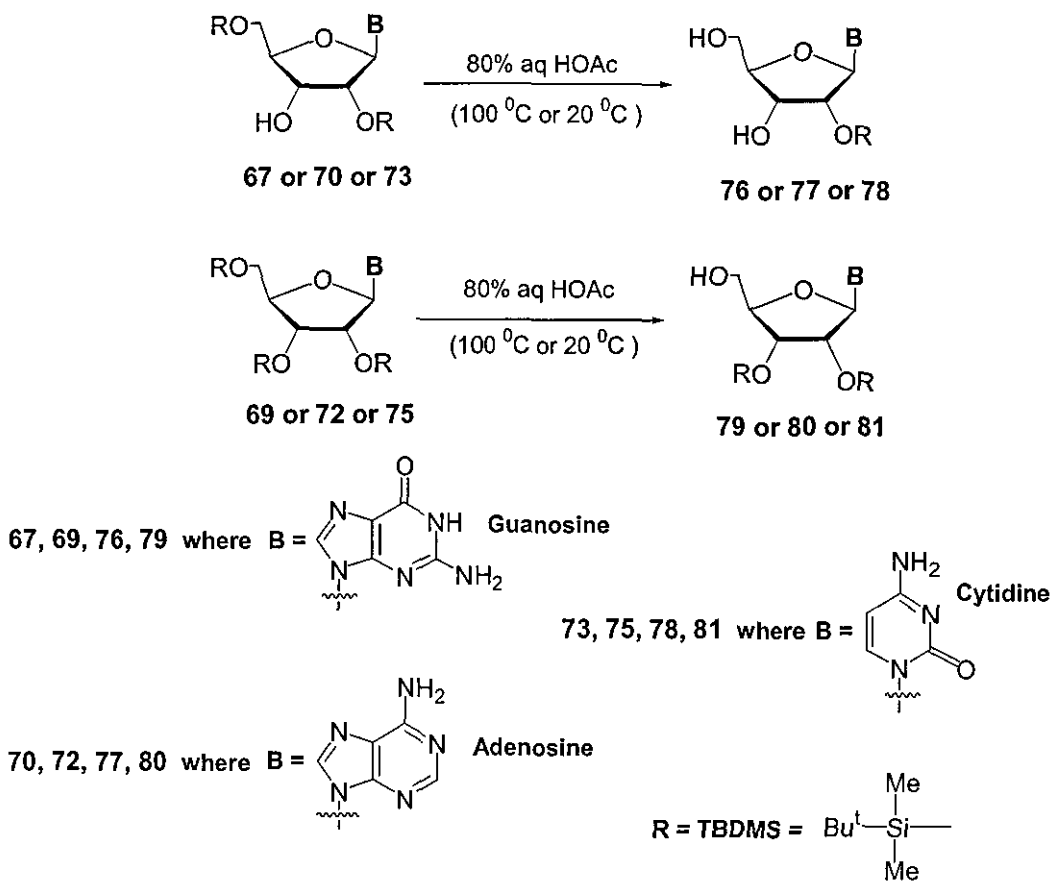
When base was used as the deprotecting reagent[305] (9 M NH_4OH in ethanol at 70 ^{0}C), it was found that 2'-and 3'-silyl groups are more sensitive to base hydrolysis than the 5'-position. This resulted in the removal of the primary TBDMS group being more difficult when both of the 2'-and 3'-positions are protected.

Cleavage of the primary TBDMS group using Lewis acids such as zinc bromide suffered from long reaction times, higher temperatures, low yields and complicated procedures.[306-308] In the case of TBAF, the 2' and 3'-silyl groups were cleaved more rapidly than 5'-silyl, which demonstrated that the fluoride ion based reagent, possesses a lack of primary preference. This can be due to the strong affinity of fluoride ions for silicon.

A useful feature of the silylated nucleosides is the fact that the 5'-silyl group is much more labile towards acid than either the 2'- or 3'-silyl. Therefore the primary silyloxy groups are cleaved under acidic conditions more easily than secondary ones.[304]

It was decided that acetic acid with a pka value of 4.76 seemed the appropriate candidate for selective deprotection, as is a moderate acid that can offer good selectivity. Consequently the 2',5'-derivatives of the nucleosides were treated with 80% aqueous acetic acid (HOAc) either at room temperature or on heating in order to remove the 5'-OH protection. Also the 2',3'-silylated nucleosides needed to be synthesized and they were prepared via the selective 5'-desilylation of the corresponding 2',3',5'-trisylilated derivatives.

The 5'-desilylation of 2',5'-and 2',3',5'-silylated guanosine, adenosine and cytidine using HOAc, is shown in **Scheme 56** and the optimized results are summarized in **Table 6** below.



Scheme 56

Table 6

Nucleoside	Temperature (^{0}C)	Time (h)	Yield (%)
67	20	24	**76** (74%) + guanosine (10%)
67	100	0.7	**76** (70%) + guanosine (14%)
70	20	22	**77** (73%) + adenosine (12%)
70	100	0.5	**77** (68%) + adenosine (19%)
73	20	22	**78** (80%) + cytidine (5%)
73	100	0.5	**78** (72%) + cytidine (13%)
69	20	30	**79** (79%) + 76 (8%)
69	100	3	**79** (71%) + 76 (13%)
72	20	28	**80** (77%) + 77 (12%)
72	100	2	**80** (70%) + 77 (14%)
75	20	28	**81** (83%) + 78 (3%)
75	100	3	**81** (76%) + 78 (10%)

When the 2',5'-derivatives of guanosine, adenosine and cytidine **67**, **70** and **73,** were treated with HOAc at room temperature the reactions were longer but the yields obtained were better (up to 80%), compared to heating where the reactions were complete within 30min with yields up to 68-74%.

The crude 5'-desilylated derivatives of guanosine and cytidine were re-crystallized from ethyl acetate and hexane, to give the pure form of **76** and **78** as white solids. In the case of adenosine re-crystallization from dichloromethane and petrol provided the purified **77** as white solid.
In both cases though, along with the 5'-desilylated derivatives **76**, **77** and **78** guanosine, adenosine and cytidine were also furnished and especially when the reactions were heated, the yields of the fully deprotected nucleosides as by-products, were increased.

The 2',3',5'-tri-O-TBDMS guanosine **69**, adenosine **72** and cytidine **75** were also selectively deprotected to give very good yields of the corresponding 2',3'-O-disilylated derivatives **79**, **80** and **81** (76-86%).

The crude 2',3'-O-disilylated derivatives of guanosine and cytidine were subjected to flash chromatography using chloroform and ethanol (4:1) and then to provide the pure forms of **79** and **81** as white solids. In the case of guanosine diethyl ether and hexane (2:1) and then diethyl ether was used as the eluent to afford the purified **80** as white solid. Again reactions at room temperature afforded higher yields with longer reaction times.

Treatment of the globally protected nucleosides with the deprotecting reagent also resulted in the removal of the 3'-O-TBDMS group to produce the 2'-O-derivatives **76**, **77** and **78** as by-products. The 5'-silyl group in adenosine appears to be slightly more resistant to acid than in guanosine and cytidine.

Steric effects can dominate the selectivity of desilylation. Although it was found by calculation of the Geistiger-Huckel charges[309] that the 2', 3' and 5'-O-TBDMS groups have almost the same charge on their oxygen atoms, their steric environments are different.
The 5'-O-TBDMS groups of trisilylated nucleosides are obviously much less hindered than the 2'-group, leading to excellent selectivity in favor of the removal of the 5'-O-TBDMS group. These steric effects are somewhat reduced for the disilylated nucleosides in which the 3'-positions have smaller OH moieties leading to slightly poorer desilylation selectivity and yields.

Our experiments revealed that although selective 5'-desilylation can be accomplished using 80% aqueous acetic acid with high yields up to 86%, it also resulted in side reactions and the production of by-products. Ideally this has to be avoided.

Around the time that attempts were made to optimize the selective deprotection conditions in order to avoid by-product formation, A. Ian Scott and his co-workers[309] published a paper about the 5'-desilylation of multisilylated nucleosides. They discovered that a TFA-H_2O-THF (1 :1 : 4) system selectively deprotected the 5'-O-TBDMS group with very satisfactory results at 0 ^{0}C.

Employment of this system for our deprotection purposes, resulted in the 2',3',5' and 2',5'-O-TBDMS nucleosides being quantitatively transformed into the expected 2',3' and 2'-O-TBDMS derivatives, as shown in **Scheme 57**. The results obtained from our experiments are shown in **Table 7** below

The use of the THF as the solvent afforded the following benefits:

(i) Increase of solubility of nucleoside substrates and hence acceleration of the reaction time.

(ii) Significantly improved selectivity of 5'-desilylation even when the reaction was carried out at room temperature.

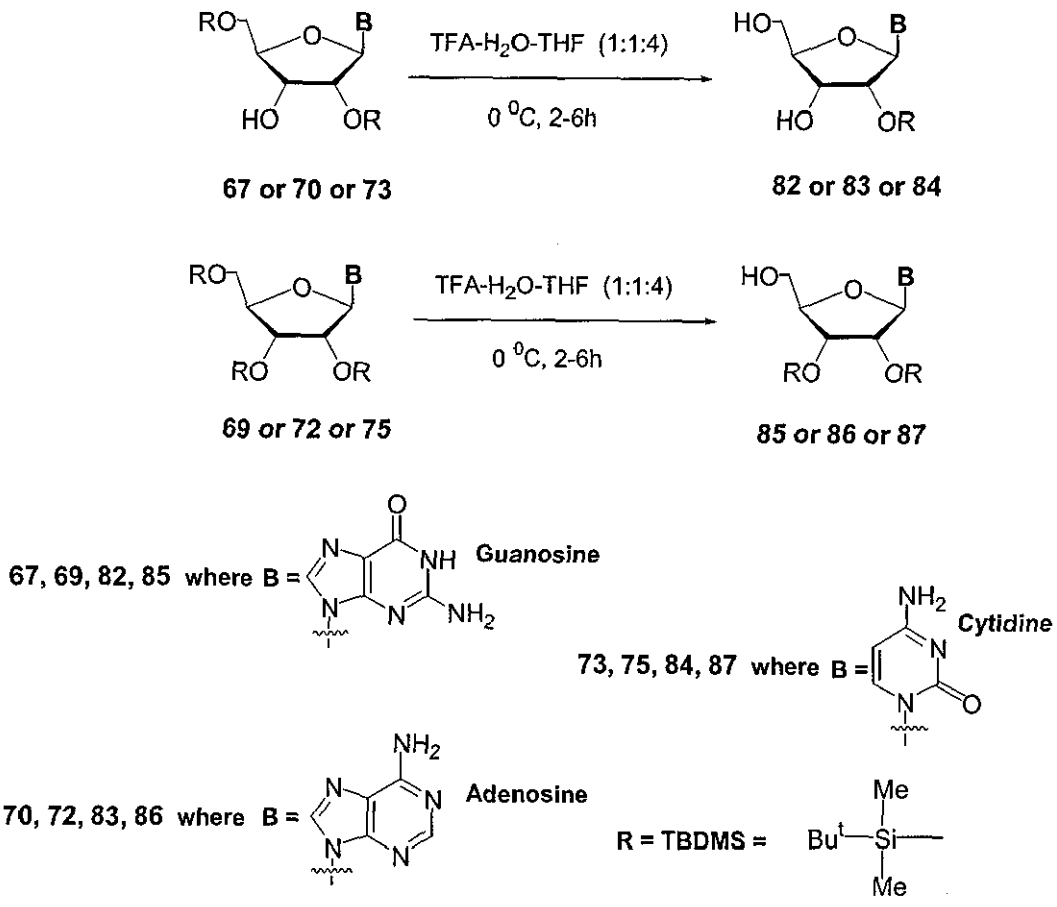
(iii) Complete absence of depyrimidination and depurination, which are common side reactions of the acidic hydrolysis of nucleosides.

Table 7

Nucleoside	Time (h)	Yield (%)
67	2	**82** (88%)
70	2	**83** (91%)
73	2	**84** (93%)
69	3	**85** (95%)
72	3	**86** (92%)
75	6	**87** (97%)

The corresponding 2',3'-O-disilylated derivatives of guanosine **85** and cytidine **87** were afforded after purification on column chromatography using chloroform and ethanol (4:1) while the adenosine derivative **86** was provided using diethyl ether (2:1) as the eluent.

The 2'-O-silylated derivatives of guanosine and cytidine **82** and **84** were recrystallized from ethyl acetate and hexane while **83** was recrystallized from dichloromethane and petrol. The yields obtained for the guanosine **85** and cytidine **87** derivatives were slightly improved compared to Scott's while the yields for **82, 83, 84,** and **86** were similar.



Scheme 57

In comparison with the results obtained from the selective 5'-desilylation using 80% aqueous acetic acid, Scott's system seemed to be more effective as the 2',3',5' and 2',5'-O-TBDMS nucleosides were quantitatively transformed into their 2',3' and 2'-O-TBDMS derivatives. Along with yield improvement another advantage of this system was that deprotection of the 5'-OH group was performed without any side reactions or production of by-products.

The synthesized 5'-desilylated derivatives of the nucleosides will be employed in the future formation of lipophilic nucleoside conjugates and subsequent synthesis of CpG dinucleotides.

3. SYNTHESIS OF LIPOPHILIC-NUCLEOTIDE CONJUGATES

Modification of oligonucleotides with lipophilic groups to provide more potent antisense agents, has been considered as one possible method to improve their pharmacokinetics. It has been shown that such modifications of *oligonucleotides can enhance the uptake and cellular efficacy.*[172] They have increased nuclease resistance,[158,160] are more active in blocking the viral replication in cell cultures and more efficacious in antisense experiments, compared to non-derivatized oligonucleotides.[178,188]

For modified nucleotides to be suitable as diagnostic systems was postulated that certain structural criteria are required to effect efficient hybridization of the modified nucleoside with its targeted genetic sequence. These criteria include a stereoregular backbone (required for a homogeneous binding constant between the analogue and its complementary genetic sequence) and affinities of the oligonucleotide analogues for targeted genetic sequences that are sufficient to effect the desirable biological or physical action.

Certain nucleoside linkages containing carbonyl moieties satisfy the above criteria and they can constitute a valuable class of nucleotide analogues. Our interest in this class was stimulated by the fact that their synthesis could be readily achieved while; these analogues can have important biochemical properties such as template activity and resistance to enzyme-catalysed degradation. Subsequently it was decided to synthesize lipophilic-nucleotide *conjugates containing such linkages.*

The previously synthesized carbonate (**10** and **11**), carbamate (**13** and **14**), and ester (**16** and **17**) linkers were employed to link the lipophilic molecules to the 3'-terminus or the amino base of the nucleosides through internucleoside linkages.

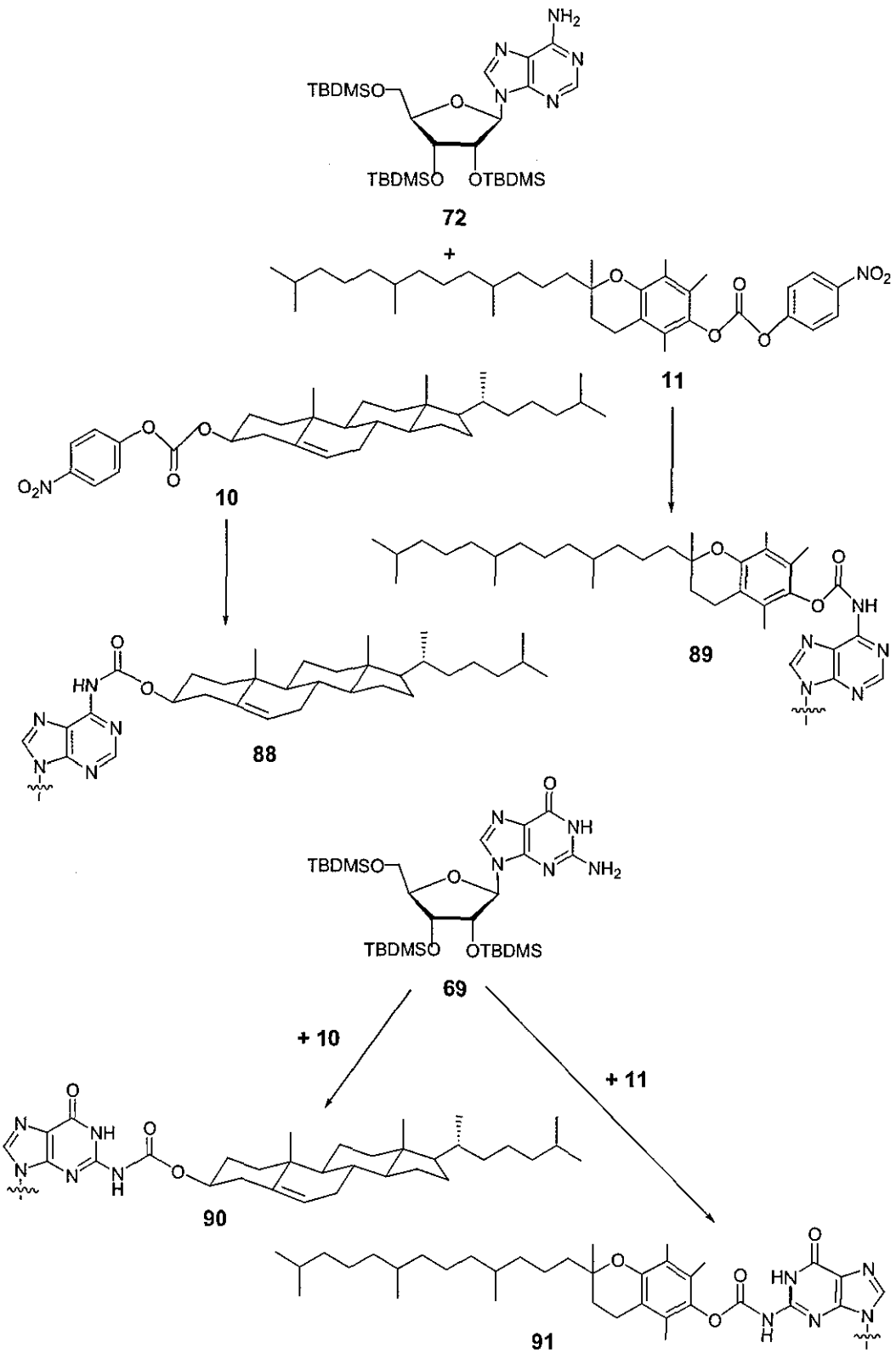
(A) Lipophilic N-carbamate analogues of nucleosides

Initially the modifications were performed at the exocyclic amino group of the nucleobases of adenosine and guanosine. This was done in order to form carbamate analogues of the nucleosides as they have shown to have excellent resistance to both chemical and enzymatic degradation and *nuclease hydrolysis*.

The 2',3',5'-TBDMS-protected guanosine **69** and adenosine **72** were reacted with both the cholesterol **10** and tocopherol **11** carbonate linkers, as shown in **Scheme 58** below. The reactions were first carried out using 1 equivalent of each of the reactants, 1.1 equivalents of triethylamine as the base, dichloromethane as the solvent and heating under reflux. This did not facilitate the formation of the amide bond and starting material was recovered unchanged.

It had been reported that 1,4-dioxane is an effective solvent for the formation of amide bonds in satisfactory yields. This solvent was used but the reaction was unsuccessful and no other identifiable product was isolated except for starting material.

It was then decided to investigate other high boiling solvents such as DMF and THF to try and increase the solubility factor and force the reaction by heating at higher temperatures, However, disappointingly under the new conditions used, the yields afforded were low, between 26-35%. In most cases other unidentifiable products were recovered along with starting material while, only a small percentage of nucleobases underwent modification to form carbamate bond linkages. This may be due to triethylamine not being strong enough to deprotonate the amine to allow for the displacement of the 4-nitrophenyl group to occur.


TBDMSO
TBDMSO
OTBDMS
72
+
11
10
89
88
TBDMSO
TBDMSO
OTBDMS
69
+ 10
+ 11
90
91

Scheme 58

Repetition of the reactions using pyridine in the place of triethylamine and refluxing in dichlromethane and 1,4-dioxane overnight, did not facilitate the synthesis of the desired lipophilic conjugates. When DMF and THF were employed as solvents in refluxing for 24h, the N-cholesteryl and tocopheryl carbamate derivatives of 2',3',5'-protected adenosine and guanosine **88-91** were obtained in poor yields with the recovery of starting material.

Table 8 below shows the outcome of the above conjugation reactions. This may infer that modification at the amino base of nucleosides does not work effectively under the conditions described, as the bases employed may not be strong enough to generate the amide bond.

Table 8

Base	Solvent	Reaction time	Yield (%) 88...	89...	90...	91
Et_3N (1.1eq)	DCM	reflux 6h	no react.	no react.	no react.	no react.
	1,4 dioxane	reflux 6h	No react.	No react.	No react.	No react.
	DMF	reflux 12h	31	35	29	33
	THF	reflux 12h	31	36	30	35
Pyridine (1.1eq)	DCM	reflux overnight	no react	no react	no react	no react
	1,4 dioxane	reflux overnight	no react	no react	no react	no react
	DMF	reflux 24h	31	38	32	35
	THF	reflux 24h	33	37	32	35

It was then decided to increase the equivalents of the protected nucleoside and base to 1.1 and 1.5, respectively in an effort to force the reaction to completion.

Anhydrous pyridine was employed as the base, THF and DMF as the solvents and the reactants were refluxed overnight, as these systems gave better results. The protected nucleosides **72** and **69** had under gone modification to give the N-carbamate analogues **88-91** in improved yields, between 40-47%. However characterization showed that carbonate linker and was still present as starting material. Increase of the equivalents to 1.2 only led to undesirable reactions and other products were isolated that could be characterized.

When pyridine was used both as solvent and base and heated under reflux conditions for 24h, the reaction led to the consumption of the starting material and formation of the desired products. In all cases the products were isolated after separation and purification by flash column chromatography using a 20:1 mixture of chloroform and methanol. ^{1}H-NMR spectroscopic analysis confirmed the formation of amide as evidence of a broad singlet at 7.68-7.73 ppm for CONH while IR analysis showed two peaks at 1674-1697 cm^{-1} indicating the presence of a secondary amide.

The 2',3',5'-TBDMS-N-carbamate tocopheryl conjugates of adenosine **89** and guanosine **91** were afforded as off yellow products in 64 and 66% yield, respectively, while the 2',3',5'-TBDMS-N-carbamate cholesteryl of adenosine **88** and guanosine **90** were furnished as off-white products in 60 and 61% yield, respectively. A summary of the results obtained is shown in **Table 9** below.

Table 9

Base	Solvent	Reaction Time	Yield (%)			
			88	89	90	91
Pyridine (1.5eq)	DMF	reflux overnight	44	43	46	40
	THF	reflux overnight	45	43	47	40
Pyridine[a]	Pyridine[a]	reflux 24h	60	64	61	66

a: Conditions were 4 ml of pyridine per 1mmol of reactant

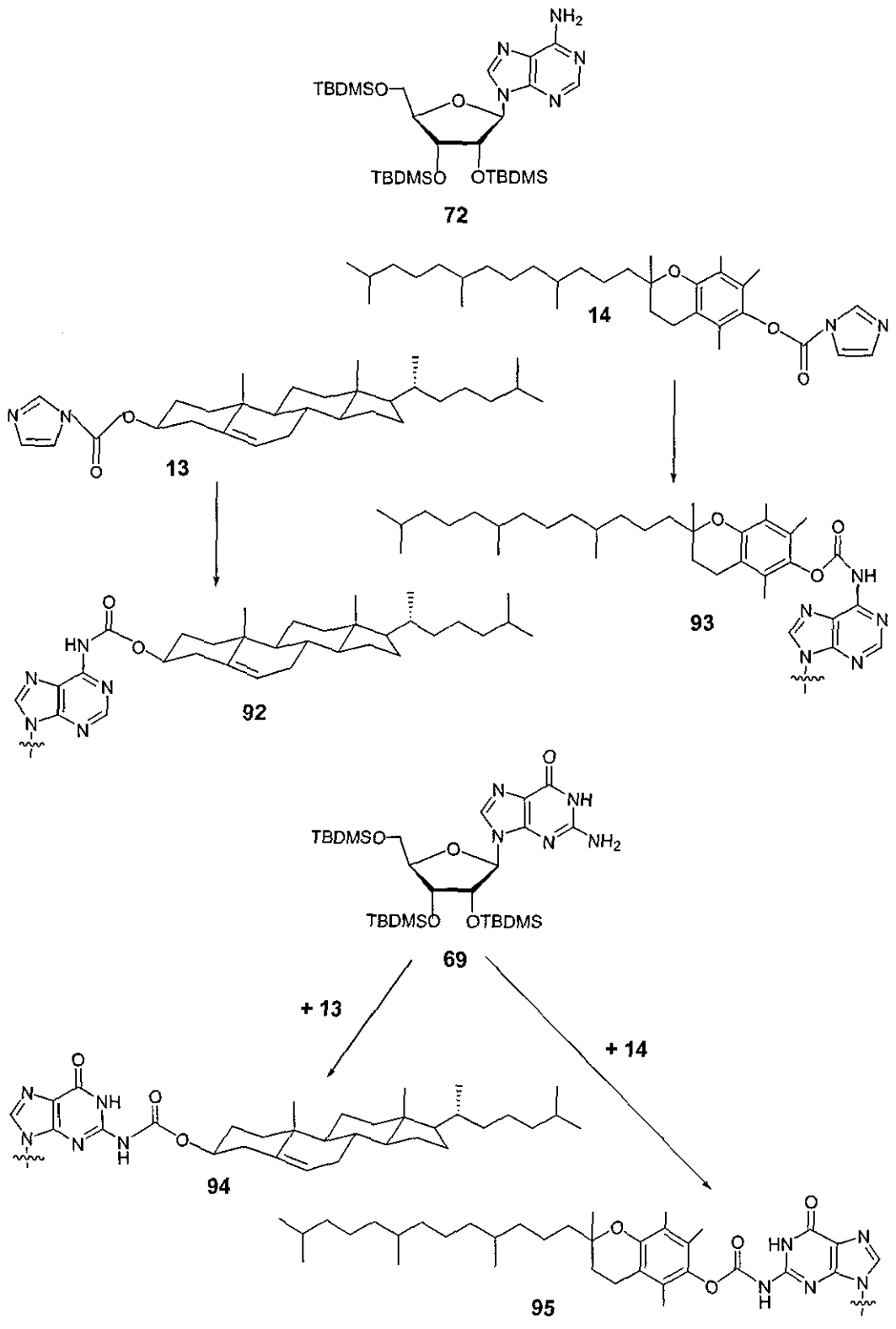
The successful development of the procedure using pyridine as base and solvent prompted an investigation into extending the amino modification of 2'-3',5'-TBDMS-protected guanosine **69** and adenosine **72** using the synthesized carbonylimidazolide linkers of cholesterol **13** and tocopherol **14.** The reactions are shown in **Scheme 59** below. However, disappointingly, this approach was not very successful and it led to undesirable reactions, as conjugates formed with the N-H bonds present in the protected nucleosides were isolated. The pyridine could be removing the electron density from the imidazole ring making it less susceptible to electrophilic attack.

It was decided to repeat the reactions under acidic conditions so that the imidazole can be protonated and therefore converted into a better leaving group. Thus small amounts of concentrated hydrochloric acid were added and the reaction was promoted but it was found that the yields of the conjugates varied greatly with seemingly small changes in the amount of acid incorporated. The results are shown in **Table 10** below.

Table 10

Acid (ml)	Solvent	Reaction Time	Yield (%) 92....	93...	94....	95
Conc HCl (2ml)	THF	reflux 24h	53	55	54	58
Conc HCl (3ml)	THF	reflux 24h	43	47	44	49
Acetic acid (5ml)	THF	15h at r.t	63	68	66	70

When acetic acid that has a lower pka value was used, instead hydrochloric acid, the protected nucleosides **72** and **69** under went modification to give the N-carbamate cholesteryl derivatives **92** and **94** as off-white products in 63 and 66% yield respectively. The N-carbamate tocopheryl conjugates of the corresponding nucleosides were furnished as off-yellow products in 68 and 70% yield as shown in **Table 10** above.


NH2
TBDMSO
TBDMSO
OTBDMS
72
14
13
93
92
TBDMSO
TBDMSO
OTBDMS
69
+ 13
+ 14
94
95

Scheme 59

The crude products were purified as in the case of the lipophilic analogues deriving form 4-nitrophenyl carbonate linkers. The structures of the purified compounds were confirmed by spectroscopic and elemental analysis and were found to identical to the previously synthesized conjugates **88-91**.

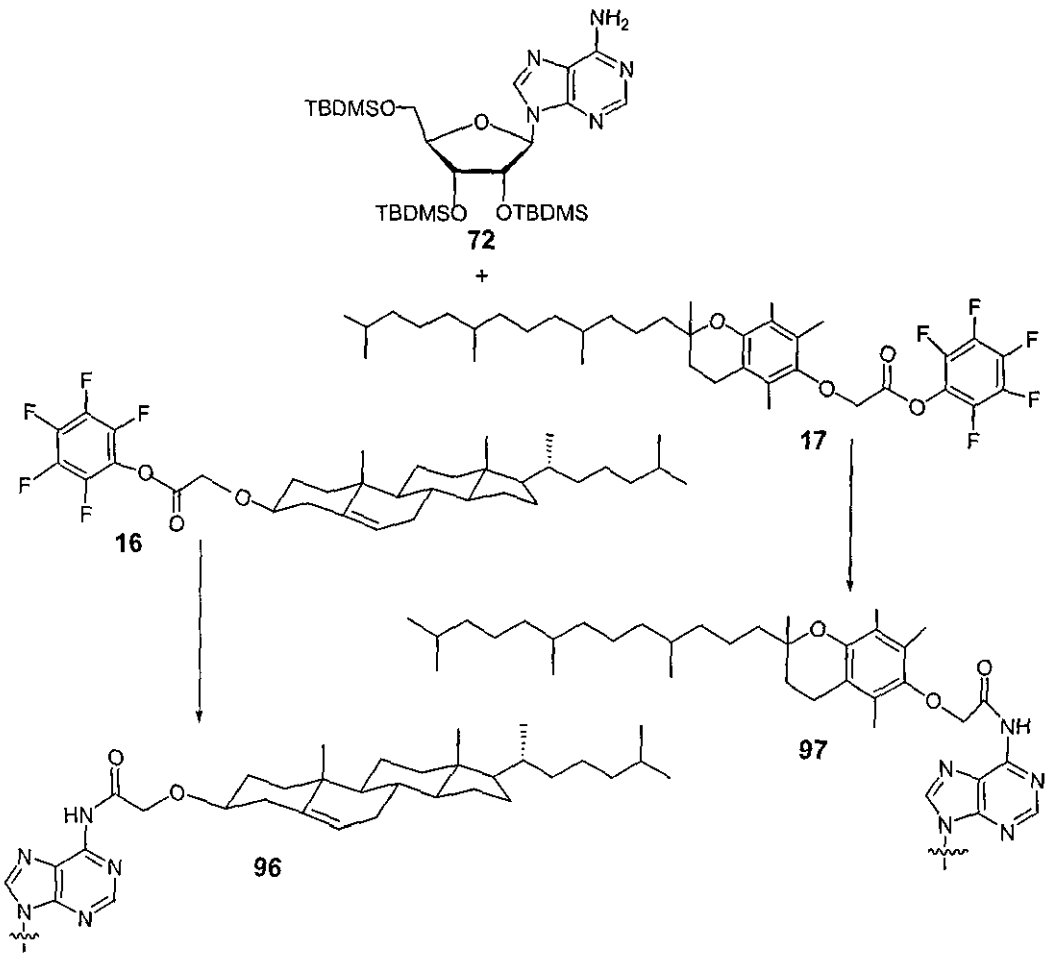
Carbonylimidazolide lipophilic derivatives in anhydrous pyridine have shown a high reactivity towards N-H bonds present in nucleoside building units. As a result several side reactions have been observed including degradation of guanosine and adenosine derivatives. In the presence of acid though carbonylimidazolide carbamate linkers managed to modify the exocyclic amino groups and form the desired carbamate conjugates in very good yields. A comparison of the results acquired using basic and acidic conditions showed that both of the methods were successful, as the yields obtained were similar.

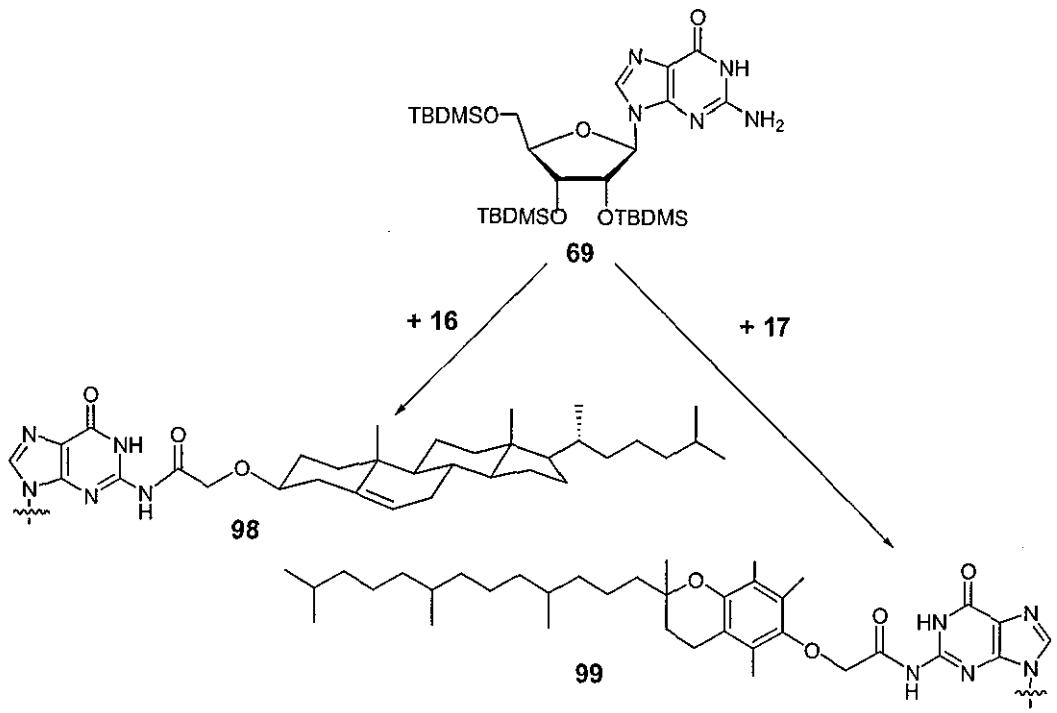
On the other hand, lipophilic derivatives containing 4-nitrophenyl-carbonate linkage are capable to transform, in aqueous pyridine, the free exo-NH_2 groups of adenosine and guanosine residues to the corresponding lipophilic carbamate nucleoside analogues, which were afforded in good yields.

(B) Lipophilic N-acetamide analogues of nucleosides

Next in our investigation of the modification on the exocyclic amino group of the nucleosides was the formation of acetamide-linked analogues of the corresponding nucleotides. Acetamido-linked analogues are attractive due to their stability over a wide pH range and their enzymatic degradation. Furthermore, they possess linkages that are achiral, thus avoiding the difficult separation of diastereomers during their synthesis and purification. The purpose of this coupling is to form a different kind of amide linkage containing a more stable CH_2 group that can stand up the conditions of DNA

In this case, the pentaflurorophenyl ester linkers of cholesterol **16** and tocopherol **17** were employed and reacted as previously with 2',3',5'-TBDMS-protected guanosine **69** and adenosine **72**. The reactions are shown in **Scheme 60** below.





Scheme 60

In order to deprotonate the amino group of the nucleobases stronger bases with high nucleophilicity, such as lithium bis (trimethyl silyl) amide and *t*-butyl lithium, were utilized. After treating 1.1 equivalent of the protected nucleosides with the same equivalent of bases at -78 ^{0}C in THF, the reactions were carried out at 0 ^{0}C for 1h and then refluxing for 30 h. However, this approach was not successful as it gave very poor yields (15-23%) of the desired products. Various attempts were also made using an increased 1.2 equivalent of the bases. Disappointingly did not give the expected results, as the yields did not improve greatly (19-29%) even after refluxing for 30h.

When pyridine was used as both solvent and activator after refluxing for 30h, the yields were improved (38-44%) but still they were not satisfactory. In some cases other unidentifiable products were recovered along with desired acetamide conjugates. The results obtained are summarized in **Table 11** below.

Table 11

Base	Solvent	Reaction Time	Yield (%) 96	97	98	99
Lithium bis (trimethyl silyl) amide (1.1 eq)	THF	0° C 1h reflux 30h	17	18	20	15
***n*-butyl lithium (1.1 eq)**	THF	0° C 1h reflux 30h	23	22	26	23
Lithium bis (trimethyl silyl) amide (1.2eq)	THF	0° C 1h reflux 30h	22	21	22	19
***t*-butyl lithium (1.2 eq)**	THF	0° C 1h reflux 30h	22	27	29	25
Pyridine[a]	Pyridine[a]	reflux 30h	38	42	44	39

a: *Conditions were 4 ml of pyridine per 1mmol of reactant*

The next step in our investigation of the acetamide conjugate synthesis was the employment of acidic conditions. For that reason acetic acid was used as previously but reactions didn't proceed as expected as the yields only improved slightly (43-50%). However, when *p*-toluenesulfonic acid was used as the reagent of choice, it adequately protonated the amino group of the protected nucleosides in order for the conjugation to take place.

The coupling of the pentafluorophenyl linkers with the tosylate of **69** and **72** was effected after refluxing overnight in DMF in the presence of diisopropylamine. It occurred selectively at the amine terminus giving the N-acetamide-linked lipophilic analogues **96-99** in very good yields, between 68-72%. Purification was accomplished by flash column chromatography eluting initially with 1% MeOH / 99% $CHCl_3$ and then with 10% MeOH / 90% $CHCl_3$, to provide the N-acetamide cholesteryl and tocopheryl derivatives as off-white and off-yellow products, respectively. **Table 12** shows the outcome of these reactions.

The synthesized compounds were assigned to be the desired N-acetamides by the presence in ^{1}H-NMR spectrum of a broad singlet at 7.68-7.75 ppm for CONH and a singlet at 2.75-2.77 ppm corresponding to the two hydrogens of the CH_2-CONH. Also evidence of two peaks at 1686-1695 cm^{-1} in Infa Red spectral analysis indicated the formation of a secondary amide.

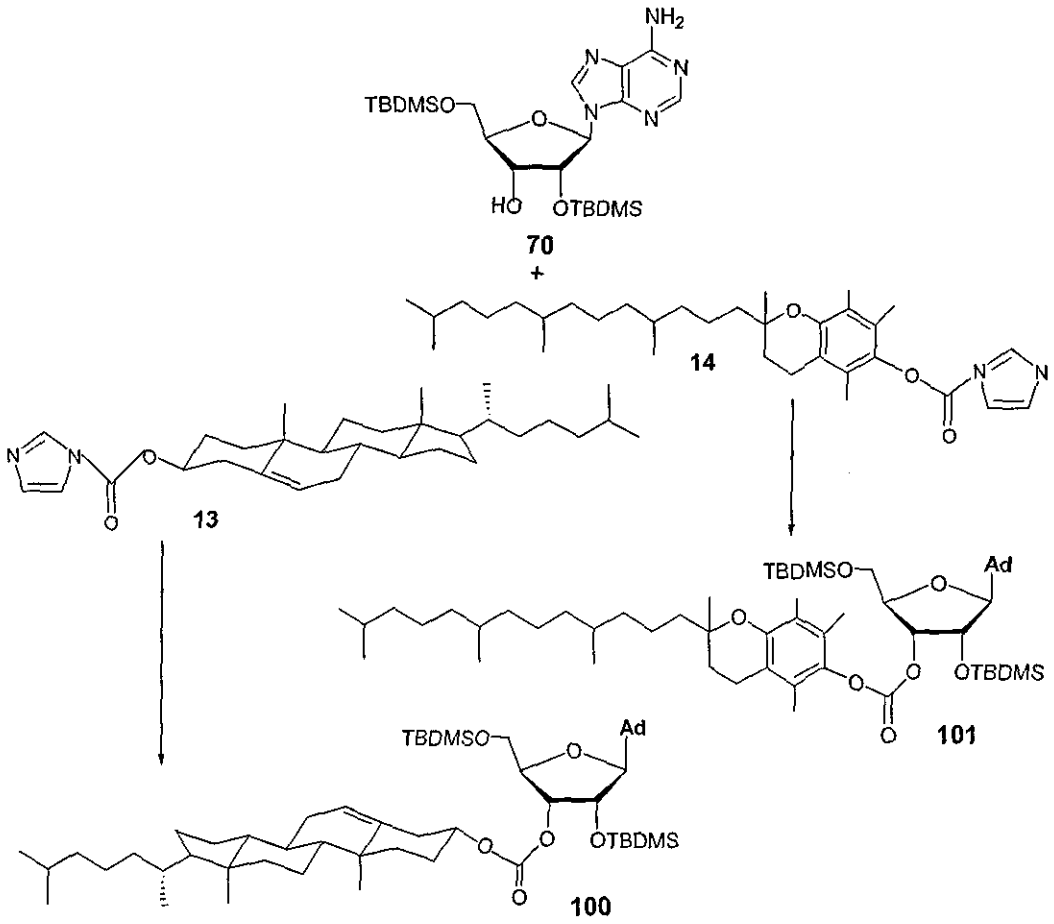
Table 12

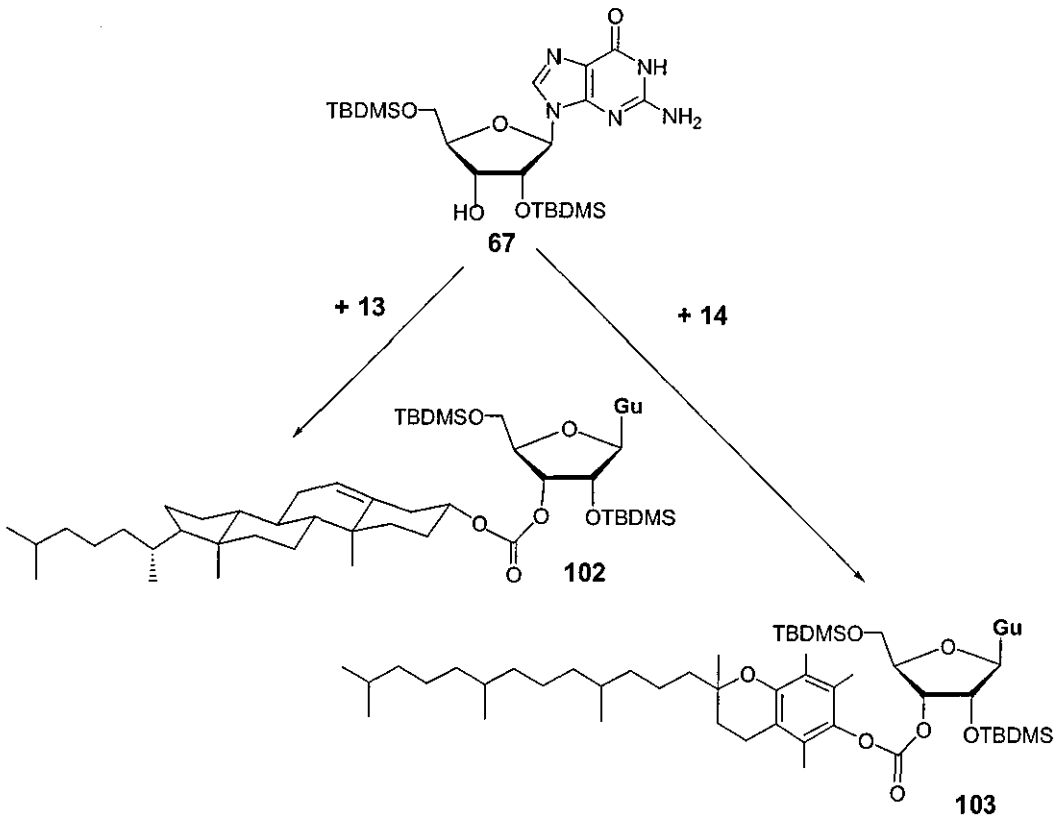
Acid	Solvent	Reaction Time	Yield (%) 96	97	98	99
Acetic acid (1.1 eq)	MeOH: THF (5:1)	15h at r.t	44	48	50	43
***p*-toluenesulfonic acid**	DMF	0° C 1h reflux 24h	68	70	69	72

(C) Lipophilic O-carbonate analogues of nucleosides

Modifications can also be carried out at the 3'-terminus of the nucleosides and it has been shown that incorporations at the 3'-end of a CpG dinucleotide potentiated the immunostimulatory activity and increases its stability against nucleases.

Nucleotide analogues containing carboxymethyl linkages at the 3'-end have proved to have two disadvantages: the relative instability of the linkage under physiological conditions and the low solubility of the compounds that makes it difficult to test their biological activity. In view of that, our next step was the synthesis of analogues in which the 3'-OH of the nucleoside is joined to the lipophilic compounds by a carbonate linkage, as such compounds would not suffer from these disadvantages. In this case, the 2',5'-O-disilylated derivatives of guanosine **67** and adenosine **70** were reacted with the carbamate linkers of cholesterol **13** and tocopherol **14**. The reactions are shown in **Scheme 61** below.





Scheme 61

The formation of the lipophilic-carbonate analogues relies on the activation of the nucleoside hydroxyl functions using basic conditions. For this reason the strong bases, lithium bis (trimethyl silyl) amide and *t*-butyl lithium were utilized. 1 Equivalent of the 3'-OH-disilylated nucleosides **86** and **69** were treated with 1 equivalent of the bases at -78 ^{0}C in THF. The formed alkoxide was subsequent exposed to 1 equivalent of the carbonylimidazolide linkers and the reactions were carried out at 0 ^{0}C for 1h and then refluxing for 12 h.

The free hydroxyls of the 2',3'-protected adenosine underwent smooth modification to provide the O-lipophilic carbonates in very good yields as shown in **Table 13** below. The 3'-O-cholesteryl carbonate derivatives of adenosine **100** and guanosine **102** were obtained in 59-64 and 59-65% yield respectively, while the 5'-O-tocopheryl analogues of the corresponding nucleosides were attained in 63-67 and 62-69% yield

t-butyl lithium gave better yields than lithium bis (trimethyl silyl) amide as it has with stronger nucleophilicity. The synthesized compounds were used without further purification as attempts to purify them, resulted in decomposition due to the instability of the active carbonates to chromatography

Table 13

Base	Solvent	Reaction time	Yield (%) 100	101	102	103
Lithium bis (trimethyl silyl) amide (1eq)	THF	0° C 1h reflux 12h	59	63	59	62
***t*-butyl lithium (1eq)**	THF	0° C 1h reflux 12h	64	67	65	69
Pyridine[a]	Pyridine	reflux 30h	30	35	32	36
Pyridine[b]	1.4 dioxane-pyridine	reflux 12h	59	64	62	65

a: Conditions were 4 ml of pyridine per 1mmol of reactant

b: Conditions were and 4.5 ml of dioxane:pydine (8:1) per 1mmol of reactant

The next step in our investigation was employment of pyridine, as for the carbamate analogues. Various attempts to prepare the carbonate analogues in anhydrous pyridine resulted in slow reactions and poor yields (30-39%) owing to the decomposition of the carbonates in this solvent. It was found though that when a mixture of 8:1 of 1.4 dioxane: pyridine was used as the solvent and activator and 1.1 equivalents of 3'-OH-disilylated nucleosides were treated with 1 equivalent of carbonylimidazolide linkers, the reactions proceed at a much faster rate. The desired carbonate conjugates were obtained in 59-65% yield, without detectable decomposition. No effort was made to purify these compounds.

The preparation of these conjugates demonstrates the utility of the synthetic sequences followed for the preparation of lipophilic carbamate and carbonate conjugates without the need for a protecting group on the base.

(D) Deprotection of the lipophilic-nucleosides conjugates

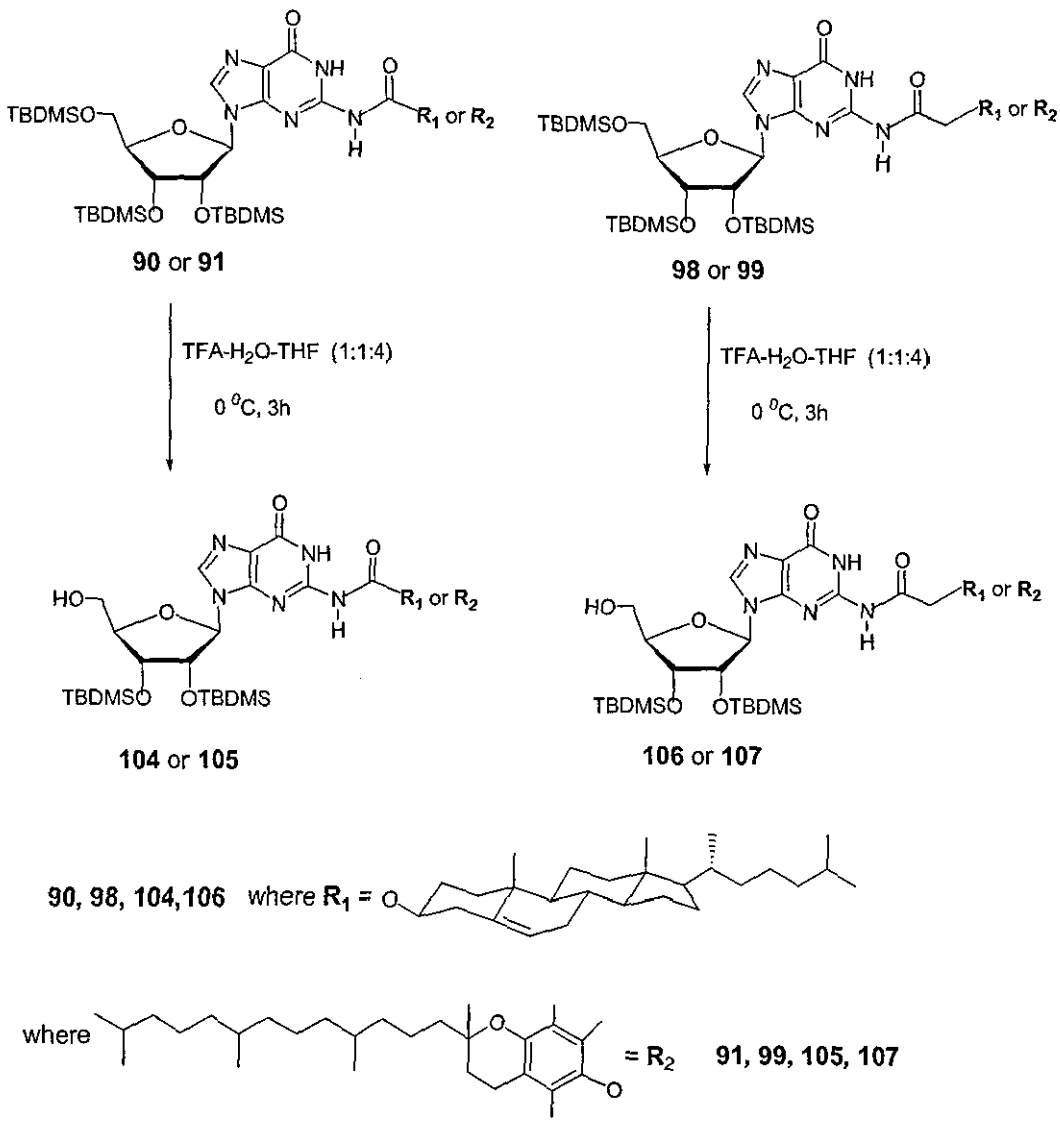
The previously synthesized derivatives of guanosine containing the novel carbamate and acetamide lipophilic linkages can be used as useful building blocks for the formation of CpG dinucleotides. Future employment of those derivatives though, requires for the 5'-terminus to be accessible for coupling with the N-free-3'-phosphoramidites of cytidine. For that reason, the N-carbamate **90-91** and the N-acetamide **98-99** lipophilic analogues of guanosine were engaged in selective cleavage of the 5'-O-TBDMS group.

Previous efforts to selectively remove the 5'-protective group using 80% acetic acid either at 20 or 100 ^{0}C resulted in side reactions and the production of by-products. In turn the TFA-H_2O-THF (1 :1: 4) system, which was used before to selectively deprotected the 5'-O-TBDMS group with satisfactory results, was employed as the desilylating agent. The compounds **90-91** and **98-99** were treated with the above system for 3h at 0 ^{0}C, as shown in **Scheme 62** below, to furnish the 2',3'-O-TBDMS carbamate and acetamide conjugates of guanosine in very good yields. The results are summarized in **Table 14.**

Table 14

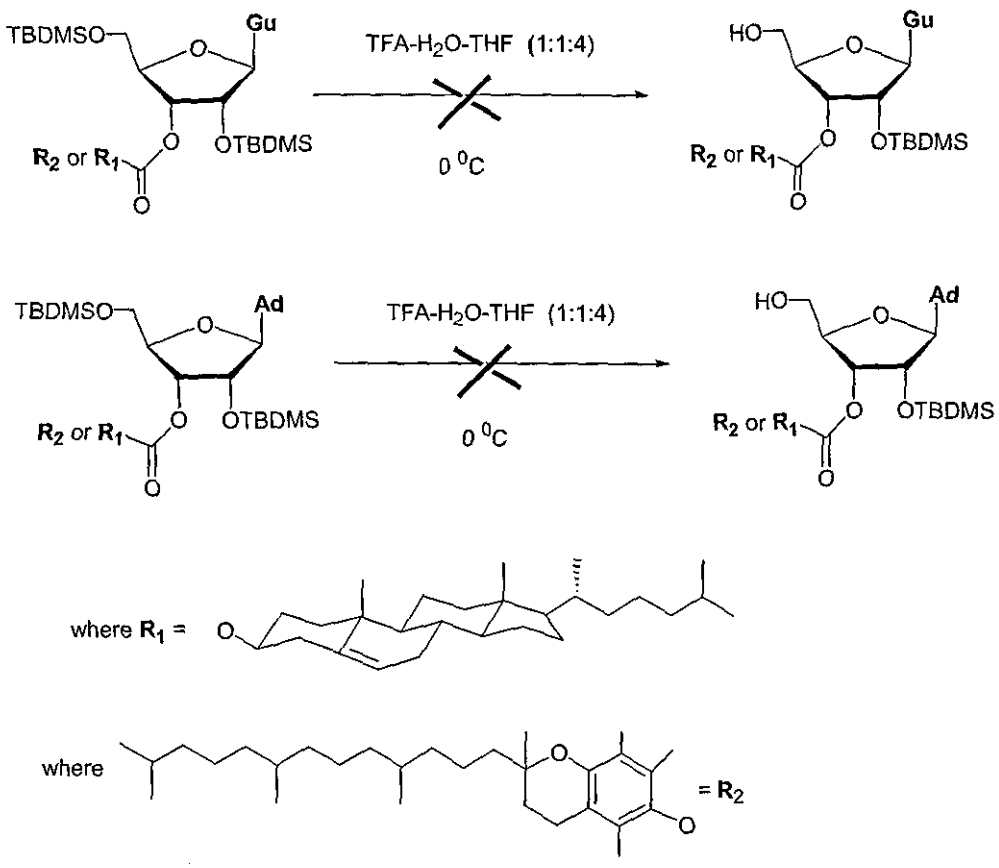
Nucleoside	Time (h)	Yield (%)
90	3	**104** (72%)
91	3	**105** (77%)
98	3	**106** (72%)
99	3	**107** (75%)

Both the carbamate and acetamide linkages were proved to be stable to the acetic conditions employed and the desired 5'-OH free analogues of guanosine were furnished without detectable degradation of the linkages.



Scheme 62

The lipophilic O-carbonate conjugates of adenosine **100-101** and guanosine **102-103** were also employed for selective desilylation. In a similar set of reactions various attempts were made to cleave the 5'-TBDMS group using the TFA-H_2O-THF system as shown in **Scheme 63** below. Disappointingly, these experiments were not successful and starting material was recovered. Removal of the protecting group utilizing hydrolytic conditions is a difficult reaction in the presence of a carbonate. The O-carbonate analogues proved to be unstable and were hydrolysed to give 2',5'-O-disilylated nucleosides.



Scheme 63

Part of our investigation was also to test the synthesized modified lipophilic nucleotides to perceive whether they are taken up into the cell membrane more efficiently. In order for that to take place the compounds need to be released of their protecting groups.

The results obtained in our desilylation experiments showed that although treatment with 80% aqueous acetic acid at 100 ^{0}C after 30-70 min or room temperature after 24-28h resulted in the selective 5'-desilylation, it also produced as a side reaction the fully deprotected nucleosides (see **Table 6**).

It was anticipated that by prolonging the reaction time complete deprotection would be achieved. In the case of conjugates aqueous conditions were best be avoided so acetic acid was employed in methanol.

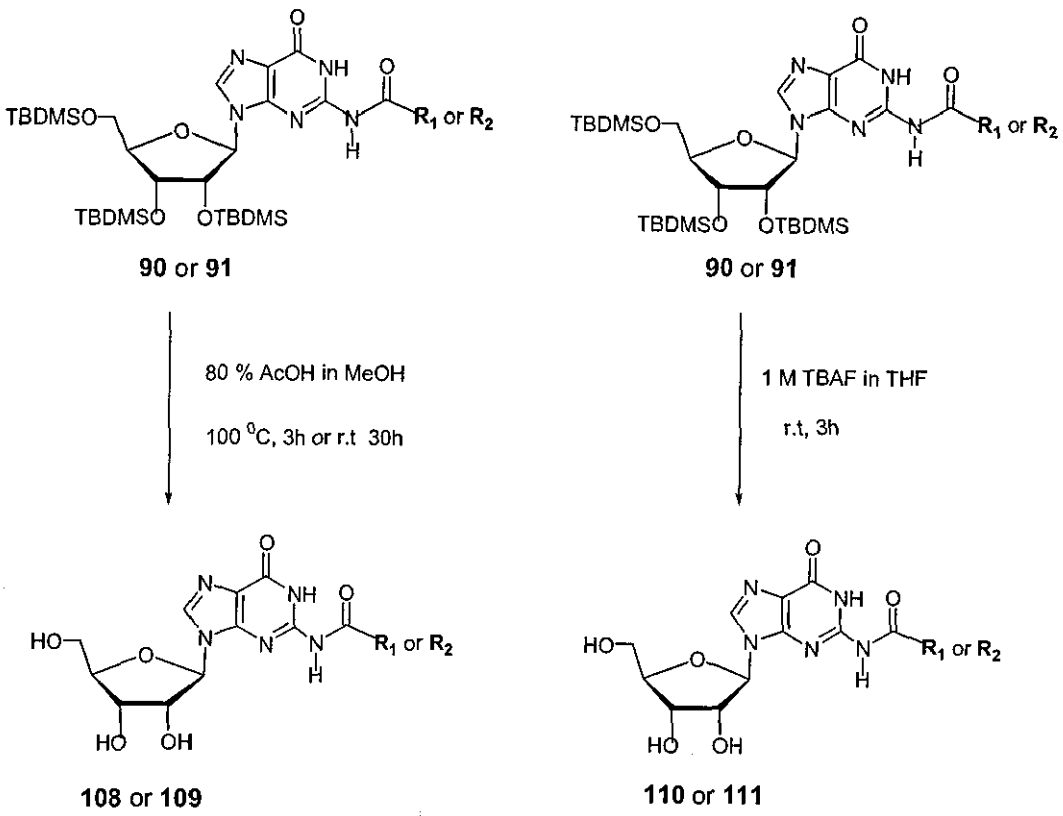
The N-carbamate lipophilic analogues **90-91** were subjected to 80% acetic acid in methanol and after 3-4h at 100 ^{0}C or 30-32h at room temperature, complete removal of the *tert*-butyldimethyl silyl groups protective groups was effected. The reactions are shown in **Scheme 64** below. The products were isolated in over 68% yield as shown in **Table 15** below. Elemental and spectroscopic analysis confirmed the structure of the products.

The use of tetrabutylammonium fluoride (TBAF) as a desilylating agent was also explored. The carbamate conjugates **90-91** were treated with 5 equivalents of TBAF in THF, as shown in **Scheme 64.** The cleavage of the protecting groups was rapid and proceeded smoothly, giving the fully deprotected conjugates after 3h at room temperature in very good yields, as shown in **Table 15** below.

Table 15

Nucleoside	Deprotecting reagent	Temperature (^{0}C)	Time (h)	Yield (%)
90	80% AcOH	22	32	**108** (70%)
90	"	100	4	**108** (67%)
91	"	22	30	**109** (74%)
91	"	100	3	**109** (68%)
90	1 M TBAF	R.T	3	**110** (76%)
91	"	R.T	3	**111** (80%)

As it can be seen from the tables the yields furnished when TBAF was employed (76-80%.) were improved in comparison to the ones obtained with 80% acetic acid. This can only be explained in terms of TBAF being a stronger acid resulting in successfully removing the silyl groups. In agreement with expectations, the nucleoside carbamate linkages were very stable to hydrolytic conditions and they appeared to have great pH stability.
Desilylation employing 80% acetic acid or tetrabutylammonium fluoride occurred without detectable degradation of the carbamate linkage.



90, 108, 110 where R_1 =

where = R_2 **91, 109, 111**

Scheme 64

(D) Alternative methods for the synthesis of lipophilic-nucleoside conjugates

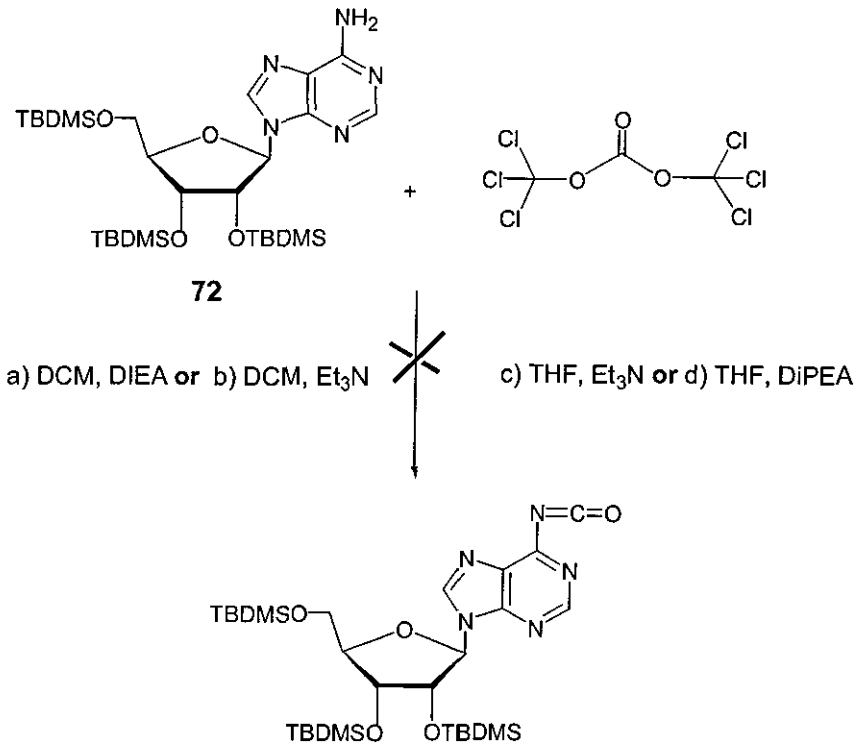
Due to the harsh conditions used for the formation of the lipophilic conjugates of nucleosides, an interest was drawn towards developing a fast and efficient method for the lipophilic modification of the nucleosides. It was decided to study different routes to try and improve the convenience of the procedures and explore if these syntheses could be effected under milder conditions.

(I) Through the formation of an isocyanate

Originally efforts were put into trying to alter the reactivity of the free amine of the nucleobases. The transformation of the amino group to the isocyanate was proposed, as it would formulate it a more reactive group that might allow easier generation of the lipophilic analogues.

Triphosgene [bis(trichloromethyl carbonate][310] is known as an effective reagent for the preparation of isocyanates when reacted with amines in the presence of base. As a stable crystalline solid it has been used as a safe replacement to phosgene which a highly toxic gaseous chemical that form drastic conditions. Thus it was thought to investigate its use for the modification of the amino groups of the nucleobases.

Various attempts were made to prepare the isocyanate by reacting 3 equivalents of the 2,3,5'-O-TBDMS protected adenosine with 1 equivalent of triphosgene in the presence of a tertiary base diisopropylethylamine, in dichloromethane (**Scheme 65**). At the end of the reaction a compound was isolated along with starting material. Infa red analysis of this compound didn't show a peak at 2250 cm^{-1} indicating that isocyanate was not formed. Several attempts were made varying the bases (triethylamine or diisopropylamine) in DCM or THF. However, disappointingly under the new conditions used the experiments were unsuccessful, as starting material was recovered and none of the desired isocyanate was formed.



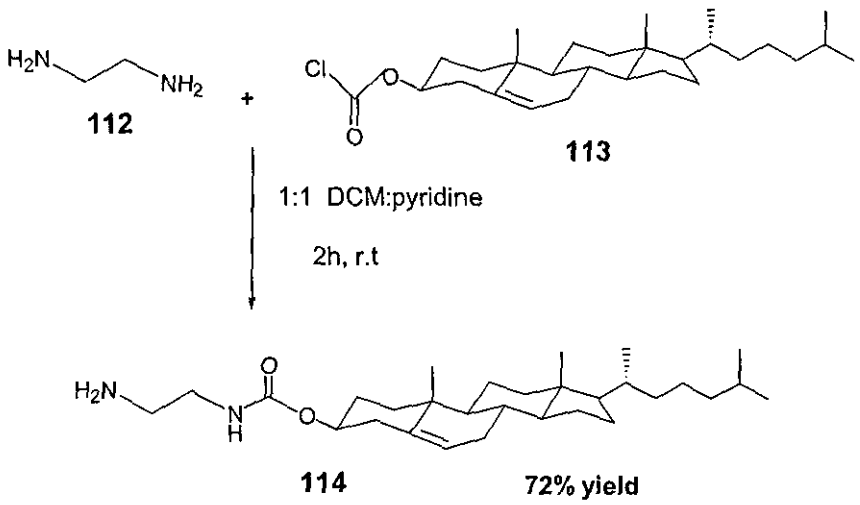
`Scheme 65

(II) Through an acid chloride

It was also proposed to investigate different methods for the modification of the amino nucleobases using the lipophilic compounds. Carbamate formation can be attained through the reaction of an acid chloride with the free amino group. The acid chloride can then be directly reacted with the amine of adenosine and displacement of chlorine atom will provide the corresponding amide. Thus, it was decided to investigate the synthesis of the cholesteryl-carbamate of globally protected adenosine through the commercially available chloroformate of cholesterol.

In order to test this type of reaction, ethylenediamine **112** (shown in Scheme **66)** was used as the model compound of an amino group. The acid chloride was used as the precursor and the nucleophilic free amine attacks and displaces the electrophile (chloride) whilst pyridine removes one of the protons to generate the amide bond.

Cholesteryl chloroformate **113** was added dropwise to a 1:1 solution of ethylenediamine in dichloromethane: pyridine and after 2h at room temperature the cholesteryl carbamate of ethylenediamine was isolated. Recrystallization from hexane afforded the 2-(chlolesteryloxycarbonyl) ethylamine **114** as crystals in 72%yield. The reaction is shown in **Scheme 65** below.

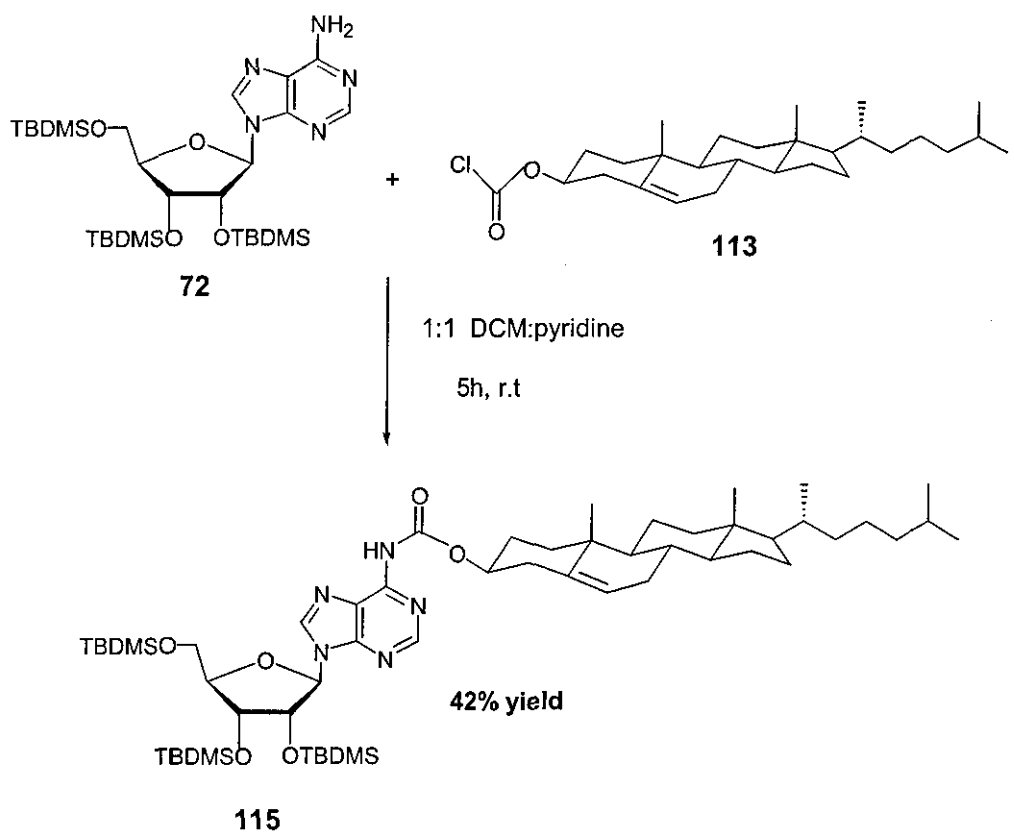


Scheme 66

The success of this reaction prompted us into applying the method for the modification of the amino base of 2',3',5,-O-TBDMS protected adenosine **72.** In a similar reaction as above **72** was treated with cholesteryl chloroformate **113** (as shown in **Scheme 67** below) and after 5h at room temperature a crude product was isolated. Separation and purification by flash column chromatography using a 1:20 mixture of chloroform: methanol as the eluent afforded an off-white solid in 42% yield.

The product was assigned to be the cholesteryl N-carbamate of globally protected adenosine **115** by evidence of the carbonyl peaks at 1684-1697 cm^{-1} in infa red spectra indicating the presence of a secondary amide. ^{1}H-NMR spectroscopic analysis confirmed the formation of amide as evidence of a broad singlet at 7.64-7.76 ppm for CONH.

This infers that the desired amide is formed under the conditions described but the yield obtained was low in comparison to the ones furnished using pyridine as both the solvent and base (60%) and acetic acid (63%) (see **Tables 9 and 10**). Another drawback of this approach was the employment of cholesteryl chloroformate, which is an expensive starting material in contrast with the already synthesized carbonate and carbamate linkers used in the conventional methods above.



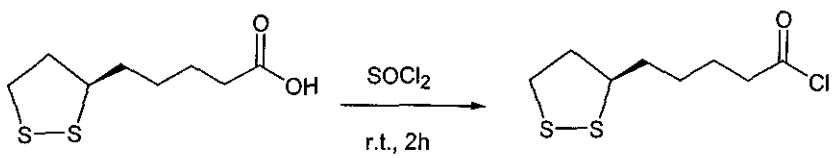
Scheme 67

(III) Through the formation of the acid chloride of thioctic acid

Part of our investigation was also the synthesis of nucleoside conjugates employing thioctic acid **3** as the lipophilic compound. As thioctic acid holds a carboxylic acid moiety the reasonable method would be through the formation of an acid chloride. Amide bond formation can be achieved through the synthesis of an acid chloride that can be readily converted to substituted amides.

The preparation of the acid chloride was performed by reaction with thionyl chloride as shown in **Scheme 68**, but the gradual formation of a black solution (within 10 min of stirring at room temperature) drew doubts concerning the formation of the acid chloride. For this reason, an IR spectrum was run showing a peak at 1796 cm^{-1} while the ^{13}C- spectrum showed a signal at 173 ppm. Both of the values are evidence of formation of an acid chloride. The black colour could be due to the fact that the thionyl chloride is attacking the sulfur atoms of thioctic acid leading to formation of decomposition products.

The acid chloride **116** was afforded after 2h at room temperature, in 72% yield as a black oil that and was used without further purification.



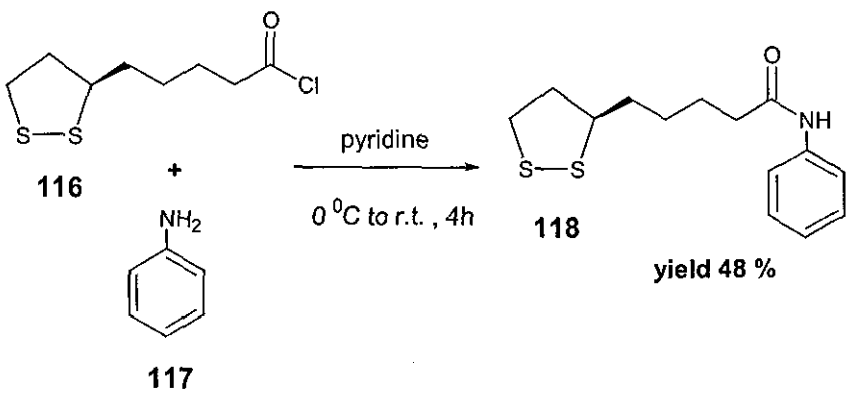
3 **116** **72% yield**

Scheme 68

In the next step the reaction of the synthesized acid chloride **116** with the 2',3',5'-O-TBDMS-protected adenosine **72** (shown in **Scheme 70** below) was performed in DMF using a 1:1 ratio of the reactants. Disappointingly the reaction failed to give the desired product. A mixture containing starting material and an unidentifiable product was formed and an attempt to separate it was unsuccessful.

The failure of this reaction can be addressed to the poor nucleophilicity of this aromatic amine and the poor quality of the acid chloride employed.

Due to the fact that there was no evidence that the amide had formed in the above reaction, a sample coupling reaction was performed using a simpler amine, aniline. A reaction of the acid chloride **116** with aniline **117** was carried out in pyridine at 0 ^{0}C and then warmed up to ambient temperature as shown in **Scheme 69**. The crude amide of aniline **118** was afforded as black oil in 48% yield.

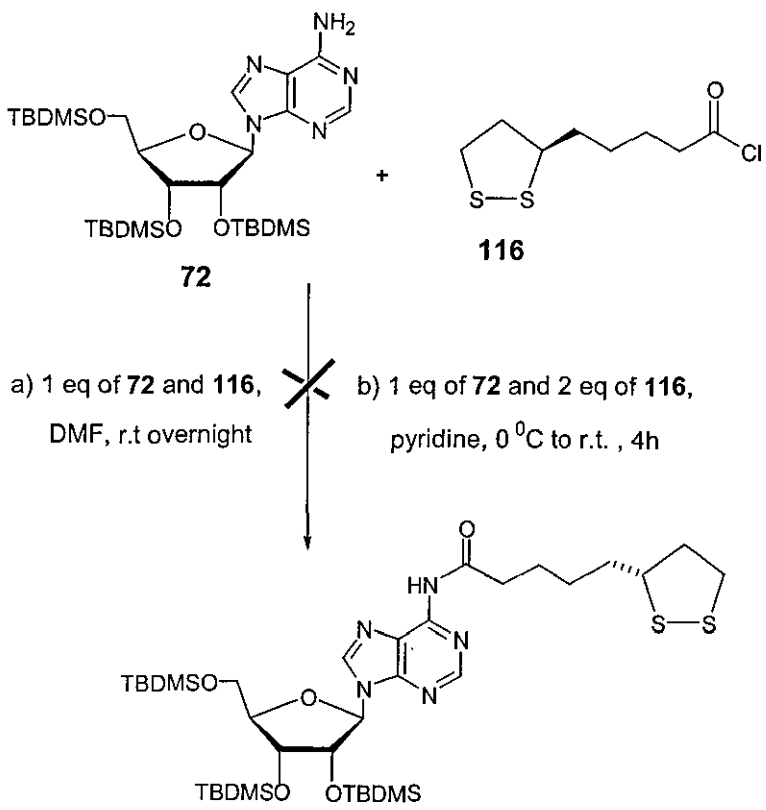


Scheme 69

The ^{1}H-NMR spectrum of **118** showed that the crude product also contained some unreacted aniline and since the reaction was performed using 1 equivalent of each of the reactants an excess of the acid chloride should be used to drive the reaction to completion. The unreacted aniline was separated from the crude product by flash column chromatography using a 1:1 mixture of petroleum ether and ethyl acetate and **118** was isolated in 48% yield. This experiment confirmed that the failed attempt of coupling the thioctic chloride **116** with the NH_2 of the TBDMS-protected adenosine **72** is basically due to the poor nucleophilicity of the aromatic amine of the adenine base.

Nevertheless, a further attempt to form the thioctic-amide conjugate of the protected adenosine was carried out in the same successful conditions used above with aniline.

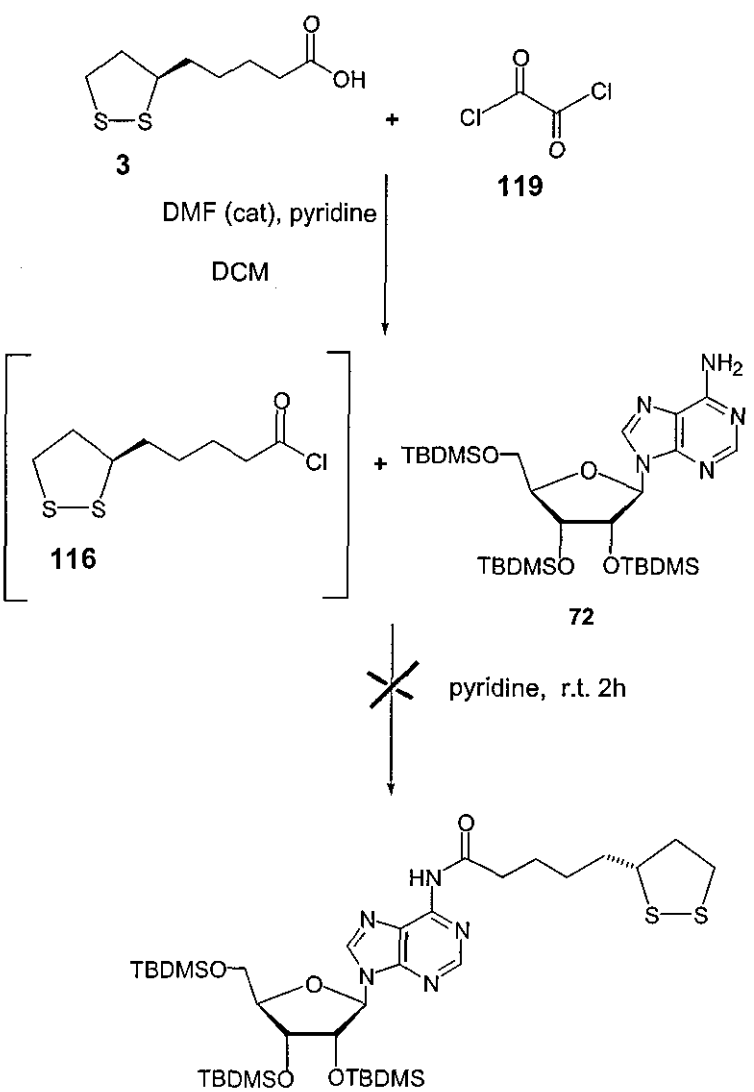
Pyridine was used as solvent but in this case the ratio of adenosine: chloride of thioctic acid was increased to 1:2 as shown in **Scheme 70** below. However, the desired thioctic-adenosine analogue did not form and starting material was recovered.



Scheme 70

Since the above reactions did not afford the desired conjugate an alternative procedure was followed based on a reported procedure[311] for the conversion of carboxylic acid to an amide, in a one-pot reaction using oxalyl chloride (shown in **Scheme 71** below). Oxalyl chloride **119** was employed instead of thionyl chloride along with a catalytic amount of DMF to generate the acid chloride. Thioctic acid **3** was treated with oxalyl chloride in the presence of pyridine. In the reaction mixture that was cooled at 0 ^{0}C, **72** was added and the reaction was stirred at room temperature for 2h.

During the reaction a black solid was formed, assumed to be pyridinium salts, which were then extracted into the aqueous layer on work-up by washing with saturated sodium bicarbonate and brine. A black oil was furnished after work up with ethyl acetate but the ^{1}H-NMR spectrum showed no sugar protons of the ribose while there was no evidence given to support the formation of the desired amide.



Scheme 71

(IV) By employing coupling reagents

Since the previous reactions performed failed to furnish the desired amide, an alternative route was proposed based on the employment of coupling reagents, typically used in peptide synthesis.

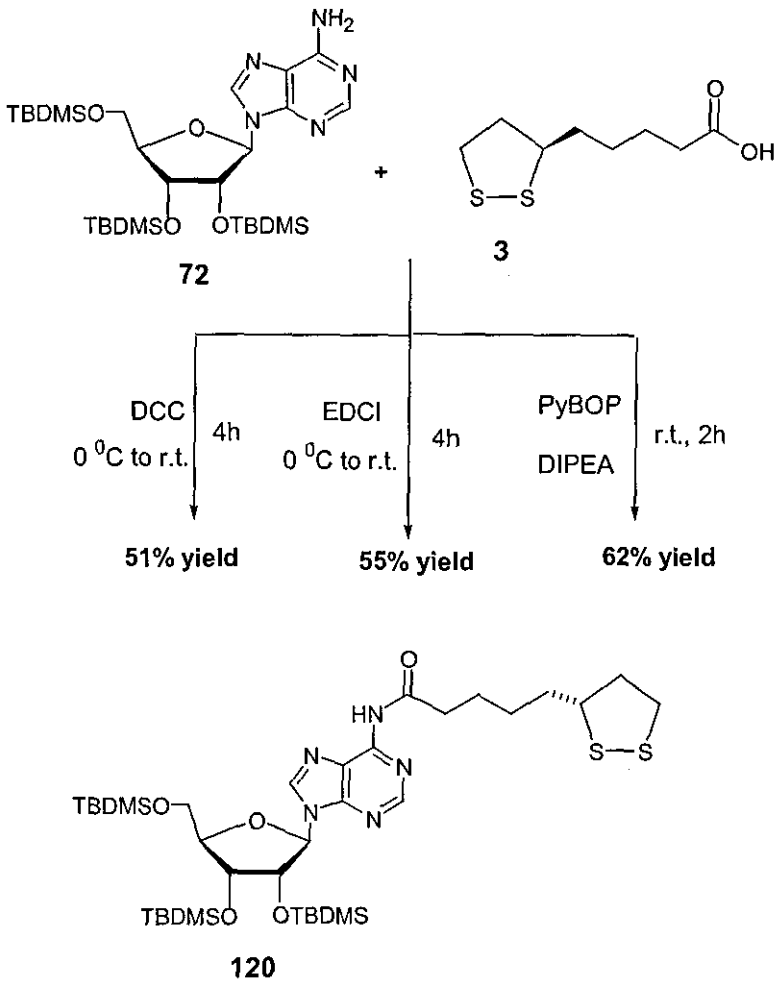
One of the most common coupling reagents in the amide bond forming reactions of amines with carboxylic acids is 1,3-dicyclohexylcarbodiimide (DCC)[294] **51**, which is shown in **Scheme 45**.

The reactivity of amines depends on their order and basicity and can affect the reaction with acids in a different manner while the acid strength also has an effect on the reactivity. The choice of the solvent for the coupling reactions performed was done on the base of the reported results,[296] as it has a distinct effect on the ratio of the reaction products. When acetonitrile is used as the solvent, the hydrated adduct dicyclohexylurea is formed and precipitating while THF is reported to reduce reaction rates and encourage the formation of the N-acylurea side product.

Dichloromethane and DMF were used as the solvents. The amine in this case is weakly nucleophilic as well as sterically hindered. The coupling was carried out using 1eq of each of the thioctic acid and 2',3',5'-O-TBDMS adenosine and 1.1eq of DCC. The coupling reagent was added proportionally to a solution of acid and amine in anhydrous DMF, cooled at 0 ^{0}C, as shown in **Scheme 72**. The desired amide **120** was afforded as yellow oil in a 51% yield after 4h of reaction.

Another coupling reaction was carried out using the water soluble 1-ethyl-3-(-3'-dimethylaminopropyl) carbodiimide hydrochloride) (EDCI)[295] **52**, as the coupling reagent (shown in **Scheme 45**). The same ratio of reagents and reaction conditions were used as for DCC and the side products were removed by simple aqueous extraction with sodium bicarbonate and water.

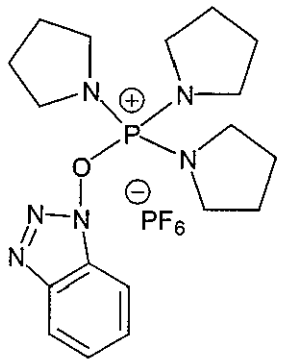
Again the amide **120** was obtained in a 55% yield as yellow oil as shown in **Scheme 72** below.



Scheme 72

In an effort to improve the yields obtained and force the reaction to completion the employment of Benzotriazolyloxy-tris(pyrrolidino)-phosphonium hexafluorophosphate (PyBOP)[312] **121**, shown in **Scheme 73**, was proposed. This is a recent coupling reagent being used in amide formation, an analog of BOP (benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate) where the dimethylamino groups are replaced by *pyrrolidino*.

PyBOP



121

Scheme 73

PyBOP is the only analog whose utilization does not involve the use or formation of cytotoxic and carcinogenic compounds like BOP does (HMPA) and also exhibits equivalent properties to it. For that reason it was decided to use PyBOP instead.

In the case of PyBOP, the same ratio of reagents and coupling reagent was used as before, but in addition the coupling reaction was performed with the assistance of diisopropylethyl amine (DIPEA) at room temperature. The crude product furnished was purified by flash column chromatography eluting with a 95: 5 mixture of DCM and methanol to afford the formed amide **120** as a yellow oil in a 62% yield (as shown in **Scheme 72**) while elemental and spectroscopic analysis confirmed its formation. The broad singlet at δ 7.90 in the ^{1}H-NMR spectrum which corresponds to the H next to amide bond (H-N-C=O) and the signal at 177.0 ppm in the ^{13}C-NMR spectrum, which corresponds, to the carbon of the carbonyl bond (C=O) indicated the presence of the amide. Also the broad signal of the NH_2 of the TBDMS-protected adenosine that appears at δ 6.46 has disappeared.

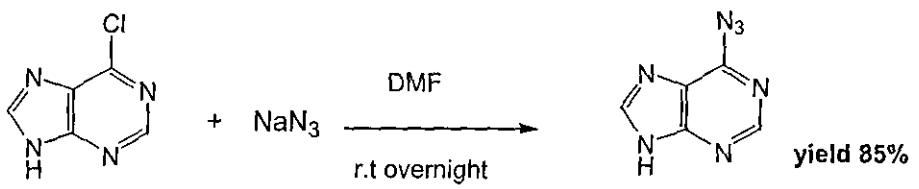
As it can be seen from the results, PyBOP proved to be most effective coupling reagent as the yield of **120** was improved over the ones obtained with EDCI and DCC, even after 2h at room temperature.

(E) Modification at the base of the nucleosides

Apart from the natural bases adenine, guanine, cytosine and thymine also incorporated in oligodeoxynucleotides have been other, less common, synthetic bases. These efforts were usually aimed at testing the effect of other modifications, such as methylation of the bases, on the action of DNA-modifying enzymes, examining alterations in hybridization characteristics. Changes at the bases are generally the most difficult chemically and require the greatest number of synthetic steps.

As part of the investigation into new types of protecting groups the displacement of the amine group with other nucleophiles was studied. We were also interested in attaching phospholipid compounds to the nucleotides in order to form their derivatives, as they are more active in blocking the viral replication in cell cultures compared to the non-derivatized nucleotides. These derivatives have found to accumulate on the cell membrane and penetrate into the cells via endosomal vesicles. The investigation first started with the reaction of 6-chloropurine with sodium azide to give the synthetically useful azide of the purine base, which can used as the precursor for the coupling with the phospholipid compounds.

6-Chloropurine **122** was treated with sodium azide (NaN_3) in DMF and through an aromatic nucleophilic displacement reaction, the azide of the purine base **123** was readily prepared as an off-white solid in 85% yield (as shown in **Scheme 74** below)



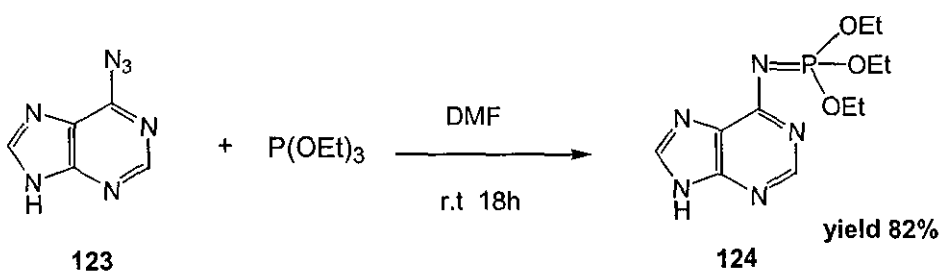
122 **123**

Scheme 74

The structure of **123** was confirmed by evidence of a peak at 2154 cm^{-1} in the IR spectral analysis, indicating the formation of the azide.

The formed azide **123** was then reacted with triethyl phosphite which is a phosphine derivative . Dropwise treatment of **123** with triethyl phosphite under nitrogen, gave successfully the phosphorimidic acid triethyl ester derivative **124**, as shown in **Scheme 75**.

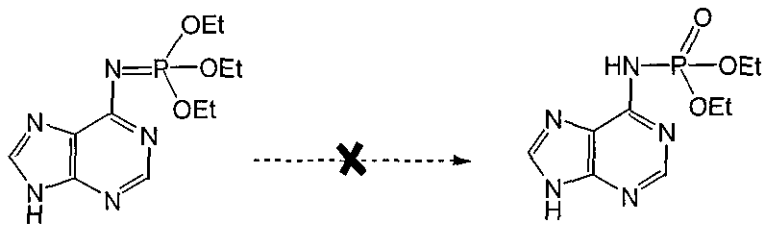
In order to avoid hydrolysis of the phosphoramidate the reaction was carried out under anhydrous conditions. The reaction proceeded smoothly in anhydrous DMF at room temperature to form the triethyl ester phosphoramidate of purine **124** as an off-white solid. Recrystallization from a mixture of dichloromethane and petroleum ether (4: 1) afforded the desired compound **124** in 82% yield.



123 **124**

Scheme 75

The ^{1}H-NMR spectrum of the phosphoramidite **124** showed signals for the three ethyl groups attached to oxygen while the ^{31}P NMR spectrum showed a singlet at δ 1.93, constitent with a trialkyl N-aryl phosphoramidate. The phosphoramidate was stable and showed no tendency to undergo dealkylation to the corresponding diethyl phosphoramidate **125,** as shown below.

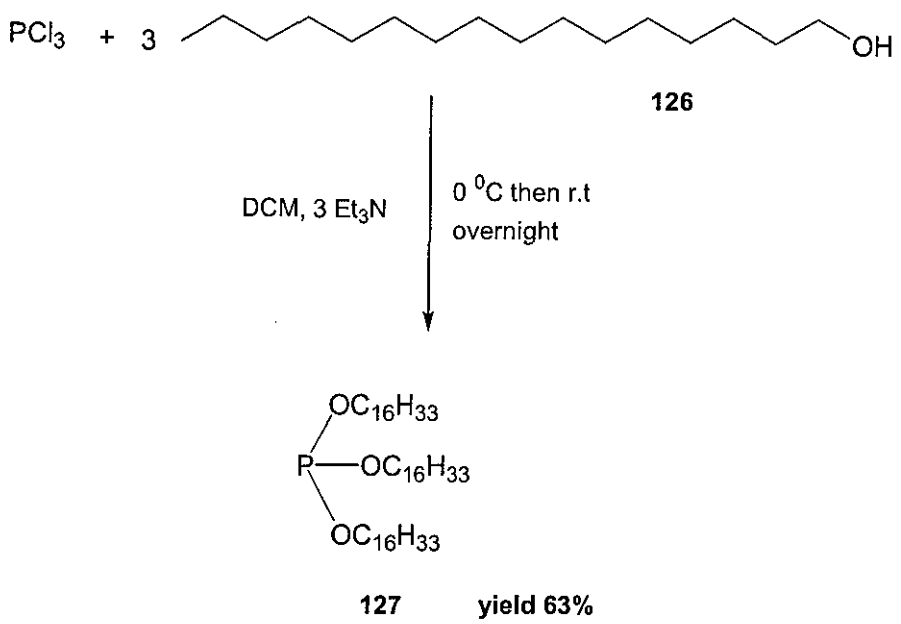


124 **125**

This reaction was then extended to show that other nucleophiles containing a phospholipid compound could react in a similar fashion to triethyl phosphite.

For this reason the tricetyl phosphite of the known phospholipid compound 1-hexadecanol (cetyl alcohol) **126,** was synthesized.

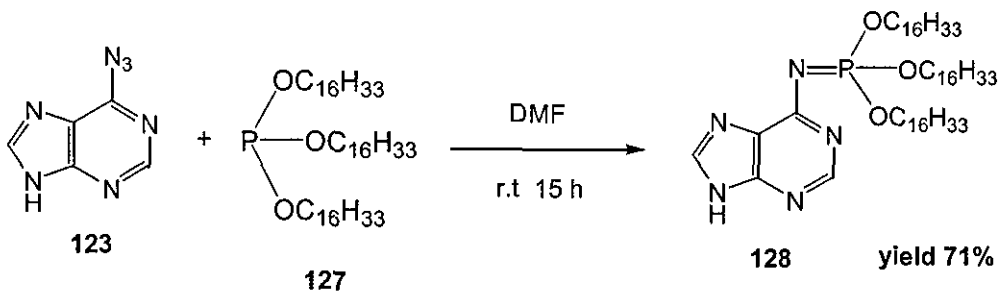
3 equivalents of cetyl alcohol in dichloromethane were treated with phosphorous trichloride n the presence of triethylamine, as shown in **Scheme 76** below. The reaction proceeded at ambient temperature to afford the tricetyl phosphite **127** as white solid in 63% yield, after recrystallization from ethanol and water.



127 **yield 63%**

Scheme 76

The synthesized azide of purine base **122** was in turn treated with the tricetyl phosphite **127** using anhydrous conditions to give the phosphorimidic acid tricetyl ester derivative, as shown in **Scheme 77.** The reaction proceeded successfully in anhydrous DMF at ambient temperature to afford the tricetyl ester phosphoramidate of purine as an off-yellow viscous liquid. Recrystallization from a mixture of ethyl acetate and hexane (3:1) afforded the desired compound **128** in 71% yield.



Scheme 77

Due to time constraints though the chemistry of this compound could not be investigated further, although it represents a useful intermediate, which could be elaborated further into biologically active materials.

4. FORMATION OF THE CpG DINUCLEOTIDES

Bacterial DNA and synthetic oligodeoxynucleotides containing CpG dinucleotides (CpG-DNAs) have been shown as potential adjuvants of the innate immune system. The goal of using of oligodeoxyribonucleotides containing CpG dinucleotides (CpG DNA) as immunomodulatory agents has been realized in recent years. Therapeutic applications of CpG DNA as antitumor, antiviral, antibacterial and anti-inflammatory agents and as adjuvants in combination with vaccines for a number of disease indications are rapidly expanding.[250-252] The safety and efficacy of several first-generation CpG DNA agents are being evaluated in human clinical trials.[254,256]

In order to improve the immunostimulatory activity of CpG phosphodiesters it was proposed to study chemical modifications in the backbone and the bases by attaching lipophilic molecules in the CpG motifs. One very important step in CpG dinucleotide formation using both solution and solid phase synthesis is the employment of 3'-phosphoramidites of cytidine. Described below is their synthesis so that they can be used as building blocks

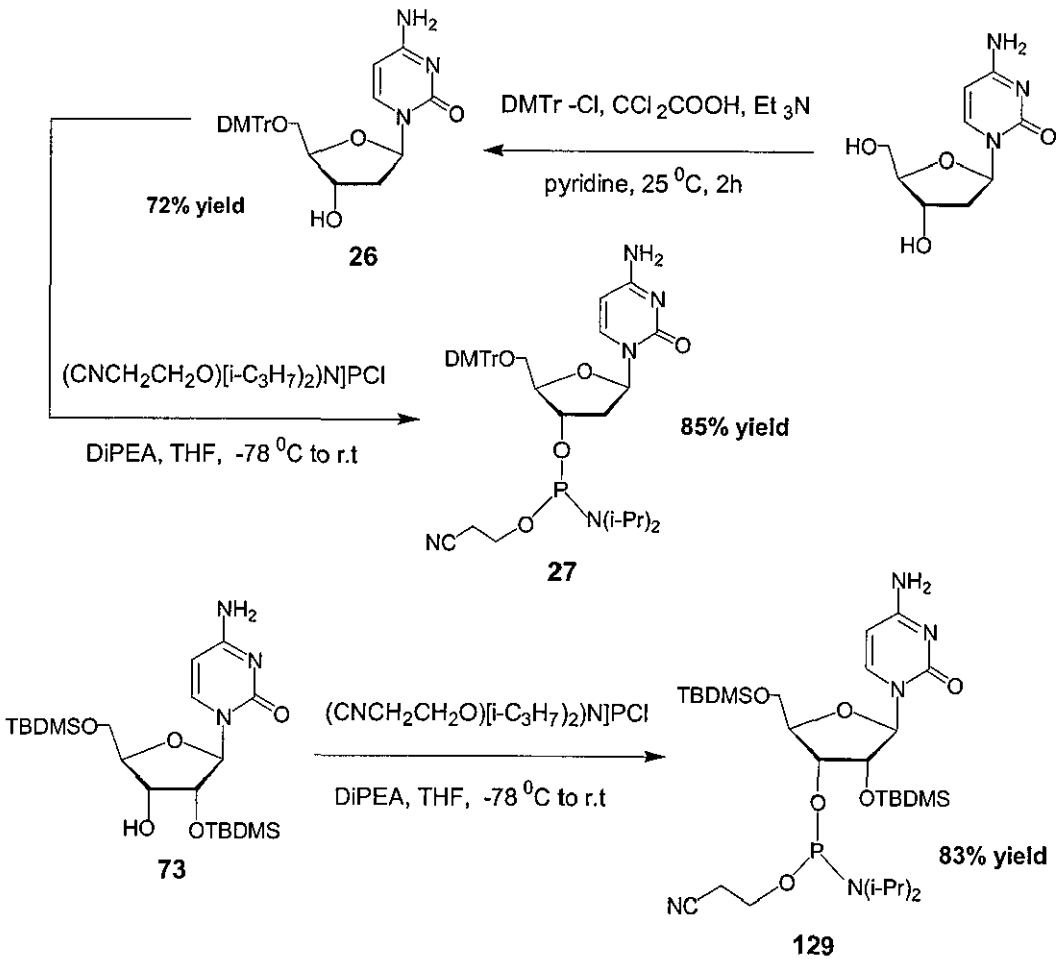
(A) Synthesis of N-free-3'-phosphoramidites of cytidine

The *N,N*-diisopropylaminophosphoramidites[313,314] of cytidine are extremely useful reagents for dinucleotide synthesis using phosphoramidite approach. In CpG polymer supported synthesis the key intermediate is the condensation of 2'-deoxyguanosine covalently joined to a glass support with the 3'-phosphoramidite of 2'-deoxycytidine.

An essential process in phosphoramidite synthesis is the phosphorylation of the hydroxyl function of the nucleoside. Phosphorylation of nucleosides such as adenosine and cytidine that have the hydroxyl and amino group in the same molecule, takes place competitively at both the functions and hence the amino groups have to be protected in order to achieve selective O-phosphorylation. However, deprotection often brings about undesired side reactions, particularly cleavage of the phosphate linkage resulting in serious loss of product. For that reason, O-phosphorylation of nucleosides without the N-protection is obviously ideal.

The *N,N*-diisopropylamino-phosphoramidites have been introduced as phosphitylating agents as they are significantly stable to moisture, can be stored for months and are easily activated using tetrazole. The removal of the protecting groups from the phosphate moiety plays an important role in the efficiency and success of dinucleotide synthesis. The protecting group should be such that it can be removed in short time under mild conditions without causing the formation of large amounts of salts and other side reactions.
A group that fulfills these criteria is the β-cyanoethyl group and for that reason the (2-cyanoethyl) (*N,N*-diisopropylamino) chlorophosphine was employed in this case as the phosphitylating agent. It has been reported that the use of cyanoethyl phosphoramidites gave rise to increased coupling efficiencies and better product yields upon deprotection.

The N-free-3'-phosphoramidites of cytidine required for dinucleotide synthesis using both solid and solution phase synthesis were prepared as monomer units. 5'-O-(Dimethoxytrityl)-2-deoxycytidine and the synthesized 2',5'-O-TBDMS cytidine both without the protection of the amino functions were employed as key intermediates and treated with the phosphitylating agent to furnish the desired phosphoramidites.
Firstly in order to synthesize 5'-O-(Dimethoxytrityl)-2-deoxycytidine, 2'-deoxycytidine was treated with 4,4'-dimethoxytrityl chloride (DMTrCl) in the presence of dichloroacetic acid and triethylamine in pyridine as shown in **Scheme 78** below. The 5'-O-protected 2'-deoxycytidine **26** was furnished as white solid in a 72% yield, after recrystallization from a mixture of toluene and acetone (5:1).
5'-O-(Dimethoxytrityl)-2-deoxycytidine **26** and 2',5'-O-TBDMS cytidine **73** were then condensed with (2-cyanoethyl) (*N,N*-diisopropylamino) chlorophosphine in THF, by the aid of ethyldiisopropylamine to afford the N-free-3'-phosphoramidites of cytidine **27** and **129** as colourless solids after trituration from petroleum ether (shown in **Scheme 78** below). The amidites **27** and **129** were obtained in 85 and 83% yield, respectively, and were used for subsequent reactions without further purification.



Scheme 78

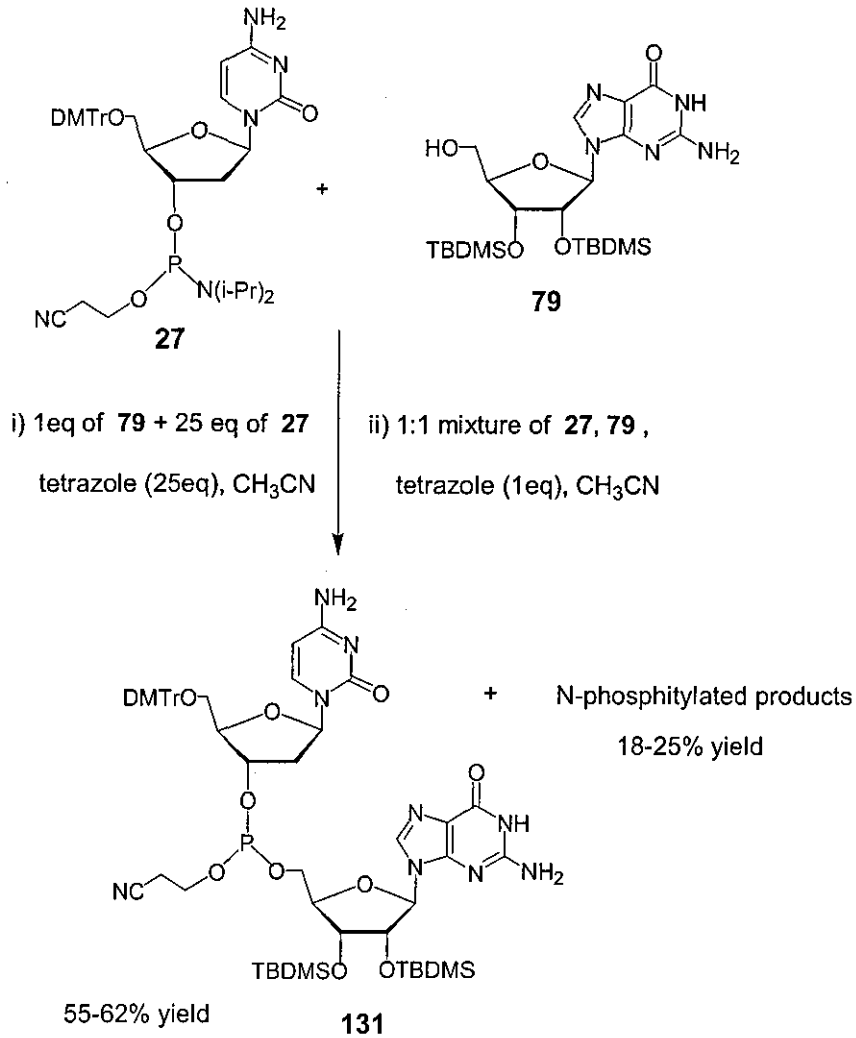
The structures of the N-free-5'-dimethoxytritylated-3'-phosphoramidites of cytidine **27** and **129** were confirmed by the ^{31}P NMR signals appearing at δ 148.07, 148.3 and 150.5 ppm, respectively (signals due to the N-phosphoramidityl products appear at δ 124-127 ppm). ^{31}P NMR also provided a diagnostic proof of the isomeric purity of **129** as it was found to be isomerically pure demonstrating the stability of silyl groups to the conditions of phosphorylation.

The stability of the N-free amidites is similar to that of the N-protected derivatives and they can be stored for several months under nitrogen at -20 ^{0}C with no apparent degradation. These phosphoramidites were obtained via a shorter pathway and in higher yields than their N-protected corresponding analogues and were utilized to enable us to synthesize dinucleotides without resort to N-protecting groups.

(B) Solution Phase synthesis of CpG dinucleotides

(I) Without the attachment of lipophilic groups

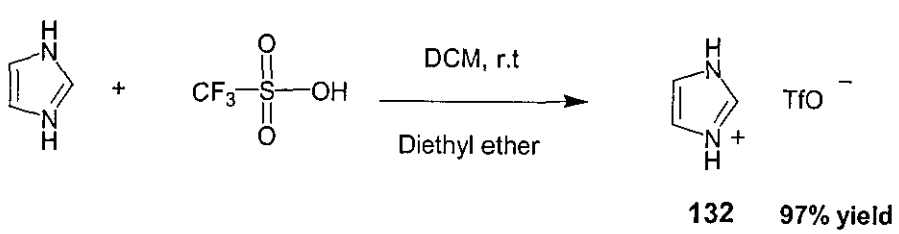
For the solution phase synthesis of CpG dinucleotides the formed N-free cytosyl phosphoramidite of cytidine **27** was reacted with the synthesized 2',3'-O-TBDMS protected guanosine **79.** Excess equivalents of the phosphoramidite and tetrazole, as the promoter, were employed which is a reaction generally used in solid-phase synthesis. Disappointingly, phosphitylation of **79** with a 1:1 mixture of tetrazole and **27** (25 equiv each to **79**) gave not only the desired phosphite **131** in a 62% yield (shown in **Scheme 79** below) but also a 25% yield of its N-phosphitylated derivatives.



Scheme 79

The presence of phosphite is supported by a signal at δ 140 ppm in ^{13}P NMR spectrum while, signals at δ 123-127 ppm suggested the formation of the N-phosphitylated products but their exact structures was not determined.
An attempt to utilize equimolar amounts **79** and **27** and condense them using 1 equivalent of tetrazole resulted again in the formation of N-phosphitylated products in 18% yield along with the production of phosphite **131** (shown in **Scheme 79** above). These experiments suggest that tetrazole is not an effective promoter for the formation of CpG dinucleotide using solution phase synthesis, thus the utilization of a new promoter was evident.

In previous research, Letsinger reported a pioneering DNA synthesis with N-unprotected building blocks that employs a mixture a pyridinium hydrochloride and aniline[226] as the activator of the phosphoramidites. However this method proved to have drawbacks and limitations as the pyridinium salt is very hygroscopic and thus not suitable for synthesis requiring anhydrous conditions.
The employment of imidazolium triflate was reported[315] as an activator, which proved to be a very effective promoter for solution-phase synthesis of dinucleotides using the phosphoramidite method without nucleoside base protection and an excellent activator towards 2-cyanoethyl *N,N*-diisopropylphosphoramidites.
This reagent was quantitatively prepared by mixing imidazole and trifluoromethanesulfonic acid in equimolar amounts in dichloromethane at ambient temperature, as shown in **Scheme 80** below. **132** was used without further purification, had good solubility in acetonitrile and high stability when stored at ambient temperature under atmospheric conditions.

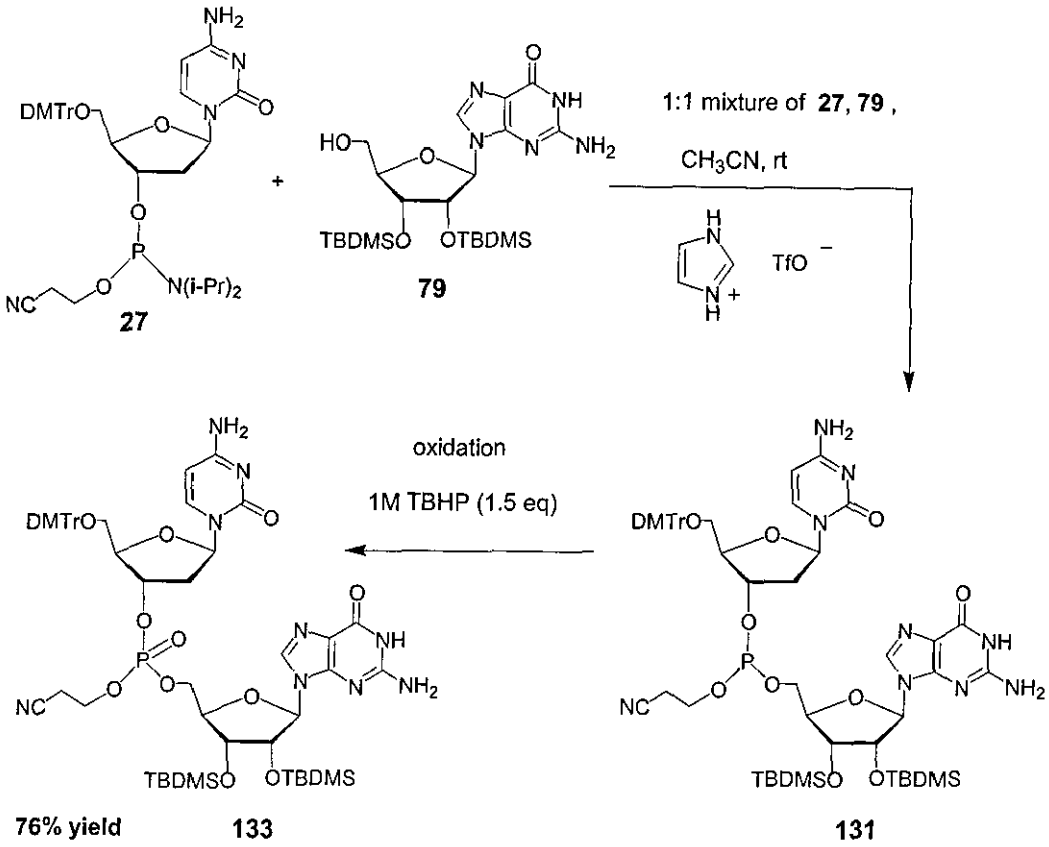


132 **97% yield**

Scheme 80

Imidazolium triflate **132** was used for the chemoselective condensation of N-free-3'-phosphoramidite of cytidine **27** and 2',3'-O-TBDMS protected guanosine **79,** when equimolar amounts of these substrates were employed in acetonitrile. The reaction (shown in **Scheme 81** below) proceed at room temperature within 20 min to give after oxidation with 1.0 M toluene solution of tert-butyl hydroperoxide (1.5 eq), **133** in 89% yield as a mixture of two diastereomers.

The formation of **133** as crude material was confirmed by ^{31}P NMR spectrum, which shows two signals at δ -1.1 and -1.0 ppm, supporting the presence of diastereomeric phosphotriester functions but no detectable signal due to N-phosphoryl products (the ^{31}P NMR signals due to N-phosphoryl products appear at δ 0-2 ppm).



Scheme 81

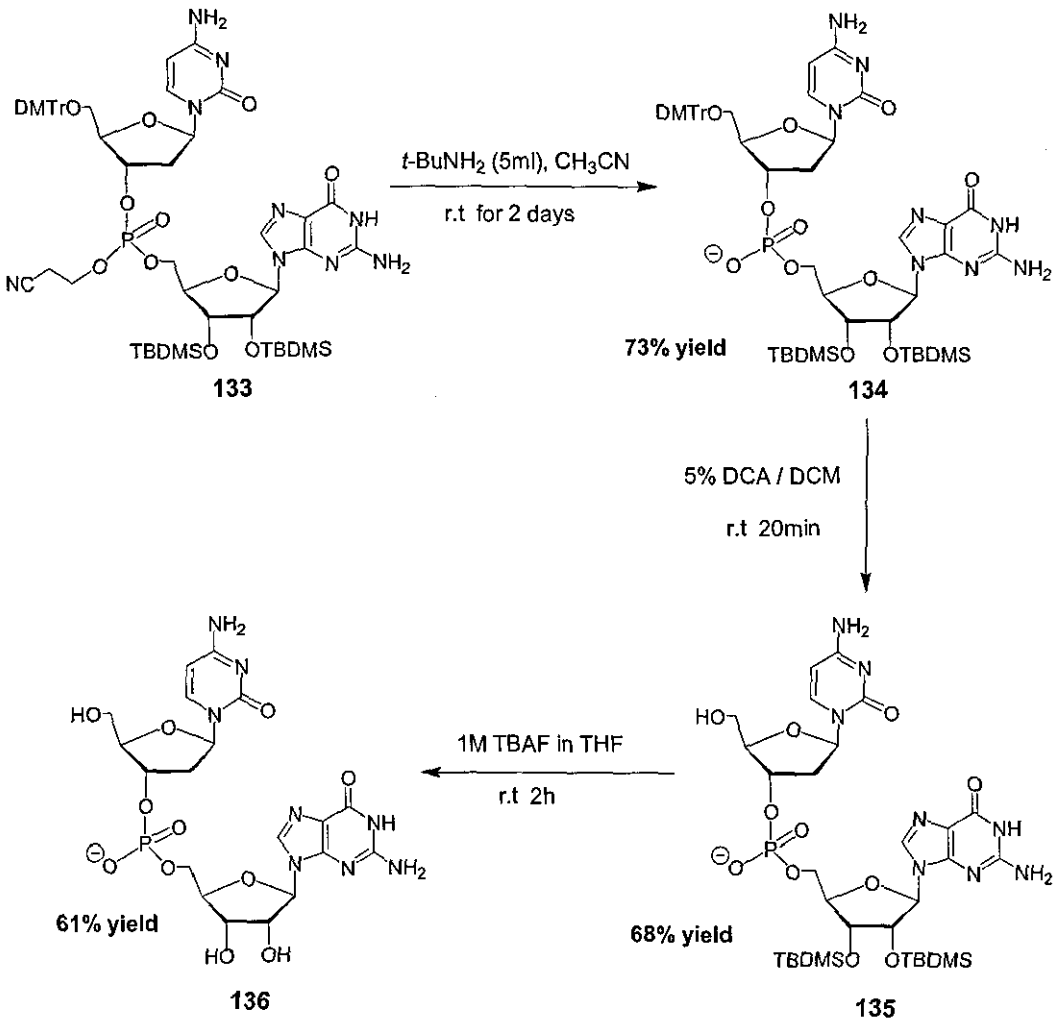
The crude product was purified on a silica gel pad eluting with a 10:1 mixture of dichloromethane and methanol to afford the pure form of **133** as a colorless amorphous solid in 76% yield. ^{31}P NMR spectrum showed two signals at δ -1.95 and -2.01 arising from purified **133.**

The removal of the cyanoethyl protection was accomplished by employing anhydrous t-butylamine (5ml) and stirring for 2 days at room temperature (shown in **Scheme 82** below). The product was isolated on a silica gel pad eluting with a 10:1 mixture of dichloromethane and methanol containing a trace amount of triethylamine to give **134** as off white foam in 73% yield. After generation of the internucleoside phosphate linkage, the dimethoxytrityl protection was removed by employing 5% dichloroacetic acid in dichloromethane (3.2eq) as shown in **Scheme 82**.

Stirring at ambient temperature for 20 min furnished **135**, after separation and purification using a 5:1 mixture of dichloromethane and methanol, in 68% yield. Silyl protection was cleaved using 2.4. equivalent of TBAF in THF (**Scheme 82**) and stirring at ambient temperature for 2h afforded the deprotected CpG **136** as colorless foam in 61% yield. The reaction sequence of the solution phase synthesis of the CpG dinucleotide is shown in **Table 16** below

Table 16

Operation	Reagents	Conditions	Yield (%)
Coupling	1: 1:1 ratio of 3'-phosphoramidite of cytidine **27**, 2',3'-O-TBDMS protected guanosine **79** and imidazolium triflate **132**	r.t for 10 min	Straight to next step
Oxidation	0.1 M toluene sol of TBHP (1.5eq)	r.t for 10 min	76
Cyanoethyl Deprotection	Anhydrous *tert*-butylamine (5ml)	r.t for 2 days	73
Detritylation	3% dichloroacetic acid in DCM (3.2eq)	r.t for 20 min	68
Desilylation	1 M THF solution of TBAF (2.4eq)	r.t for 2h	61



Scheme 82

(II) With the attachment of lipophilic groups

Dinucleotides are very small synthetic molecules resembling single stranded DNA. The presence of chemically modified nucleosides or unusual structural determinants that are rare in mammalian cells may induce immunostimulatory effect.[272,273] A number of studies have shown that modifications on the pentose sugar, phosphate backbone and nucleobases of the nucleosides has an effect on immunostimulatory activity.[269-271] In order to enhance the ability of CpG dinucleotide to penetrate into intact cells and increase their stability towards nucleases, it was suggested to investigate the synthesis of dinucleotides modified with lipophilic groups.

In the present work we will study CpG dinucleotide formation containing lipophilic analogues of cytidine and guanosine. The successful approach employed above for the solution phase synthesis of CpG was extended into utilizing the synthesized lipophilic carbamate analogues of guanosine. N-free-3'-phosphoramidite of cytidine **27** was coupled with the 2',3'-O-TBDMS-N-carbamate cholesteryl **104** and tocopheryl **105** conjugates of guanosine using a 1:1 ratio of the reactants in the presence of imidazolium triflate. The reaction (shown in **Scheme 83** below) took place in acetonitrile and the resulting phosphite intermediates were oxidized with *tert*-butyl hydroperoxide to give **139** and **140** in 87 and 84% yield respectively as a mixture two diastereomers, each.

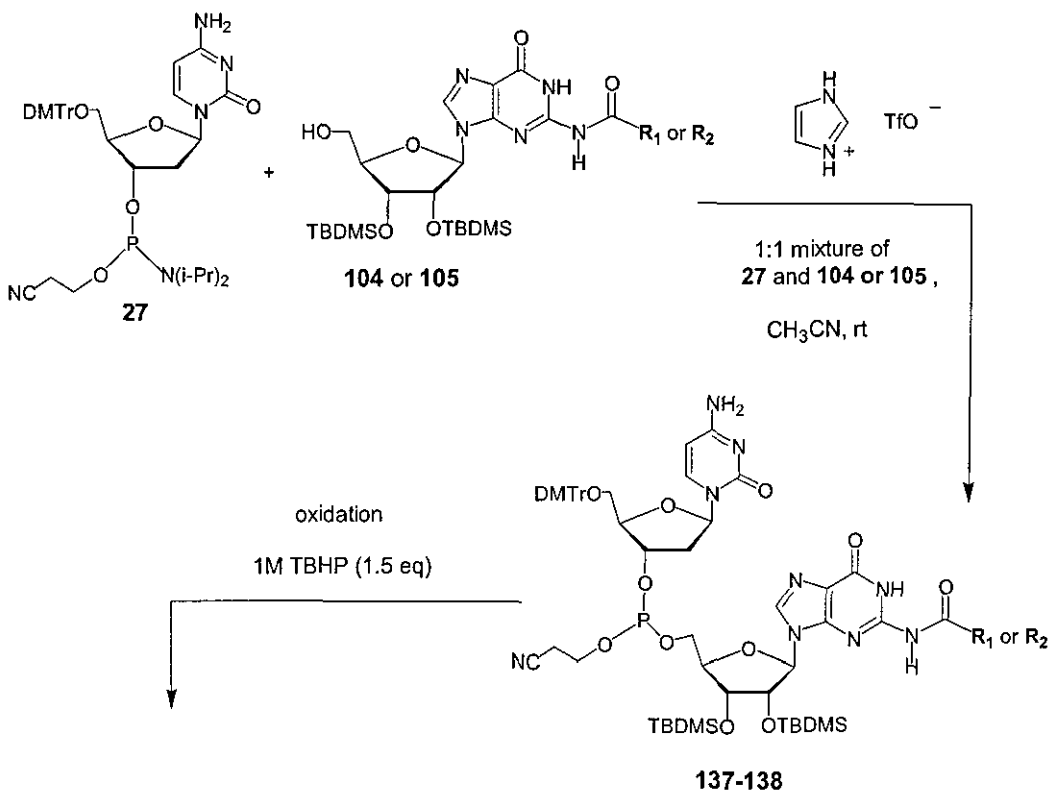
^{31}P NMR spectrum showed two signals at δ -1.24 and -1.33 ppm and δ -1.28 and -1.36 ppm arising from the diastereomeric phosphotriester functions for **139** and **140** respectively but no detectable signal due to N-phosphoryl products. The crude products were purified on a silica gel pad eluting with a 10:1 mixture of dichloromethane and methanol to afford the pure form of **139** and **140** in 72 and 75% yield, respectively. The presence of purified **139** and **140** was evident by signals at δ -2.16 and 2.24 and δ -2.19 and 2.3, respectively.

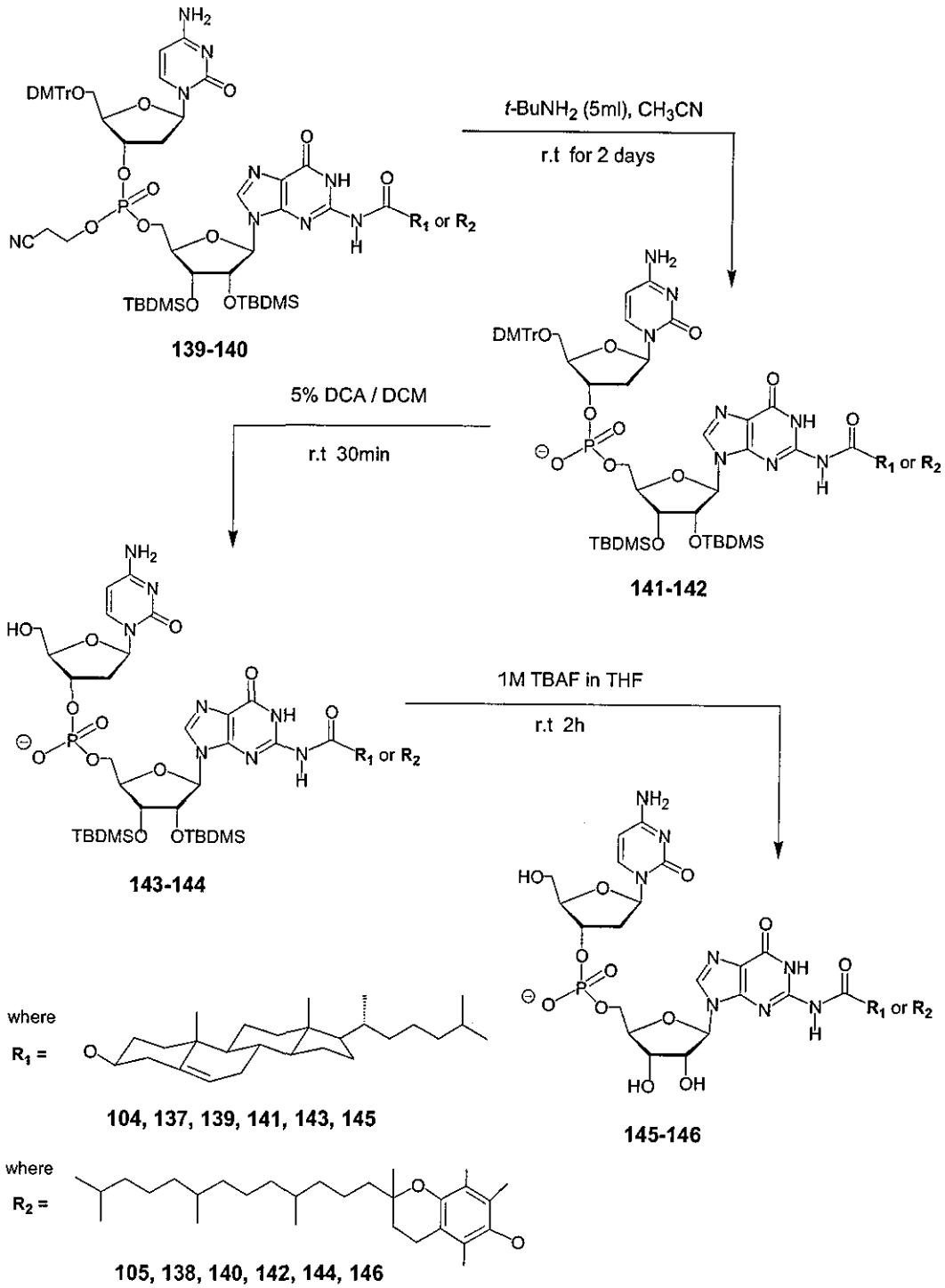
The 2-cyanoethyl protecting group was cleaved with *tert*-butylamine (**Scheme 83**) to give, after purification on a silica gel pad using a 10:1 mixture of dichloromethane and methanol containing a trace amount of triethylamine, **141** and **142** in 70 and 72% yield, respectively. The stepwise removal of protecting groups-detritylation and desilylation-was carried out as before to afford dinucleotides **145** and **146** as shown in **Scheme 83**. The reaction sequence of the solution phase synthesis of CpG dinucleotide modified with cholesterol and tocopherol is represented in **Table 17** below.

Imidazolium triflate **132** proved to be an excellent promoter that allows rapid and highly chemoselective condensation of the N-free-3'-phosphoramidites of cytidine and N-free lipophilic conjugates of guanosine when equimolar amounts of these substrates were utilized. There was no evidence of N-phosphorylation of the cytosyl base.

Table 17

Operation	Reagents	Conditions	Yield (%)
Coupling	1: 1:1 ratio of N-free-3'-phosphoramidite of cytidine **27**, 2',3'-O-TBDMS-N-carbamate cholesteryl **104** or tocopheryl **105** conjugates of guanosine and imidazolium triflate **132**	r.t for 10 min	Straight to next step
Oxidation	0.1 M toluene sol of TBHP (1.5eq)	r.t for 10 min	**139** (72) **140** (75)
Cyanoethyl Deprotection	Anhydrous tert-butylamine (5ml)	r.t for 2 days	**141** (70) **142** (72)
Detritylation	3% dichloroacetic acid in DCM (3.2eq)	r.t for 30 min	**143** (63) **144** (66)
Desilylation	1 M THF solution of TBAF (2.4eq)	r.t for 2h	**145** (57) **146** (60)





Scheme 83

The overall yields of CpG dinucleotide synthesis using N-unprotected building blocks with or without the attachment of lipophilic groups were found to be satisfactorily (57-61%) and the results confirmed the success of solution phase synthesis of the CpG dinucleotides using N-free nucleosides.

(C) Formation of novel phosphoramidites of lipophilic compounds

Phosphodiester synthetic oligonucleotides are increasingly used because of their potential activity as regulators of gene expression. One of their major drawbacks is that they are inherently prone to rapid degradation by nucleases and generally have half-lives of less than two minutes in vivo[292,316] in particular exonucleases. Recent studies suggested that an accessible 5'-end of the CpG dinucleotide is critical for the immunostimulatory activity and blocking the 5'-end of CpG by conjugation of certain ligands increases immunostimulatory activity.[253]

Based on this observation and to examine if cellular uptake is effected by 5'-conjugation phosphodiester, it was proposed to investigate terminal modifications that can enhance exonuclease resistance, such as end capping with alkylic or lipophilic chains. Similar groups have also been attached to the 3'-end of oligonucleotides by means of functionalised CPG. For that reason, CpG dinucleotides were synthesized with lipophilic moieties attached at their 5'-end.

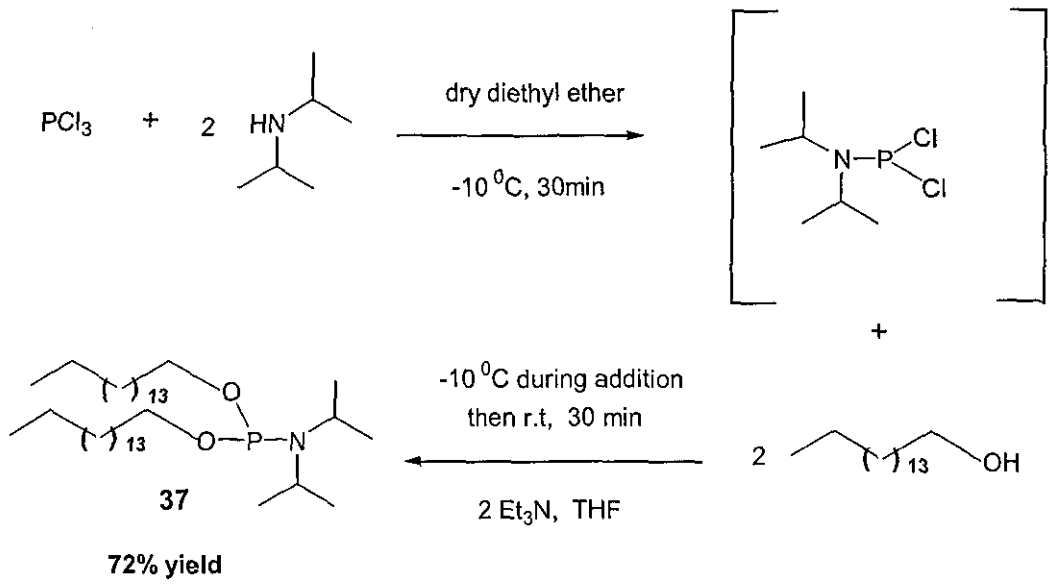
Interest was drawn towards preparing phosphoramidites of lipophilic compounds, which can subsequently be coupled to CpG through their 5'-terminus, in order to form the lipophilic phosphodiester dinucleotides. Phosphoramidites have very promising and attractive properties as reactive nucleoside intermediates for polymer support dinucleotide synthesis while; the tricoordinate phosphorus reagent enhances the attachment of lipophilic groups to the dinucleotide synthesis and improves the efficiency of uptake into the cell.

For that reason, the novel *N,N*-diisopropyl-phosphoramidites of cetyl alcohol **126** (cetyl = straight chain C_{16} group), cholesterol and tocopherol were synthesized by one-flask procedure. The diisopropylamino group was chosen because being attached to the tetra coordinate phosphorus center exhibits a high propensity to act as a good leaving group in nucleophilic displacement reaction when activated by the promoter in oligonucleotide formation. It has also been shown to furnish phosphoramidites in stable and pure form while producing higher stepwise coupling yields.

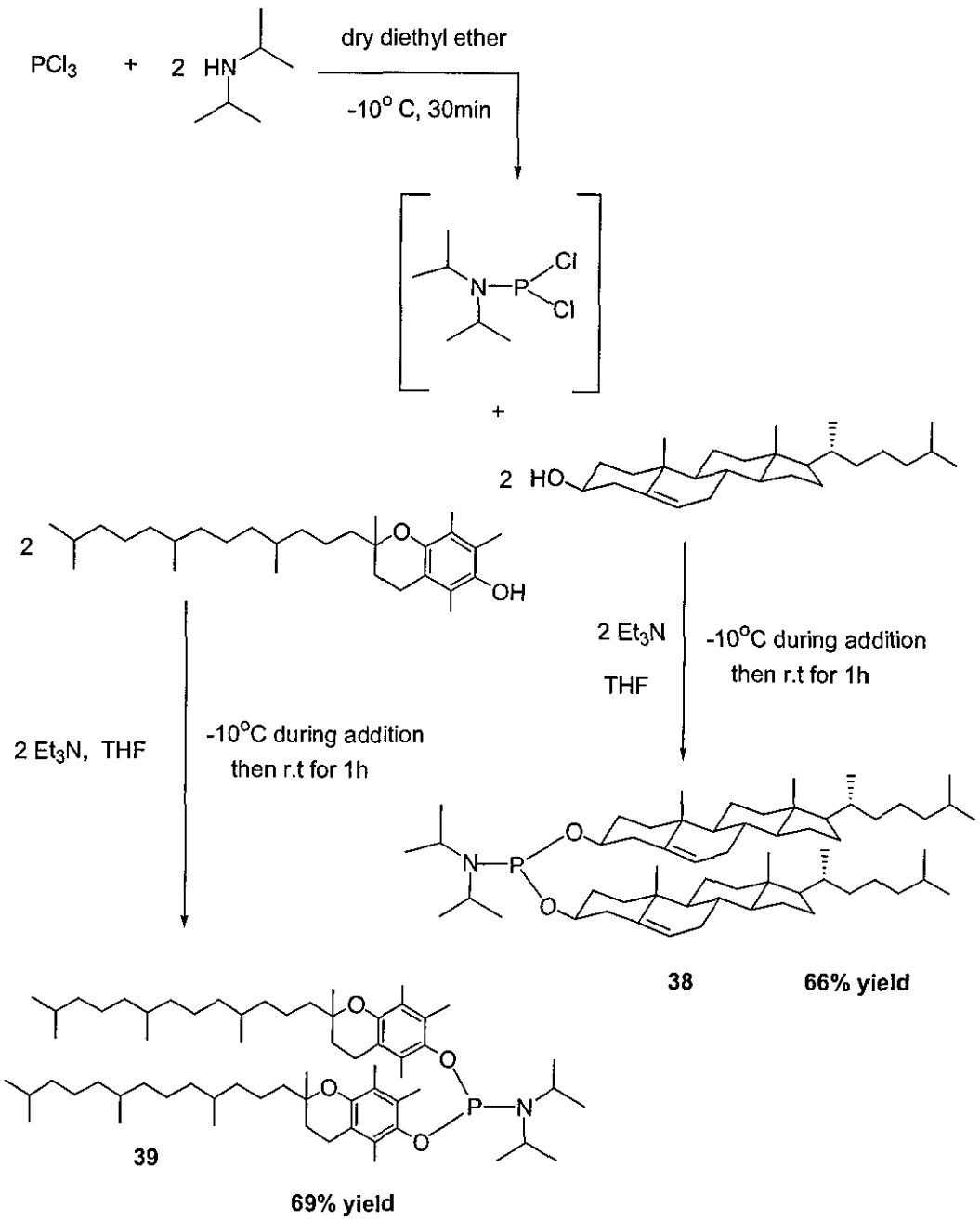
Other phosphoramidites like the *N,N*-dimethylamine and N-morpholine require stringent anhydrous and inert conditions to endure good condensation and storage while good purity can not be ensured.

N,N diisopropylamine (2eq) in dry diethyl ether, was added dropwise to phosphorous trichloride (1eq) in dry ether which was cooled at -10 ^{0}C, over the period of 30 min to 1h. After stirring at -10 ^{0}C for 30 min, the lipophilic compound in THF was added dropwise along with triethylamine while the temperature was kept constant. 2 equivalents of each of the reactants were employed in each case assuming a 70% yield of the formed diichloro-diisopropylamino phosphine intermediate. After 3 hours at room temperature amine hydrochloride was removed and the solution was concentrated under exclusion of moisture. The preparation of the *N,N*-diisopropyl-phosphoramidites is represented in **Schemes 84** and **85.**

The crude phosphoramidites of cholesterol and cetyl alcohol were purified by recrystallization from dichloromethane and petrol to furnish the purified **37** and **38** as white powdery solids in 72 and 66% yield respectively. The purified *N,N*-diisopropyl-phosphoramidite of tocopherol **39** was obtained as yellow oil in a 69% yield, after trituration with hexane. These phosphoramidites, which were sufficiently pure for our synthetic purposes, where found to be stable under dry conditions and were kept under nitrogen at -20 ^{0}C with no apparent degradation.



Scheme 84



Scheme 85

The structures of the *N,N*-diisopropyl-phosphoramidites **37, 38** and **39** were confirmed by the ^{31}P NMR signals appearing at δ 178.82, 182.54 and 184.67 ppm, respectively. These phosphoramidites will be utilized as useful building blocks for the formation of CpG dinucleotide phosphates using polymer support synthesis.

(D) Solid Phase synthesis of CpG dinucleotides

The phosphoramidite monomers **37**, **38** and **39** were employed in the preparation of antisense phosphorodiester dinucleotides, bearing 5'- polyalkyl cholesterol and tocopherol moieties, using the solid phase synthesis. Internucleotide coupling procedure is performed with the 5'-hydroxyl group being capped with the dicetyl or dicholesterol phosphate group to provide lipophilicity. The compatibility of these phosphoramidites with the established dinucleotide synthesis was studied. Commercially available 5'-DMTr 2'-deoxyguanosine **40** bearing benzoyl protection at the exocyclic amino group and derivatized at the 3'-end with Controlled Pore Glass (CPG) solid support with was used. The loading of this solid support utilized was 22 μmol/g.

The synthesis of dinucleotides was performed in a stepwise procedure according to **Table 18** using 1g of the CPG bound 2'-deoxyguanosine. In a column type reactor fitted with a sintered glass fritt, which could be maintained airtight with a serum cap. The 5'-O-DMTr group was removed using 3% trichloroacetic acid in dichloromethane and the column was shaken for 2 min. The deprotection was apparent by the appearance of a bright orange color arising from the release of the dimethoxytrityl cation.

After thorough washing with acetonitrile and drying of the glass beads *in vacuo*, phosphitylation of the free hydroxyl of guanosine attached to CPG was performed using a 1:1 mixture of the promoter and the cyanoethyl-phosphoramidite. 0.1 M of the 5'-dimethoxytritylated-3'-cyanoethyl phosphoramidite of cytidine **27** in CH_3CN (25eq) was added to the column followed by the addition of 0.1 M of imidazolium triflate **132** in CH_3CN (25eq), with a syringe under nitrogen atmosphere. After condensation (shaking for 25 min) the excess reagents were removed by washing with CH_3CN.

Standard iodine oxidation was followed using 0.1 M of iodine in 80:40:20 solution of THF: pyridine: H_2O. After shaking and thorough washing, removal of the dimethoxytrityl protector of the 5'-hydroxyl group was repeated as above.

Finally phosphitylation and subsequent capping of the free 5'-OH group was attained using a 1:1 mixture of the promoter and the synthesized dicetyl **37**, dicholesterol **38** and ditocopherol **39** phosphoramidites.

0.1 M of *N,N*-diisopropylamino-phosphoramidite (25eq) in THF was added to the column followed by 0.1 M of imidazolium triflate (25eq) in CH_3CN. After the final condensation, the excess reagents were removed by washing with CH_3CN and the iodine oxidation step was repeated as before. Deprotection of the cyanoethyl group, removal of the exocyclic amino protection and subsequent cleavage of the product form the polymer support was accomplished by treatment with a solution of concentrated aqueous ammonia in ethanol (3:1) at ambient temperature for 30min and then at 55 ^{0}C for 16h. After completion and cooling down at room temperature, the supernatant liquid was removed and the glass beads were washed with distilled water.

The combined supernatant and washings were concentrated to a small volume and the insoluble material resulting from the β-elimination and polymerization of CH_2=CH-CN was removed by filtering through a Millipore™ filter. The filtrate was applied to small column packed with celite and eluted firstly with 0.05 M of NH_4OAc for desalting and removal of all the organic impurities and finally with acetonitrile. This afforded the crude CpG dinucleotides **147**, **148** and **149** possessing a 5'-dicetyl, dicholesterol and ditocopherol phosphate, respectively. The solid phase synthesis of the 5'-di-lipophilic phosphate of CpG dinucleotide is shown in the **Scheme 86** below.

Table 18

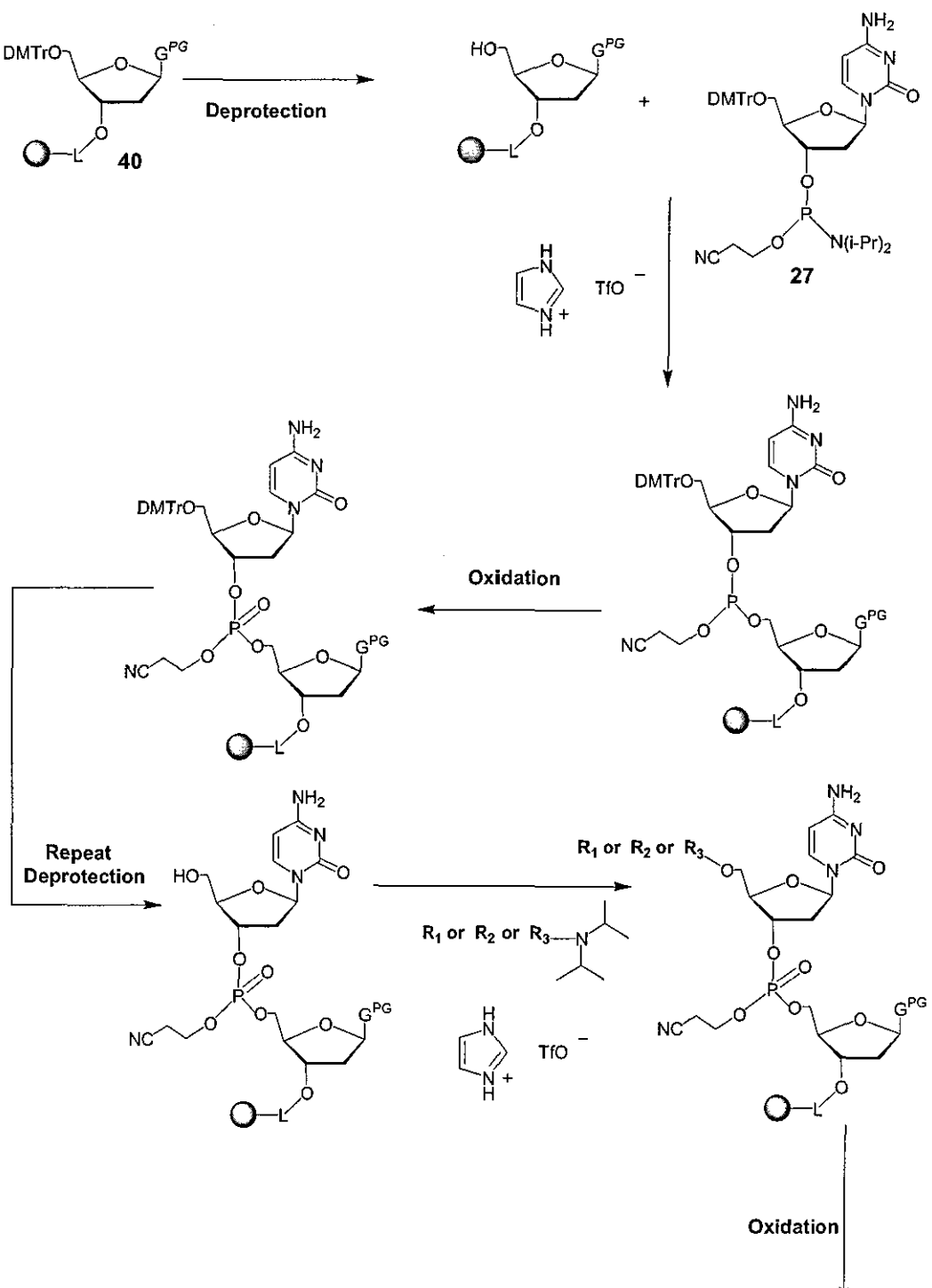
Operation	Reagents	Time (min)
Detritylation	3% trichloroacetic acid in CH_2Cl_2	5 x 2ml, shake for 2min
Washing	CH_3CN	3 x 5ml for 2min
Coupling	0.1 M cyanoethyl-phosphoramidite of cytidine (25eq) in CH_3CN + 0.1 M imidazolium triflate (25eq) in CH_3CN	Shake for 25min
Washing	CH_3CN	10 x 3ml for 2min

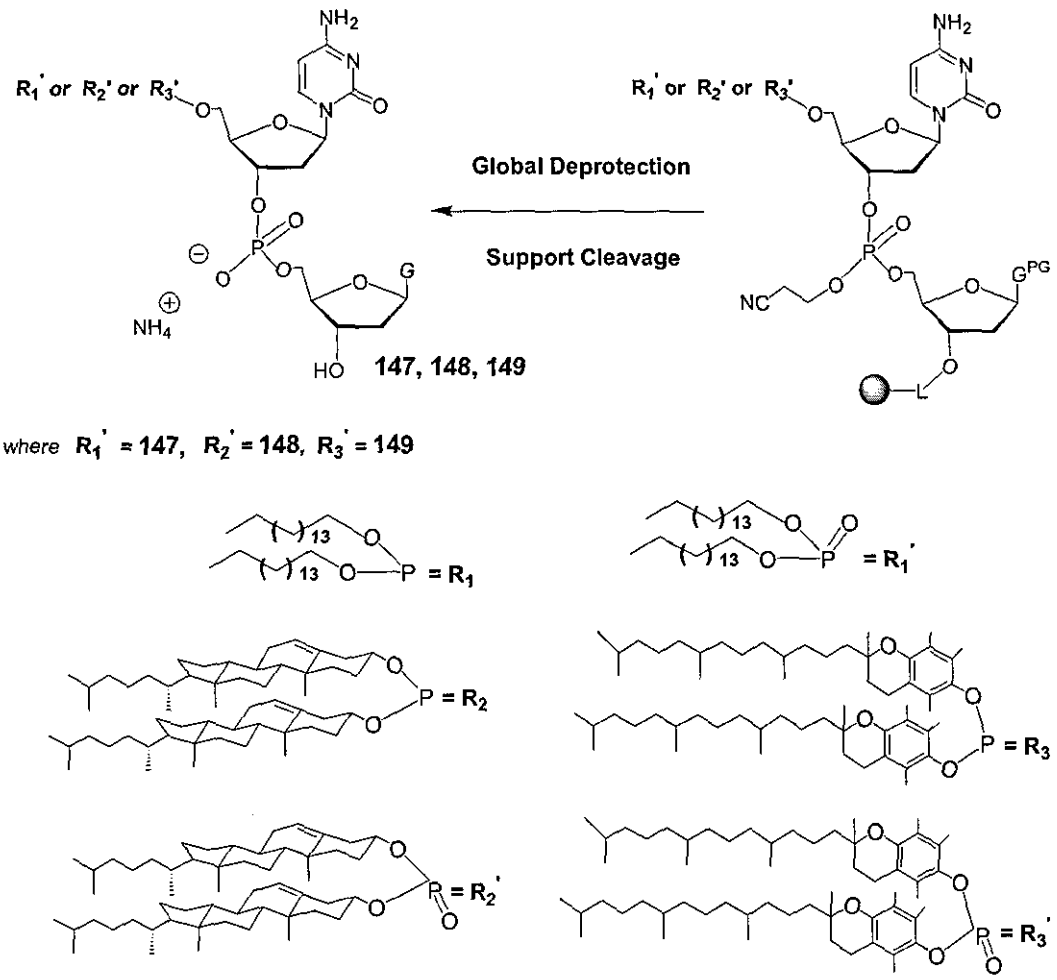
Oxidation	0.1 M J_2 in 80:40:20 solution of THF:pyridine:H_2O	2 x 2ml, shake for 5min
Washing	CH_3CN	10 x 3ml for 2min
Deprotection	As Above	As Above
Washing	CH_3CN	3 x 5ml for 2min
Coupling	0.1 M dicetyl,or dicholesterol or ditocopherol diisopropylphosphoramidite (25eq) in THF + 0.1 M imidazolium triflate (25eq) in CH_3CN	Shake for 25min
Washing	CH_3CN	10 x 3ml for 2min
Oxidation	As Above	As Above
Washing	CH_3CN	10 x 3ml for 2min
Cleaving Off	Treatment with a 3:1 solution of conc. NH_4OH:EtOH	Heat at 55 ^{0}C for 16h
Washing	Distilled water	3 x 1ml
Filtering off	Filter off controlled pore glass beads through Millipore^{TM} filter	
Desalting	0.05 M of NH_4OAc	3 x 5ml

CpG dinucleotide synthesis was repeated without capping of the free 5'-hydroxyl group using the coupling protocol as before, except for the final condensation step with the lipophilic *N,N*-diisopropylamino phosphoramidites. The solid anchored 2'-deoxyguanosine **40** was again utilized but in this case the synthesized N-free-3'-cyanoethyl phosphoramidite of 2',5'-O-TBDMS cytidine **129** was employed as the coupling amidite. After condensation and oxidation, simultaneous deblocking of the cyanoethyl protector of the internucleoside linkage and detachment of the dinucleotide from the solid support occurred, by treatment with concentrated ammonia leaving only the silyl protecting groups intact.

The TBDMS groups were removed by treating the supernatant liquid produced with 1 M TBAF in THF (5eq) for 2h. The reaction was then concentrated to a small volume and the insoluble material was removed as before by filtering.

The filtrate was applied to small column packed with celite and eluted firstly with 0.05 M of NH_4OAc for desalting and removal of all the organic impurities and finally with acetonitrile. This afforded the crude CpG dinucleotides **150** in 57% yield as shown in **Scheme 87** below.



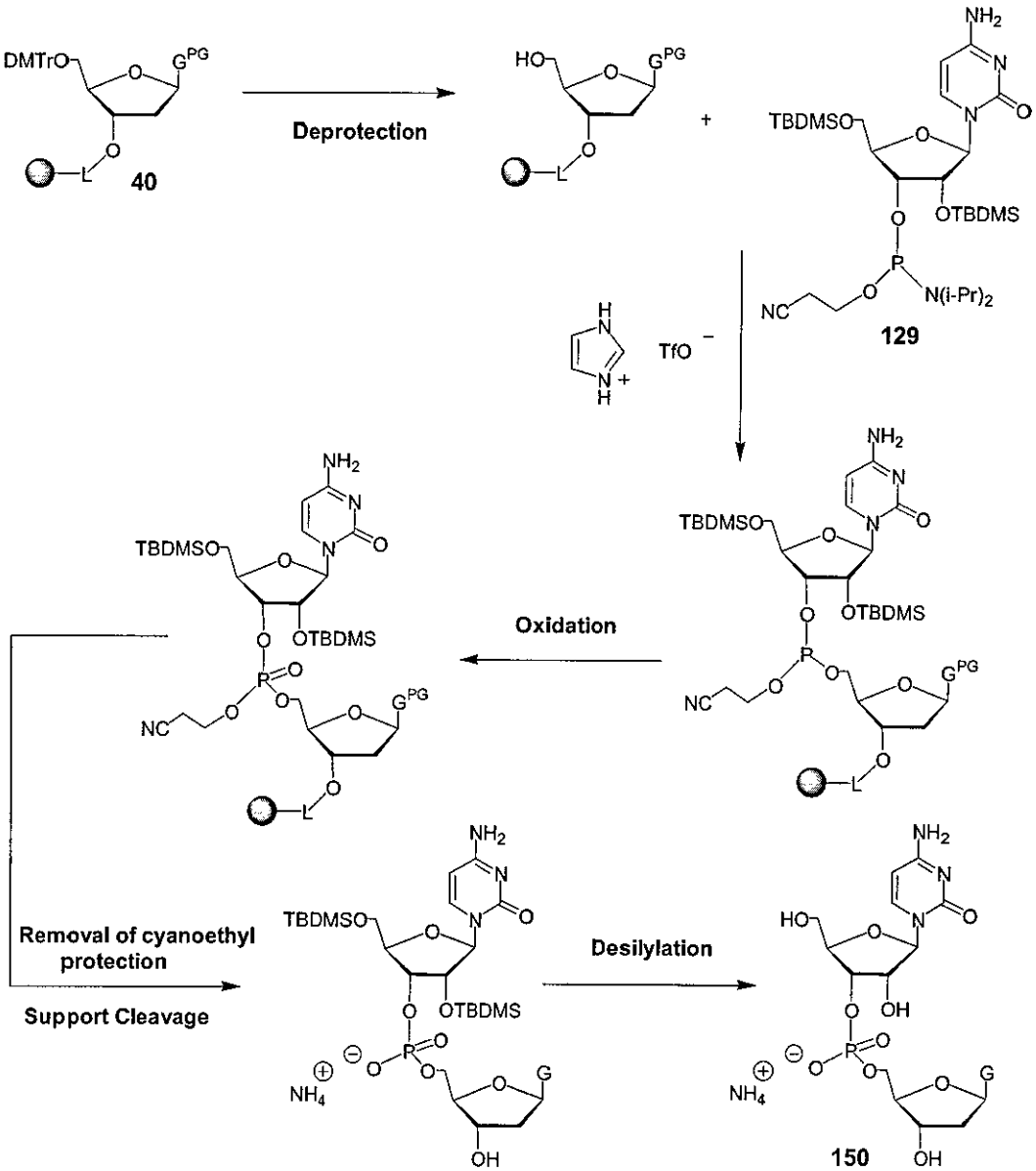


Scheme 86

The isolated yields of the crude CpG 5'-phosphates were 50% for **147**, 52% for **148** and 55% for **149.** The crude products were subjected to mass spectroscopy to confirm their structure.

A high-resolution fast atom bombardment mass spectrum (HRFAB) of **147** provided a single peak at *m/z* 1083.59968 in the region where the molecular peak appears. The result supported the structure of **147** ($C_{51}H_{89}N_8O_{13}P_2$) where the molecular weight is 1083.60244. The same mass spectroscopic analysis of **148** $C_{73}H_{113}N_8O_{13}P_2$ with calculated molecular weight of 1371.79024 gave an eminent peak at *m/z* 1371.78746 where the mass spectra of **149** ($C_{77}H_{121}N_8O_{15}P_2$) with a molecular weight of 1459.84267 showed a peak at 1459.83968.

The presence of other side products was also evident in the mass spectroscopy and elemental analysis of **148** and **149.** Purification of these products could be achieved by reverse phase HPLC analysis



Scheme 87

Nevertheless, the formed crude 5'-di-lipophilic phosphates of CpG dinucleotides **147-150** were sent to Apthon Corporation laboratories to investigate whether the conjugation of lipophilic compounds at the 5'-end of CpG affects their immunostimulatory activity.

We were advised that the immunostimulatory activity of these dinucleotides would be studied *in vitro* using BALB/c mice spleen lympocytes, measured in a lympocyte proliferation assay.

Typically mice spleen lympocytes would be cultured with the dinucleotides at different concentrations for 48h and the proliferation ^{3}H-uridine incorporation would be determined. The secretion of cytokines IL-12, IL-6 and IL-10 known to be induced by the activation of immune cells by CpG would also be studied *in vitro*. Their secretion induced by the formed CpG dinucleotides would be measured at different concentrations in BALB/c mice spleen cell cultures.

The previously synthesized N-carbamate cholesteryl and tocopheryl analogues of guanosine **108** and **109** were also sent to investigate whether the coupling of the lipophilic groups had any effect in the improvement of nucleotide uptake by the cells. Typically the interaction of **108-109** with Krebs-2 ascite carcinoma cells and L-929 mice fibroblasts would be studied and their uptake would be measured at different concentrations.

In summary, excess equivalents of the synthesized phosphoramidites and the promoter were used in the solid-phase synthesis, which can be achieved with good chemoselectivity. Phosphitylation was performed using a 1:1 mixture of the promoter and the cyanoethyl- phosphoramidite or the synthesized di-lipophilic phosphoramidites utilized for the subsequent capping of the 5'OH group

Imidazolium triflate proved to be an excellent promoter towards the reactive N,N-diisopropylphosphoramidites. It allows rapid and highly chemoselective condensation of the phosphoramidite and the corresponding nucleoside. The process was repeated using 1H-tetrazole as the promoter. Disappointingly this approach was not so successful as it produced a considerable amount of N-phosphoryl products of cytosyl derivative.

The results obtained confirmed the efficient synthesis of 5'-di-lipophilic phosphates of CpG and CpG dinucleotide by the phosphoramidite approach without cytidine base protection. The products generally have very good purity in the crude form and were obtained in satisfactory yields.

In comparison with solution phase synthesis, solid support holds an advantage, as it is easier to perform, requires less time as removal of the β-cyanoethyl group, de-N-acylation and cleavage from the polymer support were performed in one step taking 16 hours. The reaction cycle for the chain elongation required overall 75 min in contrast with solution phase, where β-cyanoethyl deprotection required up to 2 days resulting in a lengthy overall synthesis. Although the yields of the monomer units synthesized after each step in solid phase synthesis could not be obtained, the overall yields furnished using both synthetic approaches were similar.

EXPERIMENTAL

1. GENERAL EXPERIMENTAL PROCEDURES

(A) Purification of reagents and solvents

Commercially available reagents were used as supplied from Aldrich, Lancaster, Amersham Pharmacia, Calbiochem-Novabiochem, Fluka, and Fisher Scientific chemical companies without purification unless otherwise stated. Air and moisture sensitive compounds were stored in a dessicator over self-indicating silica gel, under a nitrogen atmosphere. Sodium hydride was 60% dispersion in mineral oil and was washed free from oil with petroleum ether, prior to use.

Light petroleum ether refers to the fractions boiling between 40 ^{0}C and 60 ^{0}C. Ethyl acetate and light petroleum ether were distilled from calcium chloride. Dichloromethane and chloroform were distilled from phosphorus pentoxide or calcium hydride. Methanol was distilled from magnesium methoxide. Tetrahydrofuran was distilled from sodium/benzophenone ketyl radical before use. Triethylamine and diisopropylethylamine were stored over potassium hydroxide pellets. Other solvents such as dimethylformamide, acetonitrile and toluene were purchased as anhydrous or HPLC grade solvents from the chemical companies stated above.

(B) Chromatography techniques

Analytical thin layer chromatography was carried out using either aluminium, plastic or glass plated coated with silica Merck Kieselgel 60 GF_{254} or alumina Merck neutral type E F_{254}. They were visualised under UV light (at 254 and/or 360 nm) or by staining with visualising agents such as potassium permanganate solution or an ethanolic solution of phoshomolybdic acid, (acidified with concentrated sulphuric acid), followed by heating. Flash chromatography was carried out using Merck 9385 Kieselgel 60-45 (230-400 mesh) silica.

(C) Preparation of Glassware

Air and moisture sensitive reactions were carried out using glassware that had been dried overnight in an oven at 150 °C. These were allowed to cool in a dessicator over self-indicating silica gel. All moisture and air sensitive reactions were carried out under a positive pressure of nitrogen. Reagents and solvents were introduced using syringe or cannula techniques, through a septum cap.

(D) Melting point and Elemental Analysis

Elemental analysis was performed on a Perkin-Elmer 2400 CHN elemental analyser. Melting Points were carried out on a Leica Gallin hot plate melting apparatus or an Electrothermal-IA 9100 apparatus and are uncorrected.

(E) Spectroscopic Techniques

Infa-Red (IR) spectra were recorded on a Perkin-Elmer Fourier transform paragon 1000 spectrophotometer. I.R. spectra were recorded in the range 4000-600 cm^{-1}. Samples were run as thin films or nujol mulls on sodium chloride discs.

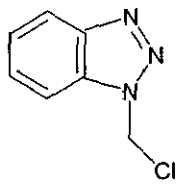
High and low resolution mass spectra (MS) were recorded on a Jeol (JMX)SX102 instrument using electron impact (EI) or fast atom bombardment (FAB) ionisation.

^{1}H , ^{13}C and ^{31}P nuclear magnetic resonance spectra were recorded using a Bruker AC-250 instrument operating at 250.13, 62.85 and 100.20 MHz respectively, or using a Bruker DPX-400 instrument operating at 400.13, 100.59 and 161.97 MHz respectively. Spectra were recorded in deuteriated solvents with reference to tetramethylsilane (TMS) as the internal standard. Chemical shifts are reported as δ values in ppm (parts per million). The coupling constants J are recorded in Hz. Spectroscopic data is annotated with the following notations: s: singlet, br s: broad singlet, d: doublet, q: quartet, m: multiplet. DEPT experiments of ^{13}C-NMR spectrums were also recorded on the same instruments.

2. SYNTHESIS OF LINKERS

(A). Acetal linkers

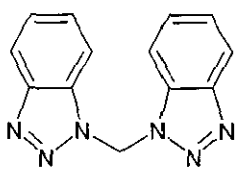
1-chloromethyl-1*H*-benzotriazole (5)



To 1-hydromethyl benzotriazole (40 mmol, 1eq) kept at ice bath temperature, thionyl chloride (240 mmol, 6eq) was added dropwise from a dropping funnel over a period of 1 h with stirring. Vigorous evolution of gas (HCl, SO_2) was manifested as the reaction proceeded. When the evolution of gas ceased, the reaction mixture was heated under reflux at 60-80 ^{0}C for 2h. The resulting solution was rotary evaporated to remove the excess thionyl chloride and the solid obtained was recrystallised from hexane to afford the desired product in a pure form as white crystals.

1-chloromethyl-benzotriazole (79% yield): m.p. 134-136 ^{0}C (lit[208] 136-138 ^{0}C); ν_{max} / cm^{-1} (nujol) 3030, 1613, 1493, 1454, 1071, 745, 692; ^{1}H-NMR (250 MHz, $CDCl_3$) 6.41 (s, 2H, CH_2-Cl), 7.43-7.49 (m, 1H, Ar), 7.60-7.70 (m, 2H, Ar), 8.10-8.13 (m, 1H,Ar), ^{13}C-NMR (100 MHz, $CDCl_3$) 70.3 (N-CH_2), 110.9, 119.1, 124.1, 127.4, 132.3, 145.6; Anal. Calcd for $C_7H_6ClN_3$: C, 50.17; H, 3.61. Found: C, 50.35: H, 3.80;

1-methylene bis-(1*H* benzotriazole) (6)



To sodium hydride dispersion 60% in mineral oil (24 mmol, 1.5eq) washed with petrol, benzotriazole (16 mmol, 1eq) dissolved in 8ml of DMF was added dropwise and a vigorous reaction took place. To the stirred reaction mixture a

solution of 1-chloromethyl benzotriazole (16 mmol, 1eq) in 10ml of DMF was added and the reaction was heated under reflux for 2h. After cooling, the reaction was poured into ice-cold water (20 ml) and the precipitate was collected by suction filtration and washed with additional ice-cold water. The bulk of water was removed through filtration and the resulting product was dissolved in dichloromethane and dried over magnesium sulphate. The drying agent was filtered off and the solvent was evaporated to dryness to afford the title compound as a white solid.

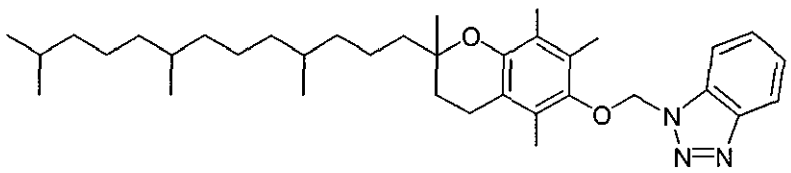
1,1-methylene bis-benzotriazole (69% yield): m.p. 189-190 ^{0}C (lit[208] 191-192.5 ^{0}C); ν_{max} / cm^{-1} (nujol) 3421, 3095, 1614, 1453, 1159, 763, 745; ^{1}H-NMR (250 MHz, $CDCl_3$) 7.21 (s, 2H, CH_2-Cl), 7.32-7.38 (m, 2H, Ar), 7.45-7.55 (m, 2H, Ar), 7.79-7.91 (m, 2H, Ar), 7.97-8.04 (m, 2H, Ar); ^{13}C-NMR (100 MHz, $CDCl_3$), 58.05 (N-CH_2), 109.8 (2C), 120.3 (2C), 124.9 (2C), 128.8 (2C), 135.5 (2C), 144.7 (2C); Anal. Calcd for $C_{13}H_{10}N_6$: C, 62.39: H, 4.03. Found C, 62.20; H, 4.02;

(I) Linkers from cholesterol and tocopherol

1 By using 1-chloromethyl benzotriazole

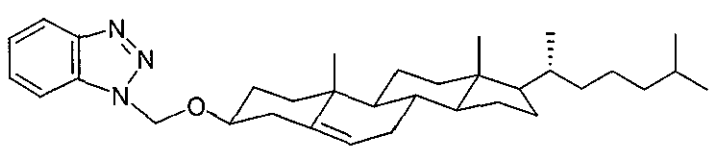
To sodium hydride dispersion 60% in mineral oil (7.5 mmol, 1.5eq) washed with petrol, the lipophilic alcohol (2 mmol, 1eq) dissolved in 6ml of THF was added dropwise and a vigorous reaction took place. To the reaction mixture, 1-chloromethyl benzotriazole (2 mmol, 1eq) dissolved in 8 ml of THF was added under nitrogen, and the reaction was heated under reflux for 4 and 5h for tocopherol and cholesterol, respectively. TLC showed the reaction to be complete. After cooling at room temperature, the reaction mixture was poured into ice water (20 ml) to remove sodium chloride and the solution slowly precipitated a solid. The crude products were isolated by suction filtration as white solid in both cases and were dried over high vacuum. The solid obtained was recrystallized from aqueous ethanol to afford the benzotriazole methyl ether of the lipophilic alcohols as white solids, in both cases.

1H-1,2,3-benzotriazol-1-ylmethyl-[2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-tridecyl) chroman-6-yloxymethyl]-ether (7)



Benzotriazole methyl ether of tocopherol (**7**): crude product 87% yield, recrystallised product 72% yield; m.p. 193-195 ^{0}C; ν_{max} / cm^{-1} (nujol) 3030, 1614, 1577, 1460, 1209, 919, 850, 744; ^{1}H NMR (250 MHz, $CDCl_3$), 0.75-0.95 [m, 12H, (CH_3-CH)$_4$], 1.0-1.6 [m, 20H, $(CH_2)_{10}$], 1.6-1.8 [m, 3H, (C**H**-CH_3)$_3$], 1.97 (s, 3H, CH_3-Ar), 2.08 (s, 3H, CH_3-Ar), 2.11 (s, 3H, CH_3-Ar), 2.32 (s, 3H, CH_3-C-O), 2.5-2.61 (m, 2H, CH_2-Ar), 7.2-7.35 (m, 4H, 2**H** Ar+N-CH_2-O), 7.55-7.6 (m, 1H, Ar), 7.8-8.1 (m, 1H, Ar), ^{13}C-NMR (100 MHz, $CDCl_3$), 11.7, 11.9, 12.8, 19.5, 19.6, 19.6 (2C), 20.3, 20.4, 21.1, 21.2 (2C), 22.9, 23.3, 25.2, 26.4, 31.1, 31.6, 35.8, 35.9, 35.9, 37.8, 73.5, 108.3 (O-CH_2-N), 115.8, 118.7, 121.5, 123.2, 125.1, 126.1, 126.9, 130.7, 139.0, 144.7, 147.9, 168.1; Anal. Calcd for $C_{36}H_{55}N_3O_2$: C, 76.95; H, 9.87; N, 7.48; Found: C, 76.87; H, 9.58, N, 7.35; HRFABMS Calcd for $C_{36}H_{55}N_3O_2$: 561.42943 found 561.4277.

1H-1,2,3-benzotriazol-1-ylmethyl-[17-(1,5-dimethylhexyl]-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-yl ether (8)



Benzotriazole methyl ether of cholesterol (**8**): *crude product 75% yield*, recrystallised form 69% yield; m.p. 128-130 ^{0}C; ν_{max} / cm^{-1} (nujol) 3092, 1613, 1493, 1452, 1157, 763, 744; ^{1}H-NMR (250 MHz, $CDCl_3$), 0.83-2.53 (m, 45H, olefinic), 3.45-3.65 (m, 1H, O-CH), 5.34 (d, 1H, *J* = 2.3 Hz, HC=C), 7.24 (s, 2H N-CH_2-O), 7.35-7.41 (m, 1H Ar), 7.48-7.55 (m, 1H, Ar), 7.84-7.90 (m, 1H,

Ar), 7.98-8.05 (m, 1H, Ar); ^{13}C-NMR (100 MHz, $CDCl_3$), 11.8, 18.5, 19.5, 22.0 (2C), 23.3, 23.5, 24.7, 24.8, 27.1, 27.8, 31.7, 32.0, 32.1, 36.1, 36.3, 36.5, 37.4, 39.2, 39.6, 42.5, 50.1, 56.3, 56.9, 72.1, 75.0 (O-CH_2-N), 109.8, 110.1, 121.6, 124.9, 128.8, 145.8, 121.1 (**C**=CH), 140.9 (CH=**C**); Anal. Calcd for $C_{34}H_{51}N_3O$: C, 78.80; H, 9.92; N, 8.11; Found: C, 78.92; H, 9.71, N, 7.95; HRFABMS Calcd for $C_{34}H_{51}N_3O$: 517.40312 found 517.4018.

2 **By using bis-benzotriazole methane**

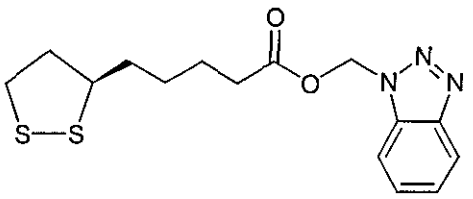
To a stirred solution of bis-benzotriazole methane (2 mmol, 1eq) dissolved in 5 ml of THF, 0.4ml trifluoroacetic acid were added dropwise and the resulting yellow solution were stirred at room temperature. After 15 min, a solution of cholesterol (2 mmol, 1eq) in 5 ml of THF was also added and the reaction mixture was heated slowly under reflux for 4h. The reaction was left to cool at room temperature and after leaving it to stir overnight, TLC showed that the reaction was completed. The solvent was removed under high vacuum to furnish an off-white solid, which was treated with water (10 ml) and neutralised to pH 7 by dropwise addition of saturated aqueous solution of sodium chloride. The mixture was then transferred into a separating funnel where it was extracted thoroughly with dichloromethane (4 x 40 ml). The organic fractions were washed with saturated sodium bicarbonate solution (2 x 20 ml), then collected and dried over magnesium sulphate. The drying agent was filtered off and the solvent was evaporated to dryness, leaving the crude product as a white waxy solid. Recrystallization of the solid from dichloromethane and petroleum ether afforded the desired linker as white solid.

Benzotriazole methyl ether of cholesterol (**8**): crude product 75% yield, recrystallised form 69% yield; m.p. 128-130 ^{0}C; ν_{max} / cm^{-1} (nujol) 3092, 1613, 1493, 1452, 1157, 763, 744; ^{1}H-NMR (250 MHz, $CDCl_3$), 0.83-2.53 (m, 45H, aliphatic), 3.45-3.65 (m, 1H, O-CH), 5.34 (d, 1H, J = 2.3 Hz, HC=C), 7.24 (s, 2H N-CH_2-O), 7.35-7.41 (m, 1H Ar), 7.48-7.55 (m, 1H, Ar), 7.84-7.90 (m, 1H, Ar), 7.98-8.05 (m, 1H, Ar); ^{13}C-NMR (100 MHz, $CDCl_3$), 11.8, 18.5, 19.5, 22.0

(2C), 23.3, 23.5, 24.7, 24.8, 27.1, 27.8, 31.7, 32.0, 32.1, 36.1, 36.3, 36.5, 37.4, 39.2, 39.6, 42.5, 50.1, 56.3, 56.9, 72.1, 75.0 (O-CH_2-N), 109.8, 110.1, 121.6, 124.9, 128.8, 145.8, 121.1 (**C**=CH), 140.9 (CH=**C**); Anal. Calcd for $C_{34}H_{51}N_3O$: C, 78.80; H, 9.92; N, 8.11; Found: C, 78.92; H, 9.71, N, 7.95; HRFABMS Calcd for $C_{34}H_{51}N_3O$: 517.40312 found 517.4018.

(II) Linkers from thioctic acid

5-[(3R)-1,2-dithiolan-3-yl]-pentanoic acid 1H-benzotriazol-1-ylmethyl ester (9)



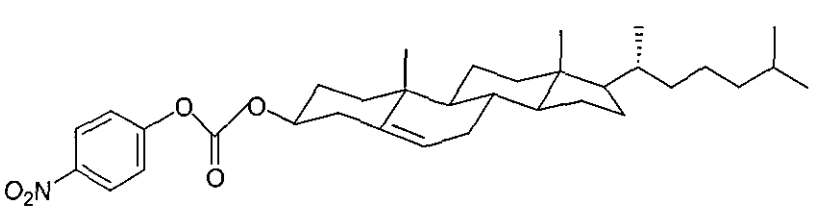
To sodium hydride (7.5 mmol, 1.5eq) washed free from oil with petroleum ether, a solution of thioctic acid (5 mmol, 1eq) in 20 ml of DMF was added dropwise and a vigorous reaction took place (colour of reaction changed to yellow). To the reaction mixture that was stirred under nitrogen, 1-chloromethyl benzotriazole (5 mmol, 1eq) dissolved in 10 ml of THF was added and the reaction was heated slowly under reflux for 4h TLC showed that the reaction was completed. After cooling at room temperature, solvents were evaporated under high vacuum to give a yellow oily residue. The residue was treated with water (20 ml) and was neutralised to pH 7 by dropwise addition of 2M solution of aqueous hydrochloric acid. The mixture was extracted with ethyl acetate (4 x 50 ml) and the organic fractions were washed with sodium bicarbonate solution (2 x 20 ml) and brine (2 x 20 ml). The organic extracts were combined, dried over $MgSO_4$, filtered and evaporated to dryness, to afford the crude product as dark yellow solid. Recrystallization from ethyl acetate and petroleum ether furnished the title compound as pale yellow solid.

Benzotriazole methyl ether of thioctic acid: crude product 77% yield, recrystallised form 62% yield; ν_{max} / cm^{-1} (nujol) 3325, 2862 1732, 1434, 788, 734, 632; ^{1}H-NMR (250 MHz, $CDCl_3$) 1.25-2.0 (m, 6H, CH_2 x 3 aliphatic), 2.05-2.10 (m, 2H, CH_2 cycle) 2.30-2.50 (m, 3H, O=C-CH_2, CH-S cycle), 3.05 - 3.25 (m, 2H, CH_2-S), 6.62 (s, 2H, N-CH_2-O), 7.30-7.45 (m, 1H,Ar), 7.56-7.68 (m, 1H, Ar), 7.72-7.86 (m, 1H, Ar), 8.02-8.18 (m, 1H, Ar); ^{13}C-NMR (100 MHz, $CDCl_3$) 24.3, 28.6, 33.1, 33.7, 38.5, 40.1, 56.2, 67.2 (N-CH_2-O), 110.8, 120.7, 125.3, 126.7, 128.5, 133.2, 178.3 (O=**C**-CH_2 quaternary); Anal. Calcd for $C_{15}H_{19}N_3O_2S_2$: C, 53.39; H, 5.67; N, 12.45; Found C, 53.51; H, 5.52; N, 12.25; HRFABMS Calcd for $C_{15}H_{19}N_3O_2S_2$: 337.09187 found 337.0888.

(B). Carbonate linkers

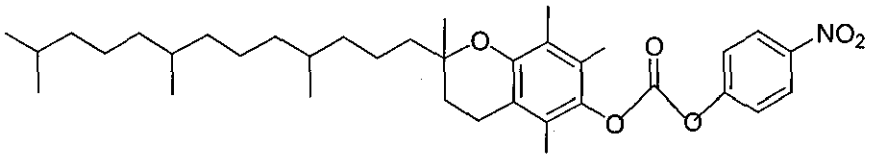
4-Nitrophenyl chloroformate (5 mmol, 1eq), dissolved in 5 ml of CH_3CN, was added dropwise to a stirred solution of tocopherol or cholesterol (5 mmol, 1eq) dissolved in 10ml of anhydrous CH_3CN and 10 ml of anhydrous THF, respectively. The solution, which turned yellow immediately owing to the formation of the p-nitrophenoxy anion, was stirred at room temperature, under N_2. After stirring for 5 min triethylamine (5.5 mmol, 1.1eq) was added dropwise and the colour of the reaction turned to dark yellow. The reaction was stirred overnight and then the solvent was removed *in vacuo*, to afford dark yellow oil in the case of tocopherol and dark yellow solid in the case of cholesterol. Both oil and solid were dissolved in 20 ml dichloromethane and 10ml of water. The residual aqueous phase was extracted with dichloromethane (3 x 20 ml) and the organic fractions were washed with saturated sodium bicarbonate solution (2 x 10 ml) and brine (2 x 10 ml). The organic extracts were combined, dried over $MgSO_4$, filtered and evaporated to dryness, to give a dark yellow oil and solid for tocopherol and cholesterol, respectively. Recrystallization of the cholesterol derivative from dichloromethane and petrol furnished the title compound as yellow solid. The tocopherol derivative was triturated from petroleum ether and the desired linker, was afforded as yellow oil.

Carbonic acid 17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-yl-4-nitrophenyl ester (10)



4-Nitrophenyl-carbonate linker of cholesterol (**10**) crude product 87% yield, recrystallised form 72% yield; m.p. 112-115 ^{0}C; ν_{max} / cm^{-1} (nujol) 1742, 1591, 1458, 1335, 850, 754; ^{1}H-NMR (400 MHz, $CDCl_3$), 0.67-2.34 (m, 45H, aliphatic), 4.51-4.71 (m, 1H, O-CH), 5.35 (d, 1H, *J* = 2.6 Hz, HC=C), 6.80 (d, 2H, *J* = 8.85 Hz), 8.10 (d, 2H, *J* = 7.65 Hz), ^{13}C-NMR (100 MHz, $CDCl_3$), 16.7, 17.3, 19.1, 20.6, 21.9, 22.3 (2C), 23.9, 26.5, 28.5, 29.9, 33.8, 34.2, 34.4, 34.5, 37.5, 37.8, 41.3, 48.3, 54.7, 58.2 70.2, 72.4, 74.8, 75.5, 122.3 (2C), 124.2 (2C), 123.2 (**C**=CH), 144.6, 149.5 (CH=**C**), 159.2, 168.1 (C=O); Anal. Calcd for $C_{34}H_{49}NO_5$: C, 74.04; H, 8.95; N, 2.53 Found: C, 74.19; H, 9.34, N, 2.45; HRFABMS Calcd for $C_{34}H_{49}NO_5$: 551.36107 found 551.3589.

Carbonic acid 2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-tridecyl) chroman-6-yl-4-nitrophenyl ester (11)

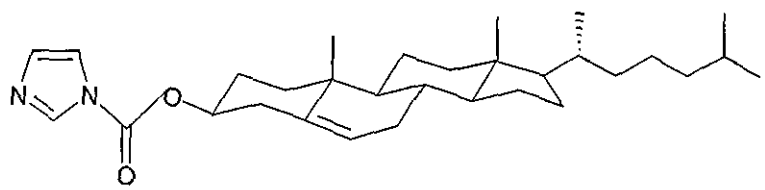


4-Nitrophenyl-carbonate linker of tocopherol (**11**) crude product 92% yield, pure form 83% yield ν_{max} / cm^{-1} (neat) 1746, 1558, 1461, 1204, 1085, 858; ^{1}H NMR (400 MHz, $CDCl_3$), 0.75-0.95 [m, 12H, ($C\mathbf{H}_3$-CH)$_4$], 1.0-1.6 [m, 20H, $(CH_2)_{10}$], 1.6-1.8 [m, 3H, ($C\mathbf{H}$-CH_3)$_3$], 1.97 (s, 3H, CH_3-Ar), 2.08 (s, 3H, CH_3-Ar), 2.11 (s, 3H, CH_3-Ar), 2.32 (s, 3H, CH_3-C-O), 2.5-2.61 (m, 2H, CH_2-Ar), 7.48 (d, 2H, *J* = 8.65 Hz), 8.31 (d, 2H, *J* = 7.42 Hz), ^{13}C-NMR (100 MHz, $CDCl_3$), 16.9, 19.5, 20.1 (2C), 20.9, 22.4 (2C), 21.1, 21.2, 22.9, 23.3, 25.3, 28.5, 31.2, 32.1, 32.4, 34.7, 35.6, 35.9, 37.5, 37.8, 39.6, 41.2, 46.2, 74.7, 121.5, 123.1, 124.2 (2C), 129.8, 130.5 (2C), 142.8, 148.5, 155.2, 168.5 (C=O); Anal. Calcd for $C_{36}H_{53}NO_6$: C, 72.60; H, 8.97; N, 2.35; Found C, 72.74; H, 9.14; N, 2.43; HRFABMS Calcd for $C_{36}H_{53}NO_6$: 595.38729 found 595.3848.

(C) Carbamate linkers

A solution of cholesterol or tocopherol (5 mmol, 1eq) in dry toluene (30 ml) was treated with N',N'-Carbonyl-diimidazole (5.5 mmol, 1.1eq). The reaction mixture was heated under reflux for 12h for cholesterol and 10h for tocopherol. After the reaction was left to cool down at room temperature, the solvent was evaporated to dryness to reveal off-brown oil and white solid for tocopherol and cholesterol, respectively. Both residues were dissolved with in ethyl acetate (40 ml) and water (10 ml). The organic layer was separated. The aqueous phase was neutralised by dropwise addition of 0.5 N HCl (5 ml) and then extracted further with ethyl acetate (4 x 40 ml). After washing the combined organic fractions with saturated sodium bicarbonate solution (2 x 10 ml) and brine (2 x 10 ml), they were dried over magnesium sulphate, filtered and evaporated to dryness. The novel carbamate linkers of tocopherol and cholesterol were furnished, as pale-yellow oil after trituration from hot hexane and white solid after recrystallization from dichloromethane and petrol, respectively.

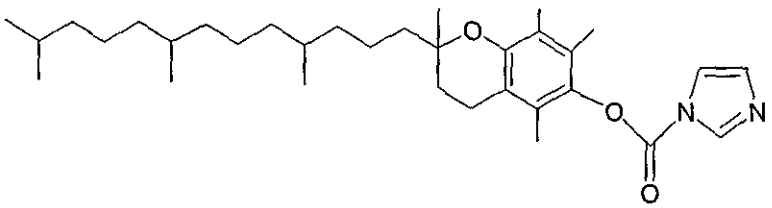
Imidazole-1-Carboxylic acid 17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-3-yl ester (13)



Carbonylimidazolide carbamate linker of cholesterol (**13**) crude product 78% yield, recrystallised form 71% yield; m.p. 108-110 ^{0}C; ν_{max} / cm^{-1} (nujol) 1748, 1591, 1484, 1407, 1301, 1011.8, 765, 650; ^{1}H-NMR (400 MHz, $CDCl_3$), 0.66-2.45 (m, 45H, aliphatic), 4.80-4.86 (m, 1H, O-CH), 5.44 (d, 1H, *J* = 2.4 Hz, HC=C), 7.05 (s, 1H, CH imid), 7.41 (s, 1H, CH imid), 8.13 (s, 1H, C**H**=N), ^{13}C-NMR (100 MHz, $CDCl_3$), 17.2, 18.3, 18.5, 19.1, 20.6, 21.2, 22.5 (2C), 23.9, 25.3, 28.5, 29.4, 30.6, 33.5, 34.4, 35.8, 35.9, 39.7, 45.7, 48.4, 51.2, 54.2, 57.8, 71.4, 113.7, 121.2, 122.3 (**C**=CH), 123.2 (2C), 136.8, 149.5 (CH=**C**),

168.5 (C=O); Anal. Calcd for $C_{31}H_{48}N_2O_2$: C, 77.45; H, 10.06; N, 5.83; Found C, 77.78; H, 9.95; N, 5.94; HRFABMS Calcd for $C_{31}H_{48}N_2O_2$: 480.37157 found 480.3698.

Imidazole-1-Carboxylic acid 2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-tridecyl) chroman-6-yl ester (14)

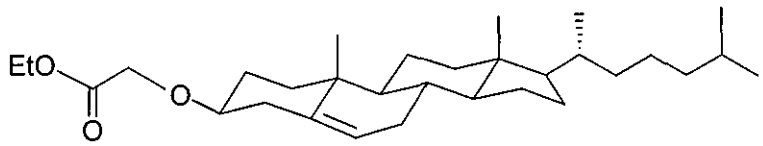


Carbonylimidazolide carbamate linker of tocopherol (**14**) crude product 84% yield, pure form 76% yield; ν_{max} / cm^{-1} (neat) 1754, 1464, 1384, 1232, 1160, 993, 763, 658; ^{1}H NMR (400 MHz, $CDCl_3$), 0.75-0.99 [m, 12H, (CH_3-CH)$_4$], 1.0-1.4 [m, 20H, $(CH_2)_{10}$], 1.6-1.8 [m, 3H, (C**H**-CH_3)$_3$], 1.96 (s, 3H, CH_3-Ar), 2.04 (s, 3H, CH_3-Ar), 2.12 (s, 3H, CH_3-Ar), 2.25 (s, 3H, CH_3-C-O), 2.51-2.64 (m, 2H, CH_2-Ar), 7.08 (s, 1H, CH imid), 7.52 (s, 1H, CH imid), 8.25 (s, 1H, C**H**=N), ^{13}C-NMR (100 MHz, $CDCl_3$), 13.3, 20.1, 20.2 (2C), 22.9 (2C), 23.2, 24.8, 25.2, 28.3, 33.2, 37.7, 37.8, 39.7, 40.3, 53.8, 74.6, 75.8, 77.1, 77.4, 77.6, 77.7, 121.5, 122.4 (2C), 123.1, 126.9, 130.6, 135.3, 137.8, 147.6, 156.2, 168.4 (C=O); Anal. Calcd for $C_{33}H_{52}N_2O_3$: C, 75.53; H, 9.99; N, 5.34; Found C, 75.64; H, 9.83; N, 5.25; HRFABMS Calcd for $C_{33}H_{52}N_2O_3$: 524.39779 found 524.3953.

(D) Ester linkers

To sodium hydride (7.5 mmol, 1.5eq) washed free from oil with petroleum ether, lipophilic alcohol (5mmol, 1eq) dissolved in 20 ml of THF was added dropwise and a vigorous reaction took place. To the reaction mixture that was stirred under nitrogen, ethyl bromoacetate (5.5 mmol, 1.1eq) was added dropwise. The reaction was stirred overnight for 12h and then allowed to cool down at room temperature. TLC showed the reaction to be complete. The solvent was removed under high vacuum, to give an off-yellow residue in both cases. The residues were treated with water (10 ml) and were neutralised to pH 7 by dropwise addition of 2M solution of aqueous hydrochloric acid. The aqueous phase was extracted with dichloromethane (5 x 30 ml) and the combined organic fractions were washed with saturated sodium bicarbonate solution (2 x 20 ml) and brine (2 x 20 ml). The organic extracts were dried over magnesium sulphate, filtered and evaporated to dryness, to afford the crude derivatives of tocopherol and cholesterol. Recrystallization of the crude ethyl ester of cholesterol from dichloromethane and petroleum ether furnished the title compound as off-white solid. The crude ethyl ester of tocopherol was triturated from hot hexane to afford the desired product as pale yellow oil.

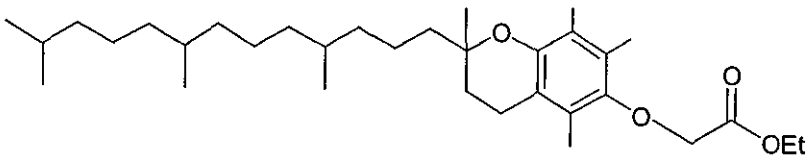
[17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-yloxy]-acetic acid ethyl ester (47)



Ethyl ester of cholesterol (**47**) crude product 88% yield, recrystallized form 78% yield; m.p. 108-110 ^{0}C; ν_{max} / cm^{-1} (nujol) 1739, 1463, 1376, 1175, 1028, 762, 683; ^{1}H-NMR (400 MHz, $CDCl_3$), 0.67-2.35 (m, 45H, aliphatic), 1.41 (t, 3H, J = 7.13, CH_2-**C**H_3,), 2.92 (s, 2H, **C**H_2-CO), 4.31 (q, 2H, **C**H_2-CH_3), 4.61-4.70 (m, 1H, O-CH), 5.36 (d, 1H, J = 2.2 Hz, HC=C), ^{13}C-NMR (100 MHz, $CDCl_3$), 17.3, 18.5, 21.2, 22.6 (2C), 23.1, 23.9, 26.4, 28.5, 29.4, 30.6, 34.2,

35.6, 35.8, 36.8, 39.6, 42.5, 43.8, 46.7, 48.8, 51.2, 59.5, 62.5, 72.2, 75.2, 113.7, 121.2, 122.8 (**C**=CH), 128.5, 148.4 (CH=**C**), 165.4 (C=O); Anal. Calcd for $C_{31}H_{52}O_3$: C, 78.76; H, 11.10 Found C, 78.84; H, 11.02; HRFABMS Calcd for $C_{31}H_{52}O_3$: 472.39164 found 472.3897.

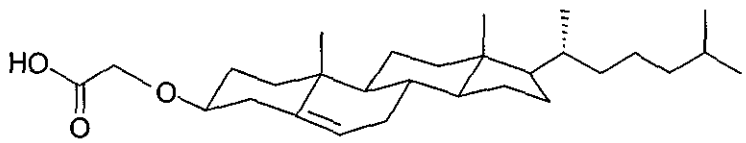
[2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-tridecyl) chroman-6-yloxy]-actic acid ethyl ester (48)



Ethyl ester of tocopherol (**48**) crude product 91% yield, purified form 82% yield; ν_{max} / cm^{-1} (neat) 1751, 1459, 1392, 1245, 1182, 993, 774, 665; ^{1}H NMR (400 MHz, $CDCl_3$), 0.73-0.97 [m, 12H, (CH_3-CH)$_4$], 1.0-1.5 [m, 20H, $(CH_2)_{10}$], 1.58 (t, 3H, J = 7.22, CH_2-CH_3,), 1.64-1.82 [m, 3H, (CH-CH_3)$_3$], 1.97 (s, 3H, CH_3-Ar), 2.08 (s, 3H, CH_3-Ar), 2.15 (s, 3H, CH_3-Ar), 2.31 (s, 3H, CH_3-C-O), 2.52-2.64 (m, 2H, CH_2-Ar), 2.98 (s, 2H, CH_2-CO), 4.42 (q, 2H, CH_2-CH_3), ^{13}C-NMR (100 MHz, $CDCl_3$), 13.6, 17.1, 19.4 (2C), 20.0, 21.2, 22.3 (2C), 24.9, 26.3, 28.52 32.2, 37.8, 39.7, 41.3, 46.3, 61.5, 74.7, 75.6, 76.1, 76.6, 77.6, 120.6, 121.7, 122.9, 123.7, 128.7, 131.7, 132.5, 134.5, 151.4, 154.0, 164.0 (C=O); Anal. Calcd for $C_{33}H_{56}O_4$: C, 76.69; H, 10.92 O 12,38 Found C, 76.81; H, 10.78; HRFABMS Calcd for $C_{33}H_{56}O_4$: 516.41786 found 516.4158.

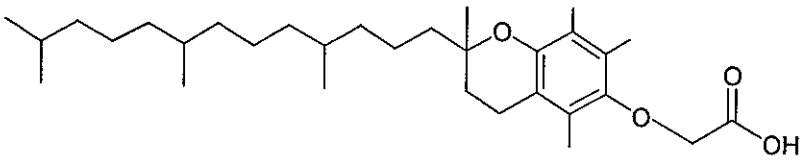
To a solution of ethyl acetate of cholesterol **47** or tocopherol **48** (5 mmol, 1eq) in 20 ml of ethanol, 10 ml of 2M solution of aqueous potassium hydroxide were added. The reaction mixture was heated under reflux for 5h and then allowed to stir overnight while attaining ambient temperature. The solvent was removed under high vacuum and the aqueous residue was acidified to pH 1 by dropwise addition of 2M solution of aqueous hydrochloric acid and then extracted thoroughly with dichloromethane (5 x 30ml). The organic extracts were combined, dried over $MgSO_4$, filtered and evaporated to dryness, to give the crude carboxylic acids of cholesterol and tocopherol. Recrystallization of the crude derivative of cholesterol from ethyl acetate and dichloromethane furnished the title compound as an off-white solid. The crude carboxylic acid of tocopherol was triturated with hot hexane to afford the desired product as an off-yellow oil.

[17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-yloxy] acetic acid (49)



Carboxylic acid of cholesterol (**49**) crude product 83% yield, recrystallized form 72% yield; ν_{max} / cm^{-1} (nujol) 2857, 1732, 1469, 1373, 1129, 1051, 958; ^{1}H-NMR (400 MHz, $CDCl_3$), 0.69-2.45 (m, 45H, aliphatic), 2.78 (s, 2H, C**H**$_2$-CO), 4.63-4.71 (m, 1H, O-CH), 5.37 (d, 1H, J = 2.4 Hz, HC=C), 8.03 (br s, 1H, OH), ^{13}C-NMR (100 MHz, $CDCl_3$), 18.9, 21.9, 22.5 (2C), 23.4, 24.4, 27.8, 29.4, 32.7, 33.2, 34.1, 34.8, 35.8, 36.8, 39.6, 41.3, 43.5, 47.4, 48.2, 52.2, 63.6, 73.6, 76.4, 115.7, 122.8, 123.1 (C=**C**H), 129.2, 149.5 (CH=**C**), 176.1 (C=O), Anal. Calcd for $C_{29}H_{48}O_3$: C, 78.33; H, 10.88 O 10,79 Found C, 78.45; H, 10.71; HRFABMS Calcd for $C_{29}H_{48}O_3$: 444.36035 found 444.3593.

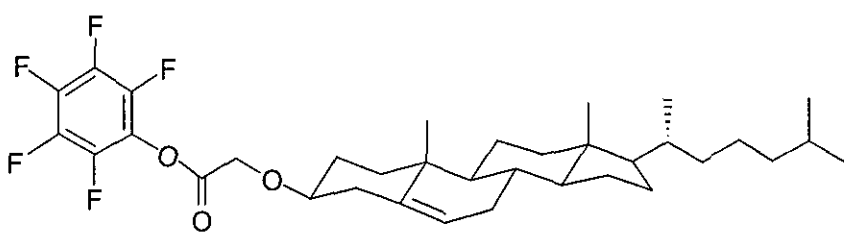
[2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-tridecyl) chroman-6-yloxy] acetic acid (50)



Carboxylic acid of tocopherol (**50**) crude product 89% yield, purified form 77% yield; ν_{max} / cm^{-1} (neat) 2875, 1752, 1463, 1385, 1252, 838, 757; ^{1}H NMR (400 MHz, $CDCl_3$), 0.75-0.98 [m, 12H, $(CH_3\text{-}CH)_4$], 1.02-1.8 [m, 20H, $(CH_2)_{10}$], 1.66-1.84 [m, 3H, $(CH\text{-}CH_3)_3$], 1.99 (s, 3H, CH_3-Ar), 2.11 (s, 3H, CH_3-Ar), 2.19 (s, 3H, CH_3-Ar), 2.34 (s, 3H, CH_3-C-O), 2.55-2.67 (m, 2H, CH_2-Ar), 2.74 (s, 2H, CH_2-CO), 8.06 (br s, 1H, OH); ^{13}C-NMR (100 MHz, $CDCl_3$), 17.5, 19.8 (2C), 21.7, 22.8 (2C), 23.4, 25.3, 28.5, 30.8, 35.2, 38.7, 40.25, 41.8, 46.4, 74.9, 75.9, 76.8, 77.4, 77.9, 78.4, 120.8, 121.9, 123.7, 124.8, 129.5, 132.6, 135.7, 152.8, 155.2, 177.0 (C=O); Anal. Calcd for $C_{31}H_{52}O_4$: C, 76.18; H, 10.72, O 13,09 Found C, 76.25; H, 10.61; HRFABMS Calcd for $C_{31}H_{52}O_4$: 488.38656 found 488.3846.

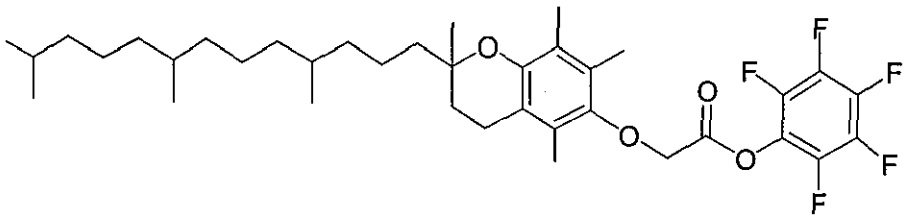
To a stirred solution of 2,3,4,5,6-pentaflurorophenol (5 mmol, 1eq) dissolved in 10ml of anhydrous DMF, a solution of the carboxylic acid of cholesterol **49** or tocopherol **50** (5 mmol, 1eq) in DMF (10ml) was added. In the resulting reaction mixture which was cooled in an ice bath to 0-5 ^{0}C and put under nitrogen, 1-ethyl-3-(-3'-dimethylaminopropyl)carbodiimide hydrochloride) (EDCI) (5.5 mmol, 1.1eq), was added neat, while 1,3-dicyclohexylcarboddimide (DCC) (5.5 mmol, 1.1eq), was dissolved in 5 ml of DMF. The addition of the coupling reagent was made in both cases proportionally every 10 min. Within 30 min the addition had concluded and the solution was left to stir at room temperature for 4h. The solvent was evaporated to dryness under high vacuum and the residue that resulted was dissolved in 40ml of ethyl acetate and 2 ml of 2M aqueous hydrochloric acid. The aqueous layer was extracted with further with ethyl acetate (4 x 20 ml) and the combined organic fractions were washed sequentially with saturated sodium bicarbonate solution (2 x 20 ml) and brine (2 x 20 ml). The organic extracts were dried over magnesium sulphate, filtered and evaporated to dryness to furnish the pentafluorophenyl ester derivatives of cholesterol and tocopherol as waxy solid and thick oil, respectively. Recrystallization from diethyl ether and hexane gave the desired purified linkers of cholesterol and tocopherol as white solid and off yellow solid, respectively.

[17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-yloxy] acetic acid pentaflurorophenyl ester (16)



Pentafluorophenyl ester linker of cholesterol (**16**): crude product 74-78% yield, recrystallized form 62-68% yield; ν_{max} / cm^{-1} (nujol) 1745, 1473, 1354, 1258, 1172, 1048, 945, 755; ^{1}H-NMR (400 MHz, $CDCl_3$), 0.70-2.47 (m, 45H, aliphatic), 2.80 (s, 2H, C**H**$_2$-CO), 4.64-4.73 (m, 1H, O-CH), 5.38 (d, 1H, J = 2.5 Hz, HC=C); ^{13}C-NMR (100 MHz, $CDCl_3$), 17.9, 18.5, 21.2, 22.6 (2C), 23.1, 23.9, 27.5, 28.2, 30.6, 32.1, 33.5, 34.2, 34.9, 35.8, 38.5, 41.1, 42.8, 47.4, 48.4, 51.4, 62.6, 71.2, 75.1, 114.5, 122.8 (C=**C**H), 129.2, 125.9, 133.7 (2C), 136.1, 140.8 (2C), 149.2 (CH=**C**), 170.2 (C=O), Anal. Calcd $C_{35}H_{47}F_5O_3$: C, 68.83; H, 7.76, O 7,86 Found C, 68.98; H, 7.61; HRFABMS Calcd for $C_{35}H_{47}F_5O_3$: 610.34454 found 610.3419.

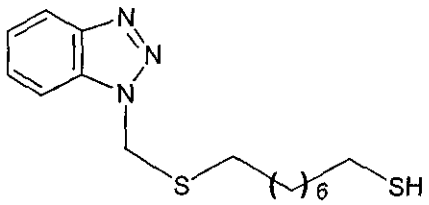
[2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-tridecyl) chroman-6-yloxy] acetic acid pentaflurorophenyl ester (17)



Pentafluorophenyl ester linker of tocopherol (**17**): crude product 80-86% yield, recrystallized form 67-73% yield; ν_{max} / cm^{-1} (nujol) 1742, 1482, 1375, 1332, 1264, 977, 832, 695; ^{1}H NMR (400 MHz, $CDCl_3$), 0.75-0.98 [m, 12H, (CH_3-CH)$_4$], 1.06-1.85 [m, 20H, $(CH_2)_{10}$], 1.69-1.86 [m, 3H, (C**H**-CH_3)$_3$], 2.04 (s, 3H, CH_3-Ar), 2.15 (s, 3H, CH_3-Ar), 2.23 (s, 3H, CH_3-Ar), 2.38 (s, 3H, CH_3-C-O), 2.59-2.71 (m, 2H, CH_2-Ar), 2.82 (s, 2H, C**H**$_2$-CO), ^{13}C-NMR (100 MHz, $CDCl_3$), 17.2, 19.4, 20.2 (2C), 20.7, 22.3 (2C), 24.1, 25.4, 28.5, 32.5, 34.3, 35.5, 37.8, 39.7, 41.2, 46.1, 74.7, 75.8, 76.4, 77.1, 77.6, 120.6, 121.2, 121.9, 123.7, 125.8, 129.5, 132.6, 134.7 (2C), 136.5, 141.2 (2C), 151.8, 154.7, 174.0 (C=O); Anal. Calcd for $C_{37}H_{51}F_5O_4$: C, 67.87; H, 7.85, O 9,77 Found C, 67.95; H, 7.92; HRFABMS Calcd for $C_{37}H_{51}F_5O_4$: 654.37075 found 654.3692.

(E) Linker for linking the antibody to DNA

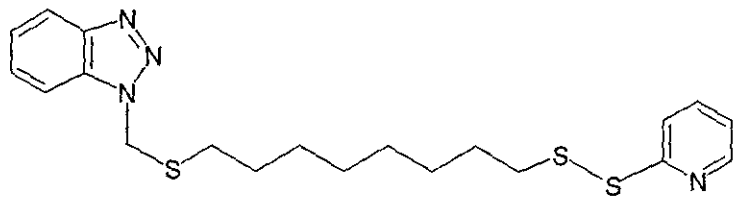
8-[(1*H*-1,2,3-benzotriazol-1-ylmethyl)thio]octyl hydrosulfide (54)



In a solution of 1,8-octanedithiol (5 mmol, 1.25eq) in 8 ml of CH_3CN, 1-chloromethyl benzotriazole (4 mmol, 1eq) dissolved in 5 ml of CH_3CN was added and the solution was stirred at room temperature. After 5min triethylamine (5 mmol, 1.25eq) was added (a change in the colour was noted) and the reaction mixture was heated under reflux for 3h. The solution was left to stand at room temperature for 30 min and white crystals started precipitating. The crystals were collected by suction filtration and the filtrate was rotary evaporated to furnish off-yellow oil. The oil was dissolved in dichloromethane (40 ml) and water (20 ml) and the aqueous layer was extracted further with dichloromethane (4 x 30ml). The combined organic fractions were washed with saturated sodium bicarbonate solution (2 x 20 ml) and brine (2 x 20 ml) and then dried over magnesium sulphate. After filtration of the drying agent and evaporation of the solvent, the title compound was furnished as a yellow oil.

Thioaminal compound (**54**): 70% yield; ν_{max} / cm^{-1} (neat) 3063, 2558, 1226, 1150, 778, 716, 661; ^{1}H-NMR (250 MHz, $CDCl_3$) 1.15-163 [m, 12H, $(CH_2)_6$], 2.47-2.55 (m, 4H, C**H**$_2$-S, C**H**$_2$-SH), 2.65 (t, 1H, *J* =4.2.1Hz, CH_2-S**H**), 5.69 (s, 2H, N-CH_2-S), 7.40 (t, 1H, *J* = 8.1Hz, Ar), 7.51 (t, 1H, *J* = 8.1Hz, Ar), 7.68 (d, 1H, *J* = 8.2Hz, Ar), 8.06 (d, 1H, *J* = 8.0 Hz, Ar); ^{13}C-NMR (100 MHz, $CDCl_3$) [24.9, 28.6, 28.7, 29.4, 29.5, 31.7, 34.3, 39.4] (8 x CH aliphatic), 49.5 (N-CH_2-S), 110.2, 120.4, 124.6, 128.4, 132.3 (C quaternary, Ar), 146.8 (C quaternary, Ar); Anal. Calcd for $C_{15}H_{23}N_3S_2$: C, 58.21; H, 7.49; N, 13.58; Found C, 57.31; H, 7.74; N, 13.87; HRFABMS Calcd for $C_{15}H_{23}N_3S_2$: 309.13334 found 309.1306.

8-[(1*H*-1,2,3-benzotriazol-1-ylmethyl)thio]octyl pyridin-2-yl disulfide (19)



In a solution of the thioaminal compound **54** (2 mmol, 1eq) in 5 ml of dichloromethane, pyridyl disulfide (4 mmol, 2eq) also dissolved in 4 ml of dichloromethane, was added. A change in colour was observed and the reaction mixture was left to stir to room temperature overnight. After allowing the mixture to cool down at room temperature, the solvent was evaporated to dryness to afford a yellow residue that was partitioned between 30 ml of ethyl acetate and 10 ml of water. The aqueous layer was extracted further with ethyl acetate (4 x 40ml) and the combined organic fractions were washed sequentially with saturated sodium bicarbonate solution (2 x 10ml) and brine (2 x 10ml). The organic extracts were dried over magnesium sulphate, filtered and evaporated to dryness, to furnish a yellow solid. The crude product was purified by flash column chromatography over silica eluting with a 3:1 mixture of petroleum ether and ethyl acetate to afford the desired product as yellow oil.

8-[(1H-1,2,3-benzotriazol-1-ylmethyl)thio]octyl pyridin-2-yl disulfide (**19**): 55% yield; ν_{max} / cm^{-1} (neat) 3043, 2552, 1571, 1225, 1139, 1116, 758, 718, 665; ^{1}H-NMR (250 MHz, $CDCl_3$) 1.15-1.30 (m, 8H, $(CH_2)_4$], 1.56-1.68 (m, 4H, $(CH_2)_2$], 2.44-2.68 (m, 2H, C**H**$_2$-S-CH_2-N), 2.72-2.79 (m, 2H, CH_2-S-S), 5.67 (s, 2H, N-CH_2-S), 7.03-7.10 (m, 1H,Ar), 7.44-7.54 (m, 2H, Ar), 7.62-7.73 (m, 2H, Ar), 8.12-8.23 (m, 2H, Ar), 8.42-8.47 (m, 1H, Ar); ^{13}C-NMR (100 MHz, $CDCl_3$) [28.2, 28.6, 29.1, 29.4, 29.6, 29.7, 31.7, 39.1] (8 x CH aliphatic), 49.3 (N-CH_2-S), [110.8, 119.7, 120.2, 120.7, 121.1, 124.7, 128.5, 132.1] (8 x CH aromatic), 137.4 (C quart, Ar), 137.6 (C quart, Ar), 149.5 (C quart, Ar); Anal. Calcd for $C_{20}H_{26}N_4S_3$: C, 57.38; H, 6.26; N, 13.38; Found C, 56.86; H, 6.13; N, 13.12 HRFABMS Calcd for $C_{20}H_{26}N_4S_3$: 418.13196 found 418.1288.

BINDING OF THE SYNTHETIC PEPTIDES AK79, THE PLASMID AND THE BISINDOLYLMALEIMIDE III HCl WITH GRE34 ANTIBODY

Stock solutions were made for each of the peptides, the plasmid, the bisindolylmaleimide III HCl, the linker, iminothiolane and the G34 antibody.

Synthetic Peptide A (LRQAGDDFC) stock solution: 9.85 mg in 10 ml of 3:1 mixture H_2O:MeOH

Synthetic Peptide B (LRRMSDEFC) stock solution: 11.35 mg in 10 ml of 3:1 mixture H_2O: MeOH

Plasmid (pSV-beta-Galactosidase Control Vector) stock solution: 40 μg=9.2 pmol in 23 μl of MeOH

Bisindolylmaleimide III HCl stock solution: 1 mg=2.4 μmol in 500 μl of H_2O

Linker 19 stock solution: 2000 nmol=2 μmol=0.8 mg of linker in 2000 μl of CH_3CN

Iminothiolane stock solution: 1 μmol=0.138 mg in 1000 μl of MeOH

CGGS-G34(16-34): Cys-23-Phe-NH_2 stock solution: 42 mg=1.57 μmol in 1000 μl of MeOH

Peptides A and B, the plasmid and the bisindolylmaleimide III HCl coupled with the existing linker, were bound to G34, which was already coupled with iminothiolane. 4 reaction mixtures were made. In the **1st mixture**: 400 μl (0.4 μmol) of linker stock solution was added to 400 μl (0.4 μmol) of peptide A stock solution while, in the **2nd mixture**: 400μl (0.4 μmol) of linker stock solution was added to 400 μl (0.4 μmol) of peptide B stock solution. In the **3rd mixture**: 400 μl (0.4 μmol) of linker stock solution was added to 83 μl (0.4 μmol) of bisindolylmaleimide III stock solution while in the **4th mixture:** 23 μl (9.2 pmol) of linker stock was added to 9.2 pmol of plasmid stock solution. The 4 mixtures were shaken gently at room temperature for 30 min.

In the meanwhile another **3 mixtures** were made and in each one, 400 μl (0.4 μmol) of iminothiolane stock solution was added to 250 μl (0.4 μmol) of G34 stock solution. A **4th mixture** was made specially in order to bound the G34 to the plasmid (already coupled to the linker) as only a pmol quantity of plasmid was available.

In this mixture 23 μl (9.2 pmol) of iminothiolane stock solution was added to 23 μl (9.2 pmol) of G34 stock solution The 4 mixtures were shaken gently at room temperature for 30 min. These were then added successively to the 4 mixtures of Peptides A and B, bisindolylmaleimide III HCl and plasmid (already coupled to the linker). The 4 reaction mixtures obtained were again shaken gently at room temperature for 40 min and kept at fridge temperature (4-6 ^{0}C).

BINDING OF THE SYNTHETIC PEPTIDES AK79 AND THE LINEARISED ANTISENSE DNA (OLIGO) TO THE GRE1 AND GRE4 ANTIBODY COLUMNS

Stock solutions were made for each of the peptides, the Linearised Antisense DNA, the linker and iminothiolane.

Linearised Antisense DNA (Oligo) stock solution: 1 μmol=1000 nmol in 400 μl of MeOH

Synthetic Peptide A (LRQAGDDFC) stock solution: 9.85 mg in 10 ml of 3:1 mixture H_2O: MeOH

Synthetic Peptide B (LRRMSDEFC) stock solution: 11.35 mg in 10 ml of 3:1 mixture H_2O: MeOH

Linker 19 *stock solution: 2000 nmol=2 μmol=0.8 mg of linker in 2000 μl of* CH_3CN

Iminothiolane stock solution: 1 μmol=0.138 mg in 1000 μl of MeOH

Peptides A and B, and the Linearised Antisense DNA (Oligo) coupled with the existing linker, were bound to GRE1 and GRE4 antibody columns, which were already coupled with Iminothiolane. 3 mixtures were made.

In the **1st mixture**: 60 μl (60 μmol) of linker stock solution was added to 60 μl of peptide A stock solution, in the **2nd mixture**: 60 μl (60 μmol) of linker stock solution was added to 60 μl of peptide B stock solution while in the **3rd mixture**: 400 μl (400 μmol) of linker stock solution was added to 400 μl of oligo stock solution. The 3 mixtures were shaken gently at room temperature for 30 min.

In the meanwhile 60 µl of the iminothiolane stock solution was added to each of the 4 columns, which were then shaken gently at room temperature for 30 min. Excess of iminothiolane was removed by washing with methanol. After that, peptides A and B and the oligo were bound to the antibody columns. The aim was to use 10 fold of oligo and linker reaction mixture for the GRE1 column and 100 fold of the same reaction mixture for the GRE4 column. 10 fold of peptide A and B and linker reaction mixtures were used for the other two GRE1 and GRE4 columns.

60 µl (10x fold) of the **1st mixture** was added to the **1st GRE1** column while; **60 µl (10x fold)** of the **2nd mixture** was added to the **1st GRE4** column. **60 µl (10x fold)** of the **3rd mixture** was added to the **2nd GRE1** column while; **600 µl (100x fold)** of the **3rd mixture** was added to the **2nd GRE4** column. The columns were shaken gently at room temperature for 40 min. After washing out excess of the reagents with methanol, the columns were kept at fridge temperature (4-6 ^{0}C).

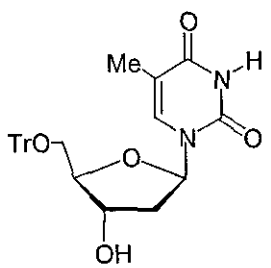
2. GLOBAL AND PARTIAL PROTECTION OF NUCLEOSIDES

(A). Protection of the 5'-position of nucleosides

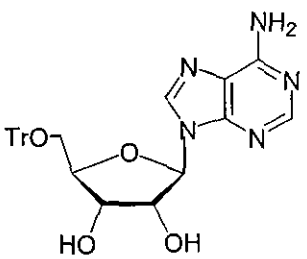
(I) Using Triphenylmethyl chloride (56)

5'-O-trityl-2'-deoxythymidine (57) and 5'-O-trityl-adenosine (58)

To a magnetically stirred solution of 2'-deoxythymidine (suspension for adenosine) (5 mmol, 1eq) in dry pyridine (10 ml), triphenylmethyl chloride (7.5 mmol, 1.5eq) was added along with a catalytic amount of 4-dimethylaminopyridine (0.125 mmol). The reaction mixture was left to stir at room temperature for 2h. The solution was evaporated to dryness under high vacuum and the residue furnished was dissolved in ethyl acetate (20 ml) and water (10 ml). The aqueous layer was extracted with ethyl acetate (3 x 25 ml) and the combined organic layers were washed with saturated sodium bicarbonate solution (2 x 10 ml). The organic fractions were dried over $MgSO_4$, the drying agent was filtered off and the solvent was rotary evaporated. A glossy residue was afforded which was recrystallized from ethanol and petrol to afford the 5'-O-trityl nucleosides as white crystalline solids.



57



58

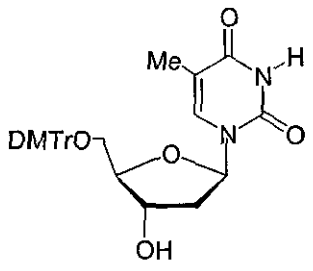
5'-O-trityl-2'-deoxythymidine **57**, 71% yield, m.p. 126-128 ^{0}C (lit[297-300] 128-130 ^{0}C); ^{1}H-NMR (250 MHz, $CDCl_3$,) 1.48 (s, 3H, CH_3), 2.30 (d, J = 4.2Hz, 1H, OH), 2.32-2.44 (m, 2H, $H_{2'2''}$), 3.38 (dd, J = 3.1, 10.5 Hz, 1H, $H_{5'}$), 3.48 (dd, J = 3.1, 10.5 Hz, 1H, $H_{5'}$), 4.06 (m, 1H, $H_{3'}$), 4.58 (m, 1H, $H_{4'}$) 6.41 (dd, J = 6.0, 7.8 Hz, 1H, $H_{1'}$), 6.94-7.22 (m, 9H trityl), 7.30-7.55 (m, 6H, trityl), 7.56 (s, 1H, H_6), 8.61 (s, 1H, NH); Anal. Calcd for $C_{29}H_{28}N_2O_5$: C, 65.89; H, 5.42; N, 5.23; Found: C, 65.91; H, 5.30; N, 5.25.

5'-O-trityl-adenosine **58**, 64% yield, m.p. 100-102 ^{0}C (lit[297-300] 104-107 ^{0}C); ^{1}H-NMR (250 MHz, $CDCl_3$, D_2O) 2.70-2.95 (m, 1H, $H_{2'}$), 3.05-3.22 (m, 2H, $H_{5'5''}$), 4.05-4.28 (m, 1H, $H_{4'}$), 4.44-4.65 (m, 1H, $H_{3'}$), 6.14 (t, $J_{apparent}$ = 7Hz, 1H, H_1), 6.30-6.76 (m, 6H, trityl), 7.05-7.44 (m, 9H, trityl), 7.88 (s, 1H, H_2), 8.14 (s, 1H, H_8); Anal. Calcd for $C_{29}H_{27}N_5O_6$: C, 68.09; H, 5.71; N, 13.69; Found: C, 68.21; H, 5.50; N, 13.85.

(II) Using 4,4'-dimethoxytrityl chloride (59)

5'-O-(Dimethoxytrityl)-2'-deoxythymidine (60) and 5'-O-(Dimethoxytrityl)-adenosine (61)

To a suspension for adenosine (solution for 2'-deoxythymidine) (5 mmol 1eq) in dry pyridine (10 ml), 4,4'-dimethoxytrityl chloride (7.5 mmol, 1.5eq) was added along with a catalytic amount of 4-dimethylaminopyridine (0.125 mmol). The reaction mixture was left to stir at room temperature for 4h. The solvent was evaporated under high vacuum to give a residue that was dissolved in ethyl acetate (20 ml) water (10 ml). The aqueous layer was then extracted with ethyl acetate (3 x 30ml) and the combined organic fractions were washed with saturated sodium bicarbonate solution (2 x 10 ml). The organic fractions were dried over magnesium sulphate; the drying agent was filtered off and evaporation of the solvent afforded a glossy residue in both cases. The crude products obtained were recrystallized from ethanol-petrol and dichloromethane-petrol for 2'-deoxythymidine and adenosine respectively, to give the 5'-O-dimethoxytrityl nucleosides as white microcrystalline solids.



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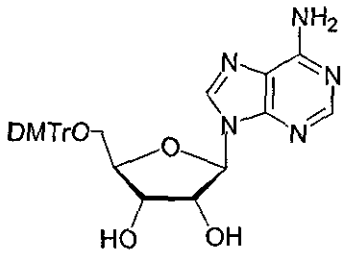


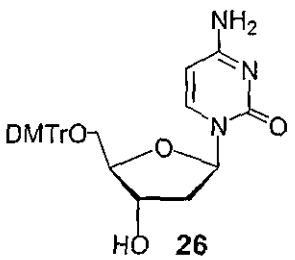
61

5'-O-(Dimethoxytrityl)-2'-deoxythymidine **(60)**, 71% yield, m.p. 118-120 ^{0}C (lit[301,302] 123-124 ^{0}C); ^{1}H-NMR (250 MHz, $CDCl_3$) 1.66 (s, 3H, CH_3), 2.48 (d, J = 5.1Hz, 1H, OH), 2.54-2.65 (m, 2H, $H_{2'2''}$), 3.56 (dd, J = 4.3, 8.5 Hz, 1H, $H_{5'}$), 3.66 (dd, J = 4.3, 8.5 Hz, 1H, $H_{5'}$), 3.96 [s, 6H, O-(CH_3)$_2$], 4.22-4.42 (m, 1H, $H_{3'}$), 4.76 (m, 1H, $H_{3'}$), 6.59 (dd, J = 6.0, 7.2 Hz, 1H, $H_{1'}$), 6.75-7.10 (m, 9H trityl), 7.26-7.55 (m, 4H, trityl), 7.38 (s, 1H, H_6), 8.45 (s, 1H, NH); Anal. Calcd for $C_{31}H_{32}N_2O_7$: C, 68.12; H, 6.27; N, 5.12; Found: C, 68.24; H, 6.15; N, 5.25.

5'-O-(Dimethoxytrityl)-adenosine **(61)**, 70% yield, ^{1}H-NMR (250 MHz, $CDCl_3$, D_2O) 2.94-3.15 (*m*, 1H, $H_{2'}$), 3.28-3.44 (*m*, 2H, 1H, $H_{5'}$), 3.65 [s, 6H, O-($C\underline{H}_3)_2$], 4.10-4.34 (m, 1H, $H_{4'}$), 4.60 (m, 1H, $H_{3'}$), 6.37 (t, $J_{apparent}$ = 9Hz, 1H, $H_{1'}$), 6.55-6.90 (m, 4H, trityl), 7.10-7.50 (m, 9H, trityl), 7.92 (s, 1H, H_2), 8.22 (s, 1H, H_8) Anal. Calcd for $C_{29}H_{31}N_5O_6$: C, 65.14; H, 5.82; N, 12.79; Found: C, 65.31; H, 5.70; N, 12.95.

5'-O-(*p,p'*-Dimethoxytrityl)-2'-deoxycytidine (26)

In a stirred suspension of 2'-deoxycytidine in pyridine (5 mmol, 1eq), dichloroacetic acid (5 mmol, 1eq) was added dropwise followed by the addition of triethylamine (5 mmol, 1eq). After stirring at ambient temperature for 10 min the suspension became a homogeneous solution. 4,4'-Dimethoxytrityl chloride (5.55 mmol, 1.11eq) was finally added and the resulting solution was stirred at 25 ^{0}C for 2h. After TLC showed completion of the reaction, the mixture was quenched with methanol (1 ml) and the solvent was removed under high vacuum. The resulting gummy residue was dissolved in dichloromethane (15 ml) and saturated sodium bicarbonate solution (10 ml). The aqueous layer was further extracted with dichloromethane (2 x 25 ml) and the combined organic fractions were washed with brine (2 x 10 ml). The combined organic extracts were dried over $MgSO_4$, filtered and evaporated to dryness to give an orange gum, as crude, in both cases. The crude product of 5'-protected 2'-deoxycytidine was recrystallized from a mixture of toluene and acetone (5:1) to afford the 5'-O-Dimethoxytritylated derivative as white solid in 72% yield.



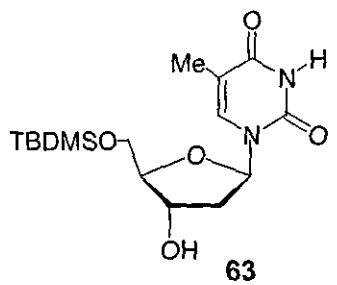
26

5'-O-(Dimethoxytrityl)-2'-deoxycytidine **(26)**, 72% yield; ^{1}H-NMR (250 MHz, $CDCl_3$, D_2O) 1.94-2.85 (m, 2H, $H_{2'2''}$), 3.24-3.37 (m, 2H, $H_{5'5''}$), 3.80 [s, 6H, O-$(CH_3)_2$], 4.12-4.23 (m, 1H, $H_{4'}$), 4.42-4.54 (m, 1H, $H_{3'}$), 5.52 (d, $J_{5\text{-}6}$ = 8 Hz, 1H, H_5), 6.17 (t, $J_{apparent}$ = 7 Hz, 1H, $H_{1'}$), 6.65-6.71 (m, 4H, trityl), 7.05-7.24 (m, 9H, trityl), 7.82 (d, $J_{5\text{-}6}$ = 8 Hz, 1H, H_6); Anal. Calcd for $C_{30}H_{31}N_3O_6$: C, 68.10; H, 5.90; N, 7.94; Found: C, 68.19; H, 5.76, N, 7.86.

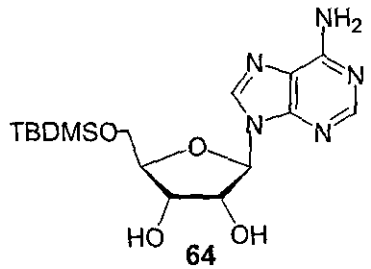
(III) Using *tert*-Butyldimethylsilyl chloride (62)

5'-O-(*tert*-butyldimethylsilyl)-2'-deoxythymidine (63), 5'-O-(*tert*-Butyldimethylsilyl) derivatives of adenosine (64), guanosine (65) and cytidine (66)

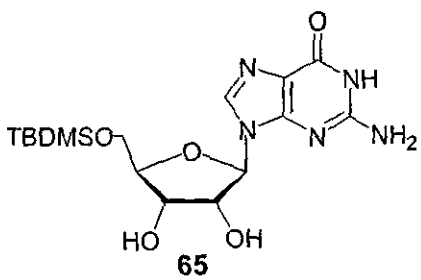
A suspension of nucleoside (5 mmol, 1eq) in dry DMF (20 ml) or anhydrous pyridine (10 ml) was heated gently with stirring at 70-90 ^{0}C for 20 min (or until the nucleoside is completely dissolved). After cooling the solution, *tert*-butyldimethylsilyl chloride (variable equivalents see Table 4 in discussion) was added neat followed by imidazole (variable equivalents see Table 4 in discussion) and the reaction mixture was left to stir at room temperature (various times see Table 4 in discussion). The solvent was then removed under high vacuum to give in all cases a viscous residue that was dissolved in ethyl acetate (40 ml). In the solution saturated sodium bicarbonate solution (20 ml) was added to extract the imidazolium salt and the layers were separated. The aqueous layer was then extracted with more ethyl acetate (3 x 40 ml) and the organic layer was washed further with aqueous ammonium chloride solution (2 x 20 ml) and brine (3 x 20 ml). The organic fractions were combined dried over sodium sulphate, filtered and evaporated. Evaporation of the solvent afforded the 5'-O protected-2'-deoxythymidine as a white powdery solid and the 5'-O protected derivatives of adenosine, guanosine and cytidine as white foam. The crude products were recrystallized from aqueous ethanol to give the 5'-O protected-nucleosides as white solids, which were dried under high vacuum.



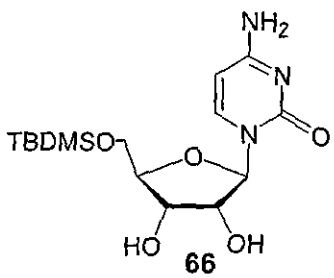
63



64



65



66

5'-O-(*tert*-Butyldimethylsilyl)-2'-deoxythymidine (**63**), 67-77% yield, mp. 186-188 ^{0}C (lit[303] 189-190 ^{0}C); ^{1}H-NMR (250 MHz, $CDCl_3$, D_2O) 0.08 (s, 6H, Si-CH_3), 0.65 (s, 9H, C-CH_3), 1.69 (s, 3H, CH_3), 2.18-2.30 (m, 2H, $H_{2'2''}$), 3.26 (dd, J = 5.4, 8.7 Hz, 1H, $H_{5'}$), 3.38 (dd, J = 5.4, 8.7 Hz, 1H, $H_{5'}$), 4.25-4.31 (m, 1H, $H_{3'}$), 4.58 (m, 1H, $H_{3'}$), 6.14 (dd, $J_{apparent}$ = 6.3Hz, 6.8Hz, 1H), 7.15 (s, 1H, H_6).

5'-O-(*tert*-Butyldimethylsilyl)-adenosine (**64**), 72-81%yield, mp. 162-164 ^{0}C (lit[303] 165-166 ^{0}C); ^{1}H-NMR (250 MHz, $CDCl_3$, D_2O) 0.11 (s, 6H, Si-CH_3), 0.62 (s, 9H, Si-CH_3), 4.01-4.10 (m, 1H, $H_{2'}$), 4.20-4.32 (m, 2H, 1H, $H_{5'}$), 4.48-4.55 (m, 1H, $H_{4'}$), 4.95-5.05 (m, 1H, $H_{3'}$), 5.92 (t, $J_{apparent}$ = 7Hz, 1H, $H_{1'}$), 7.65 (s, 1H, H_2), 8.64 (s, 1H, H_8).

5'-O-(*tert*-Butyldimethylsilyl)-guanosine (**65**), 74-86% yield, mp. 172-175 ^{0}C (lit[303] 175-177 ^{0}C); ^{1}H-NMR (250 MHz, $CDCl_3$, D_2O) 0.08 (s, 6H, Si-CH_3), 0.65 (s, 9H, C-CH_3), 2.96-3.08 (m, 2H), 3.65-3.77 (m, 1H), 4.25 (t, $J_{apparent}$ = 10.2 Hz, 1H), 4.36 (t, $J_{apparent}$ = 10.2 Hz, 1H), 5.50 (d, $J_{apparent}$ = 7.5 Hz, 1H), 7.38 (s, 1H), 7.64 (s, 1H, NH).

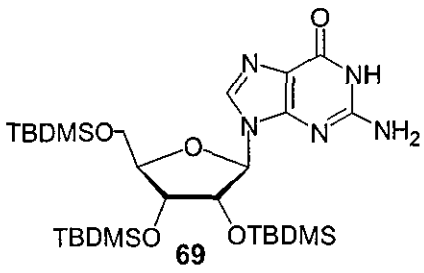
5'-O-(*tert*-Butyldimethylsilyl)-cytidine (**66**), 73-88% yield, mp. 192-195 ^{0}C (lit[303] 195-197 ^{0}C); ^{1}H-NMR (250 MHz, $CDCl_3$, D_2O) 0.65 (s, 6H, Si-CH_3), 0.81 (s,

9H, C-CH_3), 2.11-2.23 (m, 1H, $H_{2'}$), 3.02-3.13 (m, 2H, $H_{5'5''}$) 3.64-3.77 (m, 1H, $H_{4'}$), 4.15-4.20 (m, 1H, $H_{3'}$), 5.22 (d, $J_{5\text{-}6}$ = 6.6 Hz, 1H, H_5), 6.03 (t, $J_{apparent}$ = 6.3Hz, 1H, $H_{1'}$), 7.63 (d, $J_{5\text{-}6}$ = 7.5 Hz, 1H, H_6).

Procedure for the global TBDMS protection of adenosine, guanosine and cytidine.

2',3',5'-tri-O-(*tert*-Butyldimethylsilyl) derivatives of guanosine (69), adenosine (72) and cytidine (75)

A suspension of nucleoside (5 mmol, 1eq) in dry DMF (20 ml) or anhydrous pyridine (10 ml) was heated gently with stirring at 70-90 ^{0}C for 20min (or until the nucleoside is completely dissolved). After cooling the solution, *tert*-butyldimethylsilyl chloride (variable equivalents see Table 5 in discussion) was added neat followed by imidazole (variable equivalents see Table 5 in discussion) and the reaction mixture was left to stir at room temperature (various times see Table 5 in discussion). The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate (80 ml). In the solution saturated aqueous ammonium chloride solution (40 ml) and water (40 ml) was added, and the layers were separated. The aqueous layer was then extracted with more ethyl acetate (4 x 50ml) and the organic layer was washed further with saturated aqueous sodium bicarbonate (3 x 5 0ml) and brine (3 x 50 ml). The organic fractions were combined dried over sodium sulphate, filtered and evaporated. Evaporation of the solvent afforded the 2',3',5'-O-protected-nucleosides as white foam, in all cases. The white foam was further evacuated overnight under high vacuum and then was recrystallized from ethanol/water to give the 5'-O protected-nucleosides as white solids.



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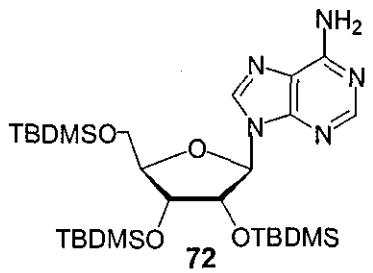

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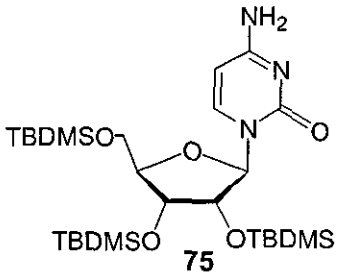


75

2',3',5'-tri-O-(*tert*-Butyldimethylsilyl) guanosine **(69)** 72-81% yield, mp. 108-110 ^{0}C (lit[303] 105-107 ^{0}C); ^{1}H-NMR (250 MHz, $CDCl_3$) 0.08 (s, 6H, Si-CH_3), 0.12 (s, 6H, Si-CH_3), 0.18 (s, 6H, Si-CH_3), 0.65 (s, 9H, C-CH_3), 0.79 (s, 9H, C-CH_3), 0.92 (s, 9H, C-CH_3), 3.58-3.75 (m, 2H) 4.01-3.14 (m, 1H), 4.35 (t, $J_{apparent}$ = 9.4 Hz, 1H), 4.46 (t, $J_{apparent}$ = 9.6 Hz, 1H), 5.82 (d, $J_{apparent}$ = 8.3 Hz, 1H), 6.52 (br s, 2H, NH_2), 7.68 (s, 1H), 7.92 (s, 1H, NH); Anal. Calcd for $C_{28}H_{55}N_5O_5Si_3$: C, 53.72; H, 8.86; N, 11.19; Found: C, 53.96; H, 8.62, N, 10.95, HRFABMS Calcd for $C_{28}H_{55}N_5O_5Si_3$: 625.35110 found 625.3487.

2',3',5'-tri-O-(*tert*-Butyldimethylsilyl) adenosine **(72)** 74-86%yield, mp. 125-127 ^{0}C (lit[303] 122-124 ^{0}C); ^{1}H-NMR (250 MHz, $CDCl_3$) 0.11 (s, 6H, Si-CH_3), 0.16 (s, 6H, Si-CH_3), 0.22 (s, 6H, Si-CH_3), 0.62 (s, 9H, C-CH_3), 0.82 (s, 9H, C-CH_3), 0.96 (s, 9H, C-CH_3), 4.13-4.19 (m, 1H, $H_{2'}$), 4.31-4.43 (m, 2H, 1H, $H_{5'}$), 4.59-4.64 (m, 1H, $H_{4'}$), 5.02-5.12 (m, 1H, $H_{3'}$), 6.03 (t, $J_{apparent}$ = 7.4 Hz, 1H, $H_{1'}$), 6.26 (br s, 2H, NH_2), 7.71 (s, 1H, H_2), 8.75 (s, 1H, H_8); Anal. Calcd for $C_{28}H_{55}N_5O_4Si_3$: C, 55.13; H, 9.09; N, 11.48; Found: C, 55.34; H, 8.87, N, 11.24, HRFABMS Calcd for $C_{28}H_{55}N_5O_4Si_3$: 609.35619 found 609.3531.

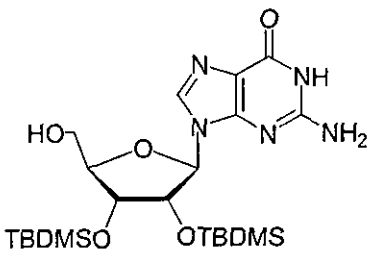
2',3',5'-tri-O-(*tert*-Butyldimethylsilyl) cytidine **(75)** 73-88% yield, mp. 144-147 ^{0}C (lit[303] 146-148 ^{0}C); ^{1}H-NMR (250 MHz, $CDCl_3$) 0.15 (s, 6H, Si-CH_3), 0.19 (s, 6H, Si-CH_3), 0.27 (s, 6H, Si-CH_3), 0.66 (s, 9H, C-CH_3), 0.85 (s, 9H, C-CH_3), 0.92 (s, 9H, C-CH_3), 2.07-2.18 (m, 1H, $H_{2'}$), 3.02-3.11 (m, 2H, $H_{5'5''}$), 3.52-3.63 (m, 1H, $H_{4'}$), 4.08-4.14 (m, 1H, $H_{3'}$), 5.12 (d, $J_{5\text{-}6}$ = 6.3 Hz, 1H, H_5), 5.95 (t, $J_{apparent}$ = 6.0 Hz, 1H, $H_{1'}$), 6.33 (br s, 1H, **N**H_2), 7.51 (d, $J_{5\text{-}6}$ = 7.1 Hz, 1H, H_6); Anal. Calcd for $C_{27}H_{55}N_3O_5Si_3$: C, 55.34; H, 9.46; N, 7.17; Found: C, 55.22; H, 9.85, N, 7.34, HRFABMS Calcd for $C_{27}H_{55}N_3O_5Si_3$: 585.34495 found 585.3426.

Procedure for the selective 5'-desilylation of 2',3',5'-tri-O-TBDMS and 2',5'-O-TBDMS nucleosides using 80% aqueous acetic acid (HOAc)

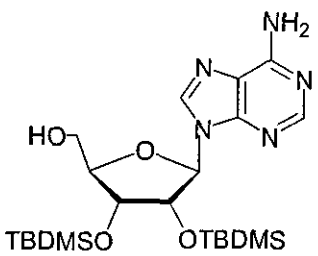
2',3',5'-tri-O-TBDMS or 2',5'-O-TBDMS nucleoside (2 mmol, 1eq) was dissolved in 20 ml of 80% aqueous acetic acid. The reaction mixture was left to either stir at room temperature or heated under reflux at 100 ^{0}C (Various times see Table 6 in discussion). After stirring the reaction mixture was neutralised (pH 8-9) with the addition of saturated aqueous sodium bicarbonate solution and then diluted with ethyl acetate (120 ml). After separation, the aqueous phase was further extracted with ethyl acetate (5 x 40 ml) and the combined organic layers were washed with water (4 x 30 ml) and brine (4 x 30 ml). The organic fractions were dried over sodium sulphate, filtered and evaporated to afford a crude products which was subjected to flash chromatography and recrystallization to provide the 2',3'-O-protected and 2'-O-protected nucleosides respectively, as white solids.

Procedure for the selective 5'-desilylation of 2',3',5'-tri-O-TBDMS and 2',5'-O-TBDMS nucleosides using TFA-H_2O-THF (1 : 1 : 4)

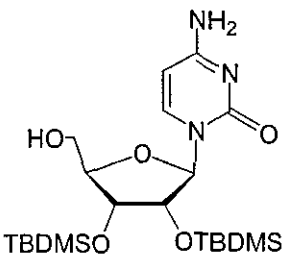
To a magnetically stirred solution of 2',3',5'-tri-O-TBDMS or 2',5'-O-TBDMS nucleoside (2 mmol, 1eq) in 40 ml of THF, kept at 0 ^{0}C, 20 ml of aqueous TFA solution (TFA:H_2O = 1:1) was added. The reaction mixture was left to stir at room temperature (Various times see Table 7 in discussion). After stirring the reaction mixture was neutralised (pH 8-9) with the addition of saturated aqueous sodium bicarbonate and then diluted with ethyl acetate (100 ml). After separation, the aqueous phase was further extracted with ethyl acetate (5 x 40 ml) and the combined organic layers were washed with water (3 x 30 ml) and brine (3 x 30 ml). The organic fractions were dried over sodium sulphate, filtered and evaporated to afford the crude products which were subjected to flash chromatography and recrystallization to provide the 2',3'-O-protected and 2'-O-protected nucleosides as white solids.



79 or **85**



80 or **86**



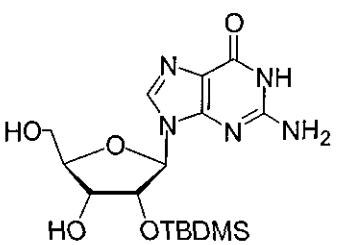
81 or **87**

2',3'-O-(*tert*-Butyldimethylsilyl) guanosine **(79 or 85),** 71-95% yield, mp. 149-152 ^{0}C (lit[303] 144-149 ^{0}C); ^{1}H-NMR (250 MHz, 20% DMSO-d_6 in $CDCl_3$) 0.14 (s, 6H, Si-CH_3), 0.19 (s, 6H, Si-CH_3), 0.81 (s, 9H, C-CH_3), 0.92 (s, 9H, C-CH_3), 3.09-3.21 (m, 2H), 3.79-3.88 (m, 1H), 4.88 (t, $J_{apparent}$ = 11.2 Hz, 1H), 5.08 (br s, 1H, OH), 5.31 (t, $J_{apparent}$ = 11.6 Hz, 1H), 5.92 (d, $J_{apparent}$ = 6.3 Hz, 1H), 6.87 (br s, 2H, NH_2), 7.67 (s, 1H), 7.89 (s, 1H, NH). Anal. Calcd for

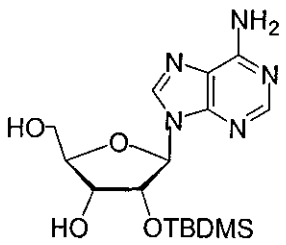
$C_{22}H_{41}N_5O_5Si_2$: C, 51.63; H, 8.08; N, 13.68; Found: C, 51.87; H, 7.88, N, 13.93, HRFABMS Calcd for $C_{22}H_{41}N_5O_5Si_2$: 511.26462 found 625.2615.

2',3'-O-(*tert*-Butyldimethylsilyl) adenosine **(80 or 86)** 70-92%yield, mp. 186-188 ^{0}C (lit[303] 185-188 ^{0}C); ^{1}H-NMR (250 MHz, 20% DMSO-d_6 in $CDCl_3$) 0.18 (s, 6H, Si-CH_3), 0.25 (s, 6H, Si-CH_3), 0.86 (s, 9H, C-CH_3), 0.98 (s, 9H, C-CH_3), 4.17-4.22 (m, 1H, $H_{2'}$), 4.34-4.47 (m, 2H, $H_{5'5''}$), 4.62-4.67 (m, 1H, $H_{4'}$), 5.08-5.20 (s, 1H, $H_{3'}$), 5.28 (br s, 1H, OH), 6.10 (t, $J_{apparent}$ = 6.6 Hz, 1H, $H_{1'}$), 6.29 (br s, 2H, NH_2), 7.76 (s, 1H, H_2), 8.79 (s, 1H, H_8); Anal. Calcd for $C_{22}H_{41}N_5O_4Si_2$: C, 53.30; H, 8.34; N, 14.13; Found: C, 53.54; H, 8.67, N, 14.28, HRFABMS Calcd for $C_{22}H_{41}N_5O_4Si_2$: 495.26971 found 495.2676.

2',3'-O-(*tert*-Butyldimethylsilyl) cytidine **(81 or 87)** 76-97% yield, mp. 190-192 ^{0}C (lit[303] 188-191 ^{0}C); ^{1}H-NMR (250 MHz, 20% DMSO-d_6 in $CDCl_3$) 0.19 (s, 6H, Si-CH_3), 0.27 (s, 6H, Si-CH_3), 0.89 (s, 9H, C-CH_3), 0.98 (s, 9H, C-CH_3), 2.10-2.21 (m, 1H, $H_{2'}$), 3.05-3.14 (m, 2H, $H_{5'5''}$), 3.56-3.68 (m, 1H, $H_{4'}$), 4.14-4.19 (m, 1H, $H_{3'}$), 4.86 (br s, 1H, OH), 5.20 (d, $J_{5\text{-}6}$ = 6.8 Hz, 1H, H_5), 5.98 (t, $J_{apparent}$ = 6.4 Hz, 1H, $H_{1'}$), 6.38 (br s, 1H, NH_2), 7.60 (d, $J_{5\text{-}6}$ = 7.4 Hz, 1H, H_6); Anal. Calcd for $C_{21}H_{41}N_3O_5Si_2$: C, 53.47; H, 8.76; N, 8.91; Found: C, 53.22; H, 8.95, N, 8.58, HRFABMS Calcd for $C_{21}H_{41}N_3O_5Si_2$: 471.25848 found 471.2553.



76 or **82**

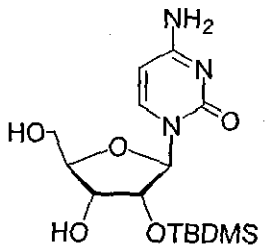


77 or **83**

2'-O-(*tert*-Butyldimethylsilyl) guanosine **(76 or 82)** 70-88% yield, mp. 135-138 ^{0}C (lit[303] 134-136 ^{0}C); ^{1}H-NMR (250 MHz, DMSO-d_6) 0.14 (s, 6H, Si-CH_3), 0.81 (s, 9H, C-CH_3), 3.11-3.24 (m, 2H) 3.85-3.94 (m, 1H), 4.50 (t, $J_{apparent}$ =

12.1 Hz, 1H), 4.64 (t, $J_{apparent}$ = 12.2 Hz, 1H), 5.12 (br s, 1H, OH), 5.24 (br s, 1H, OH), 5.92 (d, $J_{apparent}$ = 7.5 Hz, 1H), 6.84 (br s, 1H, NH_2), 7.79 (s, 1H), 8.02 (s, 1H, NH); Anal. Calcd for $C_{16}H_{27}N_5O_5Si$: C, 48.34; H, 6.85; N, 17.62; Found: C, 48.11; H, 6.98, N, 17.40, HRFABMS Calcd for $C_{16}H_{27}N_5O_5Si$: 397.17815 found 397.1754.

2'-O-(*tert*-Butyldimethylsilyl) adenosine (**77 or 83)** 68-91%yield, mp. 174-176 ^{0}C (lit[303] 175-178 ^{0}C); ^{1}H-NMR (250 MHz, DMSO-d_6) 0.18 (s, 6H, Si-CH_3), 0.98 (s, 9H, C-CH_3), 4.21-4.27 (m, 1H, $H_{2'}$), 4.38-4.52 (m, 2H, $H_{5'5''}$), 4.68-4.72 (m, 1H, $H_{4'}$), 5.10 (br s, 1H, OH), 5.16-5.27 (s, 1H, $H_{3'}$), 5.32 (br s, 1H, OH), 6.22 (t, $J_{apparent}$ = 7.4 Hz, 1H, $H_{1'}$), 6.41 (br s, 2H, NH_2), 7.89 (s, 1H, H_2), 8.92 (s, 1H, H_8); Anal. Calcd for $C_{16}H_{27}N_5O_4Si$: C, 50.37; H, 7.13; N, 18.36; Found: C, 50.08; H, 6.89, N, 18.22, HRFABMS Calcd for $C_{16}H_{27}N_5O_4Si$: 381.18323 found 381.1806.



78 or **84**

2'-O-(*tert*-Butyldimethylsilyl) cytidine (**78 or 84)** 72-93% yield, mp. 170-173 ^{0}C (lit[303] 168-171 ^{0}C); ^{1}H-NMR (250 MHz, DMSO-d_6) 0.19 (s, 6H, Si-CH_3), 0.98 (s, 9H, C-CH_3), 2.15-2.27 (m, 1H, $H_{2'}$), 3.10-3.19 (m, 2H, $H_{5'5''}$), 3.61-3.73 (m, 1H, $H_{4'}$), 4.20-4.28 (m, 1H, $H_{3'}$), 4.88 (br s, 1H, OH), 5.01 (br s, 1H, OH), 5.31 (d, $J_{5\text{-}6}$ = 6.2 Hz, 1H, H_5), 6.05 (t, $J_{apparent}$ = 6.4 Hz, 1H, $H_{1'}$), 6.44 (br s, 1H, NH_2), 7.66 (d, $J_{5\text{-}6}$ = 7.5 Hz, 1H, H_6); Anal. Calcd for $C_{15}H_{27}N_3O_5Si$: C, 50.40; H, 7.61; N, 11.75; Found: C, 50.21; H, 7.85, N, 11.57, HRFABMS Calcd for $C_{15}H_{27}N_3O_5Si$: 357.17200 found 357.1696.

3. SYNTHESIS OF LIPOPHILIC-NUCLEOTIDE CONJUGATES

(A) Lipophilic N-carbamate analogues of nucleosides

Reaction conditions that afforded poor yields

A solution of 4-nitrophenyl carbonate linker of cholesterol **10** or tocopherol **11** (3 mmol, 1eq) in 10ml of dichloromethane or 1,4-dioxane was added dropwise to a stirred solution of 2',3',5'-O-TBDMS protected guanosine **69** and adenosine **72** (3 mmol, 1eq) in dichloromethane or 1,4 dioxane (10 ml). After the addition was completed, triethylamine (3.3 mmol, 1.1eq) was added and the reaction mixture was heated under reflux for 6h. This did not facilitate the formation of the amide bond and starting material was recovered unchanged. DMF and THF were also employed as solvents and the reaction mixtures were heated for 12h. After work-up, in most cases other unidentifiable products were recovered along with starting material and the yields afforded were low, between 26-35%.

A second attempt was made using pyridine was used in the place of triethylamine and refluxing in dichlromethane or 1,4-dioxane overnight. These conditions did not facilitate the synthesis of the lipophilic N-carbamate conjugates. When DMF and THF were employed as solvents in refluxing for 24h, the N-cholesteryl and tocopheryl carbamate derivatives of 2',3',5'-protected adenosine and guanosine **88-91** were obtained in poor yields (31-38%) along with starting material.

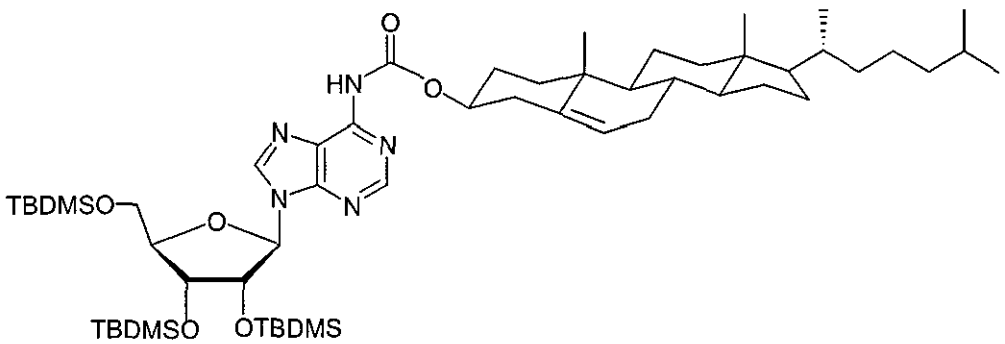
A third attempt was made where anhydrous pyridine was employed as the base, THF or DMF as the solvents and increasing the equivalents of **69** or **72** and base to 1.1 and 1.5, respectively. The N-carbamate analogues **88-91** were afforded in improved yields, between 40-47% but starting material was still present.

Procedures for the synthesis of lipophilic N-carbamate conjugates

Method A: A solution of 4-nitrophenyl carbonate linker of cholesterol **10** or tocopherol **11** (5 mmol, 1eq) in 20ml of anhydrous pyridine was added dropwise to a stirred solution of 2',3',5'-O-TBDMS protected guanosine **69** or adenosine **72** (5.5 mmol, 1eq) in 22ml of anhydrous pyridine. After the addition was completed, the reaction mixture was heated under reflux for 24h. The solvent was removed under high vacuum and the resulting residue was dissolved in 200ml of chloroform. In the solution saturated aqueous ammonium chloride solution (80 ml) and the layers were separated. The aqueous layer was then extracted with more chloroform (5 x 80 ml) and the organic fractions were washed further with saturated aqueous sodium bicarbonate (4 x 50ml) and brine (4 x 50 ml). The organic fractions were combined dried over sodium sulphate, filtered and evaporated. Separation and purification of the crude products by flash column chromatography using a 20:1 mixture of chloroform and methanol afforded the cholesteryl N-carbamate analogues of adenosine **88** and guanosine **90** as off-white foam. The tocopheryl N-carbamate conjugates of adenosine **89** and guanosine **91** were furnished as off-yellow oil.

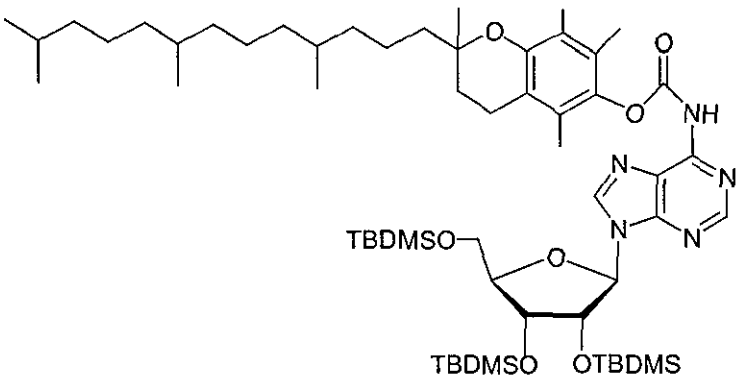
Method B: A solution of carbonylimidazolide linkers of cholesterol **13** or tocopherol **14** (3 mmol, 1eq) in 10ml of anhydrous THF was added dropwise to a stirred solution of 2',3',5'-O-TBDMS protected guanosine **69** or adenosine **72** (5.5 mmol, 1eq) also in 20ml of anhydrous THF. To the stirred solution acetic acid (5 ml) was added and the reaction mixture was left to stir at room temperature for 15h. TLC showed completion of the reaction and the reaction mixture was neutralised (pH 8-9) with the addition of saturated aqueous sodium bicarbonate solution. Ethyl acetate (100 ml) was then added and the layers partitioned. The aqueous phase was further extracted with ethyl acetate (5 x 50 ml) and the combined organic layers were washed with saturated aqueous sodium bicarbonate solution (4 x 30 ml) and brine (4 x 30 ml). The organic fractions were dried over sodium sulphate, filtered and evaporated to afford the crude products, which were subjected to flash column chromatography using a 20:1 mixture of chloroform and methanol.

The cholesteryl N-carbamate analogues of adenosine **92** and guanosine **94** were afforded as off-white foam while, the tocopheryl N-carbamate conjugates of adenosine **93** and guanosines **95** were furnished as off-yellow oil.



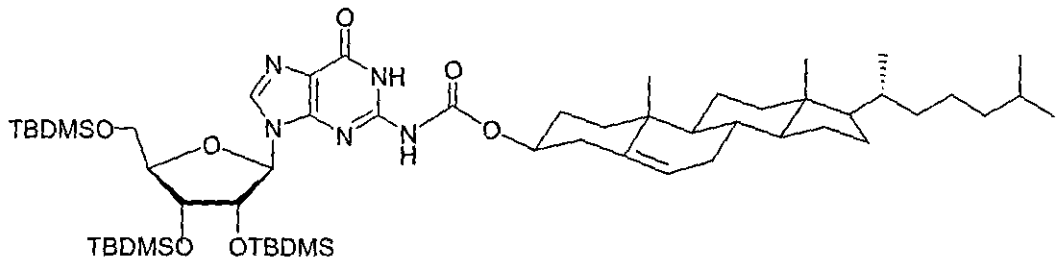
88, 92

3,4-Bis-(*tert*-butyl-dimethyl-silanyloxy)-2-(*tert*-butyl-dimethyl-silanyloxy)-[9-(tetrahydro-furan-2-yl)-9*H*-purin-6yl]- carbamic acid 17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H* cyclopenta[a]phenanthren-3-yl ester (88 and 92) 60 and 63% yield, ν_{max} / cm^{-1} (nujol) 3256, 3152, 1762, 1674, 1410, 1364, 1268, 1170, 1066, 875; ^{1}H-NMR (400 MHz, $CDCl_3$) 0.12 (s, 6H, Si-CH_3), 0.18 (s, 6H, Si-CH_3), 0.24 (s, 6H, Si-CH_3), 0.66 (s, 9H, C-CH_3), 0.85 (s, 9H, C-CH_3), 0.98 (s, 9H, C-CH_3), 0.63-2.52 (m, 45H, aliphatic), 3.48-3.66 (m, 1H, O-CH), 4.16-4.21 (m, 1H, $H_{2'}$), 4.34-4.44 (m, 2H, $H_{5'}$), 4.62-4.66 (m, 1H, $H_{4'}$), 5.08-5.16 (m, 1H, $H_{3'}$), 5.35 (d, 1H, J = 2.5 Hz, HC=C), 6.02 (t, $J_{apparent}$ = 6.7 Hz, 1H, $H_{1'}$), 7.68 (br s, 1H, CON**H**), 7.84 (s, 1H, H_2), 8.25 (s, 1H, H_8); ^{13}C-NMR (100 MHz, $CDCl_3$), -6.5 (6C), 14.7 (3C), 15.2, 17.3, 18.7, 20.7 (9C), 21.5, 22.2, 22.8 (2C), 23.9, 25.3, 28.5, 29.4, 30.6, 33.2, 34.4, 35.8, 37.5, 39.6, 40.2, 42.5, 43.2, 45.8, 48.8, 51.2, 64.7, 71.8, 73.3, 77.1, 77.7, 86.1, 123.5 (C=**C**H), 128.4, 142.8 (CH=**C**), 147.3, 144.4, 149.2, 152.0, 154.8, 171.1 (C=O); Anal. Calcd for $C_{56}H_{99}N_5O_6Si_3$: C 65.77, H 9.76, N 6.85, O 9.39 Found C 65.62, H 9.88, N 6.76; HRFABMS Calcd for $C_{56}H_{99}N_5O_6Si_3$: 1021.69032 found 1021.6887.



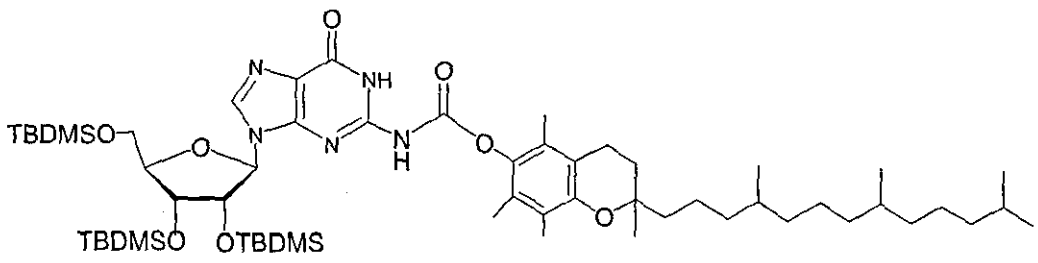
89, 93

{9-[3,4-Bis-(*tert*-butyl-dimethyl-silanyloxy)-5-(*tert*-butyl-dimethyl-silanyloxy)-tetrahydro-furan-2-yl)-9*H*-purin-6yl]-carbamic acid 2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-tridecyl)-chroman-6-yl ester (89 and 93) 64 and 68% yield, ν_{max} / cm^{-1} (nujol) 3295, 3176, 1750, 1685, 1336, 1264, 1110, 837, 778; ^{1}H-NMR (400 MHz, $CDCl_3$) 0.12 (s, 6H, Si-CH_3), 0.18 (s, 6H, Si-CH_3), 0.24 (s, 6H, Si-CH_3), 0.66 (s, 9H, C-CH_3), 0.85 (s, 9H, C-CH_3), 0.96 (s, 9H, C-CH_3), 0.70-0.90 [m, 12H, (CH_3-CH)$_4$], 0.98-1.55 [m, 20H, $(CH_2)_{10}$], 1.56-1.75 [m, 3H, (CH-CH_3)$_3$], 1.93 (s, 3H, CH_3-Ar), 2.03 (s, 3H, CH_3-Ar), 2.07 (s, 3H, CH_3-Ar), 2.27 (s, 3H, CH_3-C-O), 2.45-2.57 (m, 2H, CH_2-Ar), 4.16-4.21 (m, 1H, $H_{2'}$), 4.34-4.44 (m, 2H, $H_{5'}$), 4.62-4.66 (m, 1H, $H_{4'}$), 5.08-5.16 (m, 1H, $H_{3'}$), 6.02 (t, $J_{apparent}$ = 6.7 Hz, 1H, $H_{1'}$), 7.70 (br s, 1H, CONH), 7.84 (s, 1H, H_2), 8.25 (s, 1H, H_8); ^{13}C-NMR (100 MHz, $CDCl_3$), -6.5 (6C-silyl), 14.7 (3C), 17.5, 19.8, 20.7 (9C), 21.3 (2C), 21.9, 22.9 (2C), 21.6, 21.7, 23.3, 23.9, 25.8, 29.2, 31.6, 32.7, 32.9, 35.1, 36.0, 36.8, 38.0, 38.8, 40.2, 41.7, 46.6, 64.7, 71.8, 75.2, 77.1, 77.7, 86.1, 122.0, 123.7, 128.4, 129.9, 144.4, 149.2, 152.0, 154.8, 173.2 (C=O); Anal. Calcd for $C_{58}H_{103}N_5O_7Si_3$: C, 65.30, H, 9.73, N, 6.57; Found C 65.65, H 9.94, N 6.85; HRFABMS Calcd for $C_{58}H_{103}N_5O_7Si_3$: 1065.71653 found 1065.7137.



90, 94

3,4-Bis-(*tert*-butyl-dimethyl-silanyloxy)-2-(*tert*-butyl-dimethyl-silanyloxy)-[9-(tetrahydro-furan-2-yl)-6-oxo-6,9-dihydro-1*H*-purin-2-yl]-carbamic acid 17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H* cyclopenta[a]phenanthren-3-yl ester (90 and 94) 61 and 66% yield, ν_{max} / cm^{-1} (nujol) 3302, 3134, 1755, 1692, 1462, 1377, 1262, 1066, 836, 796; ^{1}H-NMR (400 MHz, $CDCl_3$) 0.10 (s, 6H, Si-CH_3), 0.14 (s, 6H, Si-CH_3), 0.20 (s, 6H, Si-CH_3), 0.68 (s, 9H, C-CH_3), 0.82 (s, 9H, C-CH_3), 0.95 (s, 9H, C-CH_3), 0.63-2.52 (m, 45H, aliphatic), 3.42-3.62 (m, 1H, O-CH), 3.65-3.82 (m, 2H), 4.04-4.18 (m, 1H), 4.38 (t, $J_{apparent}$ = 9.1 Hz, 1H), 4.49 (t, $J_{apparent}$ = 9.4 Hz, 1H), 5.35 (d, 1H, J = 2.5 Hz, HC=C), 5.85 (d, $J_{apparent}$ = 8.5 Hz, 1H), 7.70 (br s, 1H, CONH), 7.73 (s, 1H), 7.95 (s, 1H, NH); ^{13}C-NMR (100 MHz, $CDCl_3$), -6.2 (6C), 14.4 (3C), 15.2, 17.3, 18.7, 20.2 (9C), 21.5, 22.2, 22.8 (2C), 23.9, 25.3, 28.5, 29.4, 30.6, 33.2, 34.4, 35.8, 37.5, 39.6, 40.2, 42.5, 43.2, 45.8, 48.8, 51.2, 65.5, 72.8, 75.6, 78.2, 79.4, 123.5 (C=CH), 138.2, 139.2, 142.8 (CH=C), 148.4, 144.4, 163.6, 170.1, 172.5 (C=O); Anal. Calcd for $C_{56}H_{99}N_5O_7Si_3$: C, 64.76, H, 9.61, N, 6.74; Found C 65.02, H, 9.91, N 6.92; HRFABMS Calcd for $C_{56}H_{99}N_5O_7Si_3$: 1037.68523 found 1037.6832.



91, 95

{9-[3,4-Bis-(*tert*-butyl-dimethyl-silanyloxy)-5-(*tert*-butyl-dimethyl-silanyloxymethyl)-tetrahydro-furan-2-yl)-6-oxo-6,9-dihydro-1*H*-purin-2-yl]-carbamic acid 2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-tridecyl)-chroman-6-yl ester (91 and 95) 66 and 70% yield, ν_{max} / cm^{-1} (nujol) 3264, 3085, 1725, 1695, 1582, 1364, 1272, 1053, 848, 792; ^{1}H-NMR (400 MHz, $CDCl_3$) 0.10 (s, 6H, Si-CH_3), 0.14 (s, 6H, Si-CH_3), 0.20 (s, 6H, Si-CH_3), 0.68 (s, 9H, C-CH_3), 0.82 (s, 9H, C-CH_3), 0.95 (s, 9H, C-CH_3), 0.72-0.92 [m, 12H, (CH_3-CH)$_4$], 1.01-1.57 [m, 20H, $(CH_2)_{10}$], 1.59-1.77 [m, 3H, (CH-CH_3)$_3$], 1.95 (s, 3H, CH_3-Ar), 2.05 (s, 3H, CH_3-Ar), 2.09 (s, 3H, CH_3-Ar), 2.28 (s, 3H, CH_3-C-O), 2.46-2.57 (m, 2H, CH_2-Ar), 3.65-3.82 (m, 2H), 4.04-4.18 (m, 1H), 4.38 (t, $J_{apparent}$ = 9.2 Hz, 1H), 4.49 (t, $J_{apparent}$ = 9.4 Hz, 1H), 5.85 (d, $J_{apparent}$ = 8.3 Hz, 1H), 7.72 (br s, 1H, CONH), 7.75 (s, 1H), 7.95 (s, 1H, NH); ^{13}C-NMR (100 MHz, $CDCl_3$), -6.2 (6C-silyl), 14.4 (3C), 17.8, 20.1, 20.6 (9C), 21.6 (2C), 21.8, 22.1, 22.9, 23.4 (2C), 23.7, 24.3, 25.9, 29.5, 31.9, 32.9, 33.3, 35.5, 36.4, 37.4, 38.3, 38.9, 40.6, 42.5, 46.8, 65.0, 66.2, 73.2, 75.5, 76.3, 78.6, 79.8, 122.5, 123.9, 130.3, 138.7, 139.4, 148.6, 163.9, 170.2, 173.0 (C=O),; Anal. Calcd for $C_{58}H_{103}N_5O_8Si_3$: C, 64.34, H, 9.59, N, 6.47; Found C, 65.02, H 9.91, N 6.18; HRFABMS Calcd for $C_{58}H_{103}N_5O_8Si_3$: 1081.71453 found 1081.7114.

(B) Lipophilic N-acetamide analogues of nucleosides

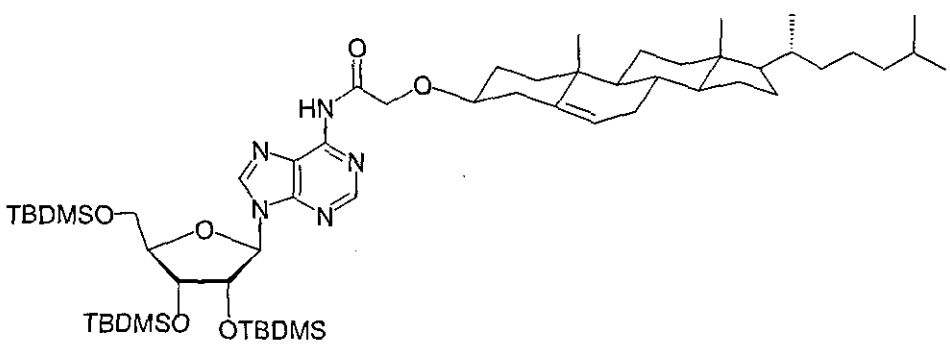
Reaction conditions that afforded poor yields

To a solution of 2',3',5'-O-TBDMS protected guanosine **69** or adenosine **72** (3.3 mmol, 1.1eq) in 10ml of anhydrous THF, a 0.6 M solution of lithium bis (trimethyl silyl) amide or *t*-butyl lithium in hexane (3.3 mmol, 1.1eq) was added dropwise at -78 ^{0}C. After the addition was completed the reaction mixture was stirred at 0 ^{0}C for h. A solution of pentaflurorophenyl ester linkers of cholesterol **16** or tocopherol **17** (3 mmol, 1eq) in anhydrous THF (10 ml) was then added dropwise to the above stirred solution and the reaction mixture was heated under reflux for 30h. The solvent was removed under reduced pressure and the residues furnished were dissolved in ethyl acetate (80 ml) and saturated aqueous sodium bicarbonate solution (40 ml). The aqueous phase was further extracted with ethyl acetate (4 x 50 ml) and the combined organic layers were washed with saturated aqueous sodium bicarbonate solution (4 x 30 ml) and brine (4 x 30 ml). The organic fractions were dried over sodium sulphate, filtered and evaporated to afford the crude products in low yields (15-23%). Various attempts were also made using an increased 1.2 equivalent of the bases, but the yields did not improve greatly (19-29%).
Another attempt was made using pyridine as both solvent and base and after refluxing for 30h, the yields were improved (38-44%). In some cases other unidentifiable products were recovered along with the furnished conjugates.

Procedure for the synthesis of lipophilic N-acetamide conjugates

A solution of pentaflurorophenyl ester linkers of cholesterol **16** or tocopherol **17** (3 mmol, 1eq) in 10ml of anhydrous THF was added dropwise to a stirred solution of 2',3',5'-O-TBDMS protected guanosine **69** and adenosine **72** (3.3 mmol, 1eq) also in 20ml of anhydrous THF. To the stirred solution kept at 0 ^{0}C, *p*-Toluenesulfonic acid (3.3 mmol, 1eq) was added and the reaction mixture was left to stir at 0 ^{0}C for 1h and then was heated under reflux for 24h. TLC showed completion of the reaction and the reaction mixture was neutralised (pH 8-9) with the addition of saturated aqueous sodium bicarbonate solution. Ethyl acetate (100 ml) was then added and the layers

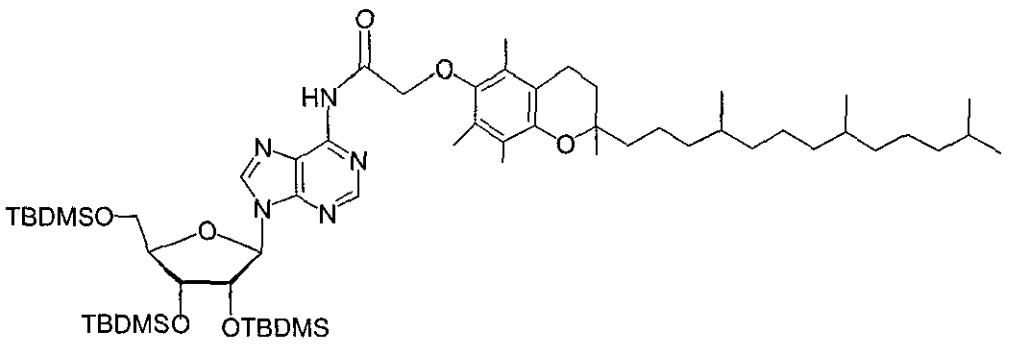
partitioned. The aqueous phase was further extracted with ethyl acetate (5 x 50 ml) and the combined organic layers were washed with saturated aqueous sodium bicarbonate solution (4 x 30 ml) and brine (4 x 30 ml). The organic fractions were dried over sodium sulphate, filtered and evaporated to afford the crude products, which were subjected to flash column chromatography eluting initially with 1% MeOH / 99% $CHCl_3$ and then with 10% MeOH / 90% $CHCl_3$. The cholesteryl N-acetamide analogues of adenosine **96** and guanosine **98** were afforded as off-white solids while, the tocopheryl N-carbamate conjugates of adenosine **97** and guanosine **99** were furnished as off-yellow oil.



96

3,4-Bis-(*tert*-butyl-dimethyl-silanyloxy)-2-(*tert*-butyl-dimethyl-silanyloxy)-[9-(tetrahydro-furan-2-yl)-2-[17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H* cyclopenta[a]phenanthren-3-yloxy-N-9*H*-purin-6-yl)-acetamide 96 60 and 63% yield, ν_{max} / cm^{-1} (*nujol*) 3236, 3104, 1742, 1690, 1575, 1416, 1272, 1131, 1066, 852, 776; ^{1}H-NMR (400 MHz, $CDCl_3$) 0.14 (s, 6H, Si-CH_3), 0.20 (s, 6H, Si-CH_3), 0.25 (s, 6H, Si-CH_3), 0.66 (s, 9H, C-CH_3), 0.87 (s, 9H, C-CH_3), 0.98 (s, 9H, C-CH_3), 0.68-2.50 (m, 45H, aliphatic), 2.77 (s, 2H, CH_2-CO), 3.52-3.70 (m, 1H, O-CH), 4.18-4.23 (m, 1H, $H_{2'}$), 4.36-4.46 (m, 2H, $H_{5'}$), 4.65-4.71 (m, 1H, $H_{4'}$), 5.10-5.18 (m, 1H, $H_{3'}$), 5.38 (d, 1H, J = 2.8 Hz, HC=C), 6.11 (t, $J_{apparent}$ = 6.3 Hz, 1H, $H_{1'}$), 7.68 (br s, 1H, CON**H**), 7.86 (s, 1H, H_2), 8.28 (s, 1H, H_8); ^{13}C-NMR (100 MHz, $CDCl_3$), -6.2 (6C), 14.9 (3C), 15.7, 17.9, 18.8, 21.1 (9C), 21.8, 22.7, 23.3 (2C), 24.4, 25.8, 29.0, 29.9, 30.8, 33.6, 34.9, 36.2, 38.1, 40.1, 40.8, 42.7, 43.8, 46.1, 49.3, 51.7, 65.0, 71.7, 72.5, 73.7,

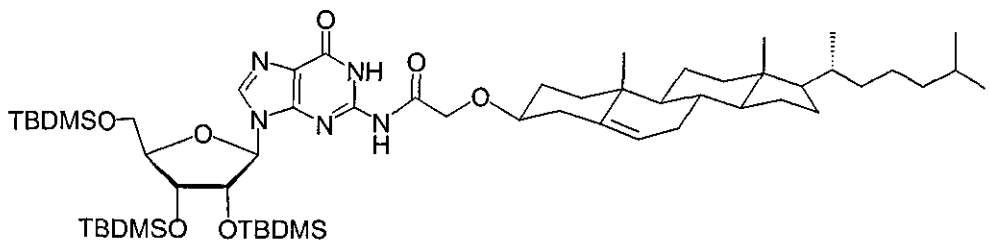
77.8, 78.2, 86.6, 123.8 (C=**C**H), 128.8, 143.2 (CH=**C**), 147.6, 144.8, 149.5, 152.4, 155.2, 171.1 (C=O); Anal. Calcd for $C_{57}H_{101}N_5O_6Si_3$: C 66.04, H 9.82, N 6.76; Found C 65.82, H 9.66, N 6.97; HRFABMS Calcd for $C_{57}H_{101}N_5O_6Si_3$: 1035.70597 found 1035.7028.



97

N-{9-[3,4-Bis-(*tert*-butyl-dimethyl-silanyloxy)-5-(*tert*-butyl-dimethyl-silanyloxymethyl)-tetrahydro-furan-2-yl)-9*H*-purin-6-yl]-2-[2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-tridecyl)-chroman-6-yloxyl]-acetamide 97

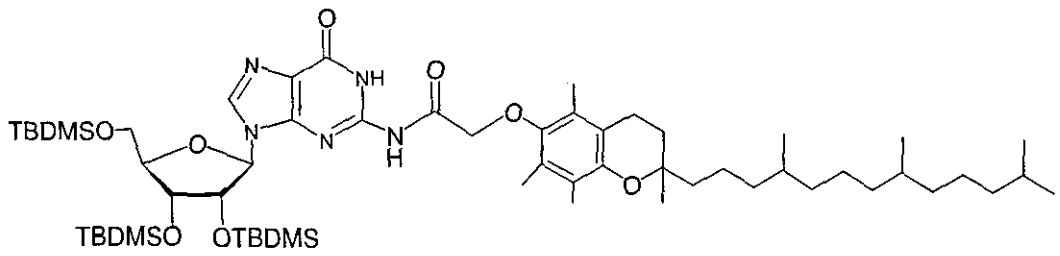
70% yield, ν_{max} / cm^{-1} (nujol) 3274, 3074, 1686, 1575, 1362, 1270, 1136, 864, 782; ^{1}H-NMR (400 MHz, $CDCl_3$) 0.14 (s, 6H, Si-CH_3), 0.20 (s, 6H, Si-CH_3), 0.27 (s, 6H, Si-CH_3), 0.68 (s, 9H, C-CH_3), 0.88 (s, 9H, C-CH_3), 0.98 (s, 9H, C-CH_3), 0.73-0.92 [m, 12H, (CH_3-CH)$_4$], 1.0-1.57 [m, 20H, $(CH_2)_{10}$], 1.59-1.78 [m, 3H, (CH-CH_3)$_3$], 1.96 (s, 3H, CH_3-Ar), 2.03 (s, 3H, CH_3-Ar), 2.07 (s, 3H, CH_3-Ar), 2.30 (s, 3H, CH_3-C-O), 2.48-2.60 (m, 2H, CH_2-Ar), 2.75 (s, 2H, CH_2-CO), 4.18-4.23 (m, 1H, $H_{2'}$), 4.36-4.47 (m, 2H, $H_{5'}$), 4.65-4.69 (m, 1H, $H_{4'}$), 5.10-5.18 (m, 1H, $H_{3'}$), 6.12 (t, $J_{apparent}$ = 6.2 Hz, 1H, $H_{1'}$), 7.75 (br s, 1H, CON**H**), 7.89 (s, 1H, H_2), 8.25 (s, 1H, H_8); ^{13}C-NMR (100 MHz, $CDCl_3$), -6.1 (6C-silyl), 15.0 (3C), 17.9, 20.3, 21.0 (9C), 21.7 (2C), 22.2, 23.4 (2C), 21.8, 22.2, 23.8, 24.3, 26.4, 29.7, 31.8, 33.0, 33.4, 35.6, 36.6, 37.2, 38.5, 39.1, 40.7, 41.9, 46.9, 65.2, 71.6, 72.1, 75.6, 77.7, 78.5, 86.6, 122.4, 123.9, 128.7, 130.2 144.7, 149.6, 152.5, 155.2, 173.5 (C=O); Anal. Calcd for $C_{59}H_{105}N_5O_7Si_3$: C, 65.57, H, 9.79, N, 6.48; Found C 65.82, H 9.96, N 6.82; HRFABMS Calcd for $C_{58}H_{103}N_5O_7Si_3$: 1079.73218 found 1079.7193.



98

3,4-Bis-(*tert*-butyl-dimethyl-silanyloxy)-2-(*tert*-butyl-dimethyl-silanyloxy)-[9-(tetrahydro-furan-2-yl)-6-oxo-6,9-dihydro-1*H*-purin-2-yl]-2-[17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H* cyclopenta[a]phenanthren-3-yloxyl]-acetamide 98

69% yield, ν_{max} / cm^{-1} (nujol) 3302, 3134, 1755, 1694, 1462, 1377, 1262, 1001, 1066, 836; ^{1}H-NMR (400 MHz, $CDCl_3$) 0.13 (s, 6H, Si-CH_3), 0.17 (s, 6H, Si-CH_3), 0.22 (s, 6H, Si-CH_3), 0.70 (s, 9H, C-CH_3), 0.84 (s, 9H, C-CH_3), 0.98 (s, 9H, C-CH_3), 0.66-2.55 (m, 45H, olefinic), 2.77 (s, 2H, OC-C**H**$_2$), 3.45-3.64 (m, 1H, O-CH), 3.67-3.85 (m, 2H), 4.06-4.20 (m, 1H), 4.40 (t, $J_{apparent}$ = 8.8 Hz, 1H), 4.51 (t, $J_{apparent}$ = 9.1 Hz, 1H), 5.37 (d, 1H, J = 2.8 Hz, HC=C), 5.88 (d, $J_{apparent}$ = 8.3 Hz, 1H), 7.73 (br s, 1H, CON**H**), 7.77 (s, 1H), 7.95 (s, 1H, NH); ^{13}C-NMR (100 MHz, $CDCl_3$), -5.9 (6C-silyl), 14.8 (3C), 15.6, 17.8, 19.1, 20.7 (9C), 21.8, 22.9, 23.2 (2C), 24.3, 25.7, 29.0, 29.8, 31.1, 33.7, 34.6, 36.2, 37.9, 39.8, 40.6, 42.9, 43.7, 44.2, 49.4, 51.5, 65.9, 72.5, 73.3, 76.0, 78.6, 79.7, 123.9 (C=**C**H), 139.1, 139.8, 143.2 (CH=**C**), 144.5, 148.7, 164.0, 170.5, 172.7 (C=O); Anal. Calcd for $C_{56}H_{101}N_5O_7Si_3$: C, 65.03, H, 9.67, N, 6.65; Found C 64.82, H, 9.93, N 6.95; HRFABMS Calcd for $C_{56}H_{101}N_5O_7Si_3$: 1051.70088 found 1051.6988.



99

N-{9-[3,4-Bis-(*tert*-butyl-dimethyl-silanyloxy)-5-(*tert*-butyl-dimethyl-silanyloxymethyl)-tetrahydro-furan-2-yl]-6-oxo-6,9-dihydro-1*H*-purin-2-yl]-2-[2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-tridecyl)-chroman-6-yloxyl]-acetamide 99 72% yield, ν_{max} / cm^{-1} (nujol) 3242, 3042, 1744, 1695, 1586, 1337, 1270, 1086, 871, 768; ^{1}H-NMR (400 MHz, $CDCl_3$) 0.12 (s, 6H, Si-CH_3), 0.16 (s, 6H, Si-CH_3), 0.22 (s, 6H, Si-CH_3), 0.70 (s, 9H, C-CH_3), 0.85 (s, 9H, C-CH_3), 0.98 (s, 9H, C-CH_3), 0.75-0.95 [m, 12H, (CH_3-CH)$_4$], 1.04-1.60 [m, 20H, $(CH_2)_{10}$], 1.64-1.80 [m, 3H, (CH-CH_3)$_3$], 1.98 (s, 3H, CH_3-Ar), 2.08 (s, 3H, CH_3-Ar), 2.13 (s, 3H, CH_3-Ar), 2.31 (s, 3H, CH_3-C-O), 2.48-2.60 (m, 2H, CH_2-Ar), 2.76 (s, 2H, OC-CH_2), 3.68-3.85 (m, 2H), 4.07-4.21 (m, 1H), 4.41 (t, $J_{apparent}$ = 8.7 Hz, 1H), 4.53 (t, $J_{apparent}$ = 9.0 Hz, 1H), 5.88 (d, $J_{apparent}$ = 7.9 Hz, 1H), 7.74 (br s, 1H, CONH), 7.79 (s, 1H), 7.99 (s, 1H, NH); ^{13}C-NMR (100 MHz, $CDCl_3$), -5.9 (6C-silyl), 14.8 (3C), 18.2, 20.5, 21.2 (9C), 21.9 (2C), 22.3, 22.7, 23.5, 23.9 (2C), 24.4, 25.1, 26.4, 30.3, 32.3, 33.5, 34.0, 36.1, 36.9, 37.8, 38.7, 39.4, 40.8, 42.9, 45.2, 65.5, 66.6, 71.9, 73.7, 75.8, 76.5, 78.4, 80.1, 122.9, 124.3, 130.8, 139.1, 139.6, 148.8, 164.4, 170.5, 173.5 (C=O); Anal. Calcd for $C_{59}H_{105}N_5O_8Si_3$: C, 64.65, H, 9.66, N, 6.39; Found C, 64.91, H 9.90, N 6.16; HRFABMS Calcd for $C_{59}H_{105}N_5O_8Si_3$: 1095.75040 found 1095.7486.

(C) Lipophilic O-carbonate analogues of nucleosides

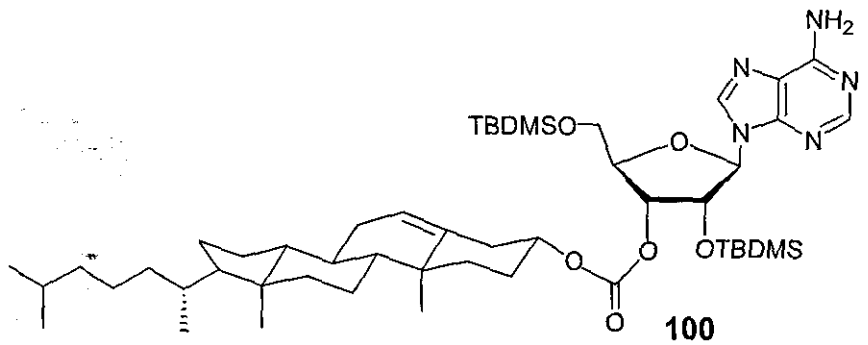
Procedure for the synthesis of lipophilic O-carbonate conjugates

Method A: To a solution of 2',5'-O-TBDMS protected guanosine **67** or adenosine **70** (5 mmol, 1eq) in 12 ml of anhydrous THF, a 0.6 M solution of lithium bis (trimethyl silyl) amide or *t*-butyl lithium in hexane (5 mmol, 1eq) was added dropwise at -78 ^{0}C. After the addition was completed the reaction mixture was stirred at 0 ^{0}C for 1h. A solution of carbamate linker of cholesterol **13** or tocopherol **14** (5 mmol, 1eq) in anhydrous THF (12 ml) was then added dropwise to the above stirred solution and the reaction mixture was heated under reflux for 12 h. The solvent was removed under reduced pressure and the residues furnished were dissolved in ethyl acetate (100 ml) and saturated aqueous sodium bicarbonate solution (50 ml). The aqueous phase was further extracted with ethyl acetate (5 x 50 ml) and the combined organic layers were washed with saturated aqueous sodium bicarbonate solution (5 x 30 ml) and brine (5 x 30 ml). The organic fractions were dried over sodium sulphate, filtered and evaporated to afford the 3'-O-cholesteryl carbonate derivatives of adenosine **100** and guanosine **102** as off-white foam while, the 5'-O-tocopheryl analogues of the corresponding nucleosides were attained as yellow oils.

Method B: A solution of carbamate linker of cholesterol **13** or tocopherol **14** (3 mmol, 1eq) in 13.5 ml of 8:1 mixture of anhydrous 1.4-dioxane: pyridine, was added dropwise to a stirred solution of 2',5'-O-TBDMS protected guanosine **67** or adenosine **70** (3.3 mmol, 1eq) in 15 ml of the same mixture. After the addition was completed, the reaction mixture was heated under reflux for 12 h. The solvent was removed under high vacuum and the resulting residue was dissolved in 240 ml of chloroform. In the solution saturated aqueous ammonium chloride solution (100 ml) and the layers were separated. The aqueous layer was then extracted with more chloroform (6 x 60 ml) and the organic fractions were washed further with saturated aqueous sodium bicarbonate (5 x 50ml) and brine (5 x 50 ml). The organic fractions were

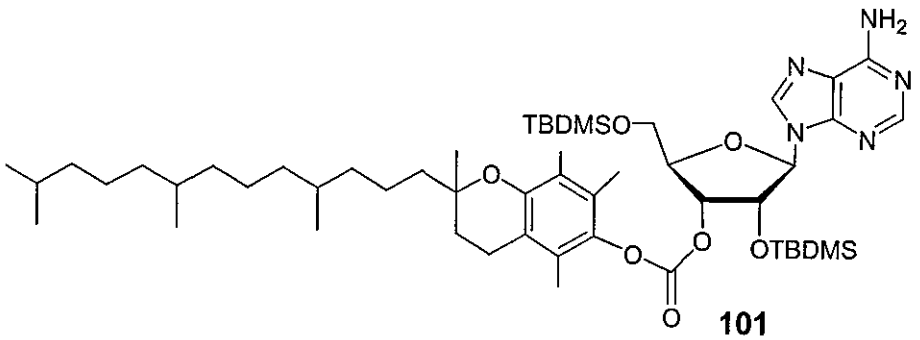
combined dried over sodium sulphate, filtered and evaporated. The 3'-O-cholesteryl carbonate derivatives of adenosine **100** and guanosine **102** were furnished as off-white foam while, the 5'-O-tocopheryl analogues of the corresponding nucleosides were attained as yellow oils.

Various attempts to prepare the carbonate analogues in anhydrous pyridine resulted in poor yields (30-39%).



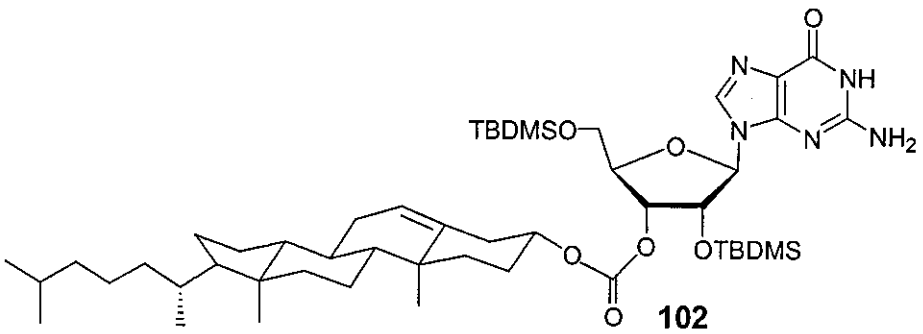
100

Carbonic acid 4-(*tert*-butyl-dimethyl-silanyloxy)-2-(*tert*-butyl-dimethyl-silanyloxy)-tetrahydro-furan-3-yl ester 17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H* cyclopenta[a]phenanthren-3-yl-9*H*-purin-6-ylamine ester 100 59-64% yield, ν_{max} / cm^{-1} (nujol) 1736, 1647, 1382, 1268, 1170, 976, 845; ^{1}H-NMR (400 MHz, $CDCl_3$) 0.11 (s, 6H, Si-CH_3), 0.18 (s, 6H, Si-CH_3), 0.62 (s, 9H, C-CH_3), 0.98 (s, 9H, C-CH_3), 0.65-2.54 (m, 45H, aliphatic), 3.49-3.67 (m, 1H, O-CH), 4.17-4.22 (m, 1H, $H_{2'}$), 4.35-4.45 (m, 2H, $H_{5'}$), 4.64-4.68 (m, 1H, $H_{4'}$), 5.10-5.18 (m, 1H, $H_{3'}$), 5.37 (d, 1H, J = 2.9 Hz, HC=C), 6.05 (t, $J_{apparent}$ = 6.9 Hz, 1H, $H_{1'}$), 6.31 (br s, 2H, NH_2), 7.86 (s, 1H, H_2), 8.27 (s, 1H, H_8); ^{13}C-NMR (100 MHz, $CDCl_3$), -6.1 (4C-silyl), 14.8 (2C), 15.5, 17.6, 19.0, 21.0 (6C), 21.8, 22.5, 23.2 (2C), 24.4, 25.8, 28.9, 29.7, 30.9, 33.5, 34.8, 35.9, 37.9, 4.01, 40.8, 42.7, 43.6, 46.1, 49.2, 51.6, 64.9, 72.2, 73.7, 77.6, 78.1, 86.6, 123.9 (C=**C**H), 128.8, 143.3 (CH=**C**), 147.5, 144.7, 149.6, 152.4, 155.1, 166.8 (C=O); Anal. Calcd for $C_{50}H_{85}N_5O_6Si_2$: C 66.11, H 9.43, N 7.71, Found C 65.84, H 9.76, N 7.98; HRFABMS Calcd for $C_{50}H_{85}N_5O_6Si_2$: 907.60384 found 907.6002.



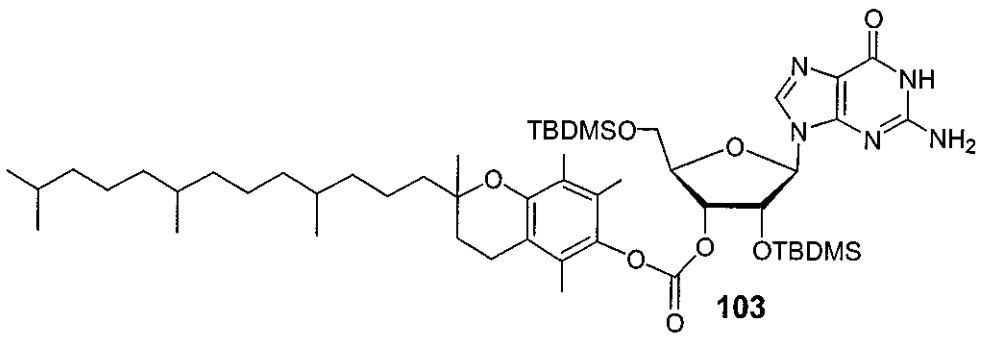
101

Carbonic acid 5-(6-amino-purin-9-yl)-4-(*tert*-butyl-dimethyl-silanyloxy)-2-(*tert*-butyl-dimethyl-silanyloxymethyl)-tetrahydro-furan-3-yl ester 2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-tridecyl)-chroman-6-yl ester 101 63-67% yield, ν_{max} / cm^{-1} (nujol) 1734, 1676, 1582, 1269, 1157, 825, 793; ^{1}H-NMR (400 MHz, $CDCl_3$) 0.13 (s, 6H, Si-CH_3), 0.16 (s, 6H, Si-CH_3), 0.64 (s, 9H, C-CH_3), 0.95 (s, 9H, C-CH_3), 0.68-0.87 [m, 12H, (CH_3-CH)$_4$], 0.95-1.52 [m, 20H, $(CH_2)_{10}$], 1.54-1.73 [m, 3H, (CH-CH_3)$_3$], 1.91 (s, 3H, CH_3-Ar), 2.0 (s, 3H, CH_3-Ar), 2.05 (s, 3H, CH_3-Ar), 2.24 (s, 3H, CH_3-C-O), 2.42-2.54 (m, 2H, CH_2-Ar), 4.14-4.19 (m, 1H, $H_{2'}$), 4.34-4.41 (m, 2H, $H_{5'}$), 4.60-4.64 (m, 1H, $H_{4'}$), 5.05-5.13 (m, 1H, $H_{3'}$), 6.0 (t, $J_{apparent}$ = 6.2 Hz, 1H, $H_{1'}$), 6.34 (br s, 2H, NH_2), 7.8 (s, 1H, H_2), 8.22 (s, 1H, H_8); ^{13}C-NMR (100 MHz, $CDCl_3$), -6.7 (4C-silyl), 14.5 (2C), 17.1, 19.2, 20.3 (6C), 20.9 (2C), 21.5, 22.6 (2C), 21.2, 21.3, 22.8, 23.4, 25.5, 28.7, 31.4, 32.3, 32.7, 34.8, 35.6, 36.5, 37.6, 38.4, 39.8, 41.3, 46.2, 64.4, 71.5, 74.8, 76.6, 77.1, 85.9, 121.8, 123.4, 128.0, 129.5, 144.0, 148.9, 151.7, 154.4, 167.2 (C=O); Anal. Calcd for $C_{52}H_{89}N_5O_7Si_2$: C, 65.57, H, 9.42, N, 7.35; Found C 65.91, H 9.81, N 7.08; HRFABMS Calcd for $C_{52}H_{89}N_5O_7Si_2$: 951.63005 found 951.6275.



102

Carbonic acid 2-amino-1,9-dihydro-purin-6-one 4-(*tert*-butyl-dimethyl-silanyloxy)-2-(*tert*-butyl-dimethyl-silanyloxy)-tetrahydro-furan-3-yl ester 17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-yl ester 102 59-65% yield, ν_{max} / cm^{-1} (nujol) 1757, 1686, 1433, 1358, 1267, 1084, 862, 785; ^{1}H-NMR (400 MHz, $CDCl_3$) 0.09 (s, 6H, Si-CH_3), 0.13 (s, 6H, Si-CH_3), 0.66 (s, 9H, C-CH_3), 0.81 (s, 9H, C-CH_3), 0.61-2.51 (m, 45H, aliphatic), 3.40-3.60 (m, 1H, O-CH), 3.64-3.80 (m, 2H), 4.02-4.16 (m, 1H), 4.36 (t, $J_{apparent}$ = 8.9 Hz, 1H), 4.47 (t, $J_{apparent}$ = 9.1 Hz, 1H), 5.32 (d, 1H, J = 2.4 Hz, HC=C), 5.83 (d, $J_{apparent}$ = 8.2 Hz, 1H), 6.71 (br s, 2H, NH_2), 7.70 (s, 1H), 7.92 (s, 1H, NH); ^{13}C-NMR (100 MHz, $CDCl_3$), -6.4 (4C-silyl), 14.1 (2C), 15.0, 17.1, 18.4, 20.0 (6C), 21.2, 21.9, 22.5 (2C), 23.7, 25.0, 28.2, 29.1, 30.3, 32.8, 34.1, 35.7, 37.4, 39.4, 39.9, 42.3, 42.9, 45.7, 48.6, 51.0, 65.2, 72.4, 75.2, 78.0, 79.3, 123.1 (C=**C**H), 137.9, 138.8, 142.4 (CH=**C**), 148.1, 144.2, 163.5, 167.7 (C=O), 169.7; Anal. Calcd for $C_{50}H_{85}N_5O_7Si_2$: C, 64.96, H, 9.27, N, 7.58; Found C 65.29, H, 9.60, N 7.97; HRFABMS Calcd for $C_{50}H_{85}N_5O_7Si_2$: 923.41010 found 923.4077.



103

Carbonic acid 2-amino-1,9-dihydro-purin-6-one 4-(*tert*-butyl-dimethyl-silanyloxy)-2-(*tert*-butyl-dimethyl-silanyloxymethyl)-tetrahydro-furan-3-yl ester 2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-tridecyl)-chroman-6-yl ester 103 62-69% yield, ν_{max} / cm^{-1} (nujol) 1744, 1682, 1585, 1372, 1270, 1076, 845, 788; ^{1}H-NMR (400 MHz, $CDCl_3$) 0.10 (s, 6H, Si-CH_3), 0.13 (s, 6H, Si-CH_3), 0.67 (s, 9H, C-CH_3), 0.81 (s, 9H, C-CH_3), 0.70-0.90 [m, 12H, (CH_3-$CH)_4$], 1.0-1.54 [m, 20H, $(CH_2)_{10}$], 1.57-1.75 [m, 3H, (CH-$CH_3)_3$], 1.92 (s, 3H, CH_3-Ar), 2.01 (s, 3H, CH_3-Ar), 2.06 (s, 3H, CH_3-Ar), 2.26 (s, 3H, CH_3-C-O), 2.44-2.54 (m, 2H, CH_2-Ar), 3.63-3.80 (m, 2H), 4.01-4.15 (m, 1H), 4.36 (t, $J_{apparent}$ = 9 Hz, 1H), 4.45 (t, $J_{apparent}$ = 9.2 Hz, 1H), 5.83 (d, $J_{apparent}$ = 8.2 Hz, 1H), 6.72 (br s, 2H, NH_2), 7.72 (s, 1H), 7.94 (s, 1H, NH); ^{13}C-NMR (100 MHz, $CDCl_3$), -6.5 (4C-silyl), 14.8 (2C), 18.1, 20.5, 21.0 (6C), 21.9 (2C), 22.1, 22.8, 23.3, 23.7 (2C), 24.1, 24.6, 26.2, 29.8, 32.2, 33.4, 33.9, 35.8, 36.7, 37.9, 38.6, 39.2, 40.8, 42.9, 45.1, 65.5, 66.6, 73.7, 75.9, 76.7, 78.9, 80.2, 122.8, 124.3, 130.5, 139.0, 139.8, 148.9, 164.4, 168.4 (C=O), 173.0; Anal. Calcd for $C_{52}H_{89}N_5O_8Si_2$: C, 64.49, H, 9.26, N, 7.23; Found C, 64.84, H 9.61, N 6.94; HRFABMS Calcd for $C_{52}H_{89}N_5O_8Si_2$: 967.62497 found 967.6219.

(D) Alternative methods for the synthesis of lipophilic-nucleoside conjugates

(I) Through the formation of an isocyanate

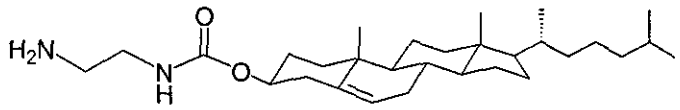
Triphosgene (2 mmol, 1eq) was dissolved in dichloromethane. A mixture of 2',3',5'-O-TBDMS protected adenosine (6 mmol, 3eq) and diisopropylethylamine or triethylamine or diisopropylamine (12 mmol, 6eq) in dichloromethane was slowly added to the stirred solution of triphosgene over a period of 30 min using a suringe pump. After the addition had finished the reaction mixture was left to stir at room temperature for 30 min. The solvent was rotary evaporated and the resulting residue was dissolved in 40 ml of ethyl acetate and 30 ml of saturated sodium bicarbonate solution. The aqueous layer was further extracted with ethyl acetate (4 x 40ml) and the combined organic fractions were washed with saturated sodium bicarbonate solution (3 x 20 ml) and brine (2 x 20 ml). The organic extracts were dried over magnesium sulphate, filtered and evaporated to dryness. Disappointingly the experiments were unsuccessful, as starting material was recovered in all cases and none of the desired isocyanate was formed.

(II) Through an acid chloride

A solution of cholesteryl chloroformate (3 mmol, 1eq) in 10 ml of dichloromethane was added dropwise to a stirred 1:1 solution of ethylenediamine or 2',3',5'-O-TBDMS protected adenosine in dichloromethane (8 ml) and pyridine (8 ml). After the addition was completed, the reaction mixture was stirred at room temperature for 2 h. The solvent was removed under high vacuum and the resulting residue was dissolved in 100 ml of dichloromethane and 80ml of water. The aqueous layer was extracted further with dichloromethane (4 x 30ml) and the combined organic fractions were washed with brine (4 x 20 ml). The organic extracts were dried over magnesium sulphate, filtered and evaporated to dryness, to give the title compounds. Recrystallization from hexane afforded 2-

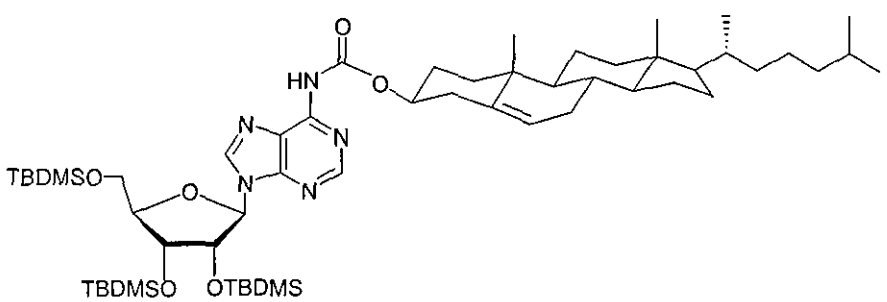
(chlolesteryloxycarbonyl)ethylamine as white crystals in 72% yield. The cholesteryl N-carbamate of globally protected adenosine was furnished as off-white solid in 42% yield after separation and purification by flash column chromatography eluting with 1:20 mixture of chloroform methanol.

(2-amino-ethyl)-carbamic acid 17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H* cyclopenta[a]phenanthren-3-yl ester 114



2-(chlolesteryloxycarbonyl)ethylamine (**114**) recrystallised form 72% yield; m.p. 152-155 ^{0}C; ν_{max} / cm^{-1} (nujol) 3355, 3252, 3148, 2456, 1755, 1306, 1461, 1170, 1047, 894; ^{1}H-NMR (250 MHz, $CDCl_3$), 0.63-2.52 (m, 45H, aliphatic), 3.04 (t, 2H, C**H**$_2$NH$_2$), 3.46-3.66 (m, 1H, O-CH), 3.71-3.82 (m, 4H, N**H**$_2$ and HNC**H**$_2$), 5.36 (d, 1H, J = 2.3 Hz, HC=C), 7.62 (br s, 1H, CON**H**); ^{13}C-NMR (100 MHz, $CDCl_3$), 17.3, 18.5, 20.5, 22.6 (2C), 23.9, 24.7, 25.3, 28.5, 29.4, 30.7, 32.0, 33.2, 34.4, 35.8, 36.2, 37.5, 39.2, 40.6, 42.5, 43.6, 48.7, 51.2, 56.4, 57.8, 73.3, 109.8, 122.8 (C=**C**H), 140.9 (CH=**C**), 159.5 (C=O); Anal. Calcd for $C_{30}H_{52}N_2O_2$: C 76.22, H 11.09, N 5.93, O 6.77 Found C 75.96, H 11.10, N 5.86; HRFABMS Calcd for $C_{30}H_{52}N_2O_2$: 472.40288 found 472.4011.

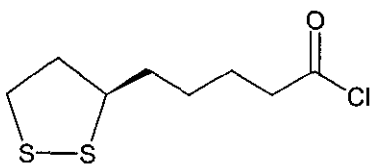
3,4-Bis-(*tert*-butyl-dimethyl-silanyloxy)-2-(*tert*-butyl-dimethyl-silanyloxy)-[9-(tetrahydro-furan-2-yl)-9*H*-purin-6yl]- carbamic acid 17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H* cyclopenta[a]phenanthren-3-yl ester 115



Cholesteryl N-carbamate of 2',3',5'-O-TBDMS-adenosine (**115**) purified form 42% yield; m.p. 145-147 ^{0}C; ν_{max} / cm^{-1} (nujol) 3256, 3152, 1762, 1684, 1268, 1410, 1364, 1255, 1170, 1066, 875; ^{1}H-NMR (400 MHz, $CDCl_3$) 0.12 (s, 6H, Si-CH_3), 0.18 (s, 6H, Si-CH_3), 0.24 (s, 6H, Si-CH_3), 0.63-2.52 (m, 45H, aliphatic), 0.66 (s, 9H, C-CH_3), 0.85 (s, 9H, C-CH_3), 0.98 (s, 9H, C-CH_3), 3.48-3.66 (m, 1H, O-CH), 4.16-4.21 (m, 1H, $H_{2'}$), 4.34-4.44 (m, 2H, $H_{5'}$), 4.62-4.66 (m, 1H, $H_{4'}$), 5.08-5.16 (m, 1H, $H_{3'}$), 5.35 (d, 1H, J = 2.5 Hz, HC=C), 6.02 (t, $J_{apparent}$ = 6.7 Hz, 1H, $H_{1'}$), 7.65 (br s, 1H, CONH), 7.84 (s, 1H, H_2), 8.25 (s, 1H, H_8); ^{13}C-NMR (100 MHz, $CDCl_3$), -6.5 (6C), 14.7 (3C), 15.2, 17.3, 18.7, 20.7 (9C), 21.5, 22.2, 22.8 (2C), 23.9, 25.3, 28.5, 29.4, 30.6, 33.2, 34.4, 35.8, 37.5, 39.6, 40.2, 42.5, 43.2, 45.8, 48.8, 51.2, 64.7, 71.8, 73.3, 77.1, 77.7, 86.1, 123.5 (C=**C**H), 128.4, 142.8 (CH=**C**), 147.3, 144.4, 149.2, 152.0, 154.8, 161.1 (C=O); Anal. Calcd for $C_{56}H_{99}N_5O_6Si_3$: C 65.77, H 9.76, N 6.85, O 9.39 Found C 65.62, H 9.88, N 6.76; HRFABMS Calcd for $C_{56}H_{99}N_5O_6Si_3$: 1021.69032 found 1021.6887.

(II) Through the formation of the acid chloride of thioctic acid

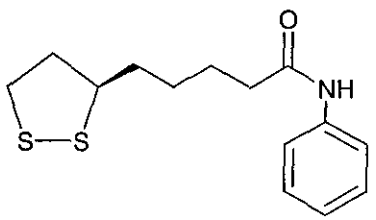
5-[(3R)-1,2-dithiolan-3-yl] pentanoyl chloride (116)



To thioctic acid (5 mmol, 1eq) kept at ice bath temperature, thionyl chloride (30 mmol, 6eq) was added dropwise with stirring (no vigorous reaction was noted). After 10 min of stirring the dark orange solution turned into dark brown and gradually into black. The reaction mixture was stirred at room temperature for 2h and then the solvent was removed under reduced pressure to furnish the acid chloride as black oil. The compound was used without further purification

Acid chloride of thioctic acid (**116**) 72% yield ν_{max} / cm^{-1} (nujol) 2929, 2556 1812, 1461, 1323, 1284, 932, 807, 735; ^{1}H-NMR (250 MHz, $CDCl_3$) 1.43-2.12 (m, 6H, $(CH_2)_3$ aliphatic), 2.18-2.31 (m, 2H, CH_2 cycle) 2.36-2.58 (m, 3H, O=C-C**H**$_2$ + C**H**-S cycle), 3.12-3.32 (m, 2H, CH_2-S), ^{13}C-NMR (100 MHz, $CDCl_3$) 25.2, 27.4, 35.3, 37.6, 38.5, 43.4, 52.8, 175.8 (O=**C**-CH_2 quaternary); Anal. Calcd for $C_8H_{13}ClOS_2$: C, 42.75, H, 5.83, O, 7.12; Found C, 42.86, H, 5.68; HRFABMS Calcd for $C_8H_{13}ClOS_2$: 224.00963 found 224.0059.

N1-phenyl-5-[(3R)-1,2-dithiolan-3-yl] pentanamide (118)



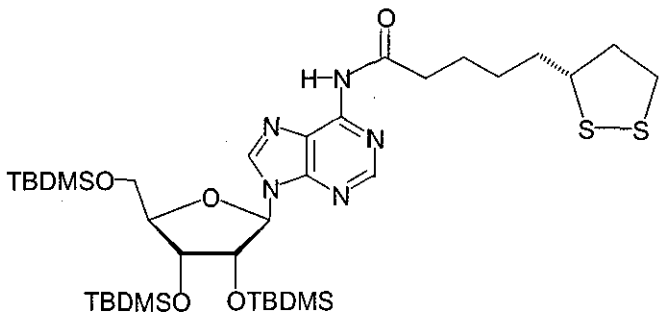
To a solution of aniline (2 mmol, 1eq) in 1 ml of pyridine kept at ice bath temperature, the acid chloride of thioctic acid 116 (2 mmol, 1eq) dissolved in 5 ml of pyridine, was added dropwise through a syringe. After the addition was completed, the reaction was allowed to warm at room temperature and then it was left to stir for 2 h. Pyridine was evaporated under high vacuum leaving a black oil which was dissolved in diethyl ether (20 ml) and water (10 ml). The aqueous layer was extracted further with diethyl ether (4 x 20 ml) while the combined organic fractions were washed sequentially with saturated sodium bicarbonate solution (2 x 10ml) and 20% aqueous hydrochloric acid (2 x 2ml) The combined organic extracts were dried over sodium sulphate, filtered and evaporated to dryness to furnish the crude amide of aniline as black oil. The crude product was purified by flash column chromatography using a 1:1 mixture of petroleum ether and ethyl acetate as the eluent to afford the desired product as brown oil

Thioctic amide of aniline (**118**) 48% yield; ν_{max} / cm^{-1} (nujol) 3354, 3072, 2935, 2586 1675, 1510, 1442, 1257, 996, 881, 693; ^{1}H-NMR (250 MHz, $CDCl_3$)

1.52-2.16 (m, 6H, $(CH_2)_3$ aliphatic), 2.23-2.35 (m, 2H, CH_2 cycle) 2.41-2.63 (m, 3H, O=C-CH_2 + C**H**-S cycle), 3.18-3.39 (m, 2H, CH_2-S), 6.57-6.64 (m, 2H, Ar), 6.72-6.81 (m, 2H, Ar), 6.87-6.96 (m, 1H, Ar), 7.62 (br s, 1H, CONH); ^{13}C-NMR (100 MHz, $CDCl_3$) 25.4, 27.8, 35.6, 37.8, 38.9, 43.6, 53.6, 118.4 (2C), 124.2, 130.5 (2C), 142.5, 174.6 (O=**C**-CH_2, quaternary); Anal. Calcd for $C_{14}H_{19}NOS_2$: C, 59.75, H, 6.80, N, 4.98, O, 5.68; Found C, 59.60, H, 6.94, N, 4.88; HRFABMS Calcd for $C_{14}H_{19}NOS_2$: 281.09080 found 281.0755.

(III) By employing coupling reagents

5-[(3R)-1,2-dithiolan-3-yl] {9-[3,4-bis-(*tert*-butyl-dimethyl-silanyloxy)-5-(*tert*-butyl-dimethyl-silanyloxymethyl)-tetrahydro-furan-2-yl-9*H*-purin-6-yl}-pentanamide 120



120

DCC and EDCI

To a stirred solution of thioctic acid (4 mmol, 1eq) in 5ml of dichloromethane, 2',3',5'-O-(*tert*-butyldimethylsilyl) adenosine (4 mmol, 1eq) was added dissolved in dichloromethane (5 ml). The resulting mixture was cooled in an ice bath at 0-5^0 C and the coupling reagent (2.2 mmol, 1.1eq) was added proportionally every 10 min. Within 30 min the addition had concluded and the yellow solution (pale yellow suspension in the case of DCC) was left to stir at room temperature for 4h. The reaction mixture was concentrated to dryness to give a yellow residue (pale yellow in the case of DCC), which was dissolved in ethyl acetate (15 ml) and 2M aqueous hydrochloric acid (2 ml) and the layers partitioned. The aqueous layer was extracted further with ethyl acetate (4 x 40 ml) and the combined organic fractions were washed sequentially with saturated sodium bicarbonate solution (3 x 10 ml) and brine (3 x 10 ml). The

combined organic layers were dried over sodium sulphate, filtered and evaporated to dryness to furnish the formed amide as yellow oil in both cases.

Thioctic carbamate analogue of 2',3',5'-O-(*tert*-Butyldimethylsilyl) adenosine (**120)** (51% yield from DCC), (55% yield from EDCI) ν_{max} / cm^{-1} (neat) 3382, 2954, 1747, 1695, 1257, 1092, 838, 735, 667; ^{1}H-NMR (400 MHz, $CDCl_3$) 0.10 (s, 6H, Si-CH_3), 0.16 (s, 6H, Si-CH_3), 0.22 (s, 6H, Si-CH_3), 0.65 (s, 9H, C-CH_3), 0.81 (s, 9H, C-CH_3), 0.94 (s, 9H, C-CH_3), 1.32-1.52 (m, 2H, CH_2 aliphatic), 1.54-1.62 (m, 4H, $(CH_2)_2$ aliphatic), 1.65-1.85 (m, 2H, CH_2 cycle), 2.23-2.45 (m, 2H, O=C-C**H**$_2$), 3.0-3.22 (m, 2H, CH_2-S), 3.35-3.52 (m, 1H, CH-S), 3.78-3.95 (m, 1H, $H_{2'}$), 4.14-4.25 (m, 2H, $H_{5'}$), 4.51-4.72 (m, 1H, $H_{4'}$), 4.78-4.94 (m, 1H, $H_{3'}$), 5.65 (t, $J_{apparent}$ = 7Hz, 1H, $H_{1'}$), 7.90 (*br* s, 1H, HNCO), 8.13 (s, 1H, H_2), 8.16 (s, 1H, H_8); ^{13}C -NMR (100 MHz, $CDCl_3$) -6.2 (6C-silyl), 14.7 (3C), 20.5 (9C), 26.5, 28.5, 34.4, 36.7, 39.8, 43.6, 54.0 (7C, thioctic), 62.3, 72.4, 74.0, 87.0, 88.6, 128,5, 144.5, 148.5, 151.7, 155.1 177.0 (O=**C**-CH_2 quaternary). Anal. Calcd. for $C_{36}H_{67}N_5O_5S_2Si_3$: C, 54.16; H, 8.46; N, 8.77; Found C, 54.55; H, 8.18; N, 8.59; HRFABMS calcd for $C_{36}H_{67}N_5O_5S_2Si_3$: 797.38914 found 797.3869.

PYBOP

To a stirred solution of thioctic acid (2 mmol, 1eq) in 5ml of dichloromethane, 2',3',5'-O-(*tert*-Butyldimethylsilyl) adenosine (2 mmol, 1eq) was added dissolved in dichloromethane (5 ml). After 5 min of stirring a solution of PyBOP coupling reagent (2.2 mmol, 1.1eq) in 5ml of dichloromethane was added along with ethyldiisopropylamine (6 mmol, 3eq) and the reaction was stirred at room temperature for 2 h. The solvent was evaporated to dryness to give a yellow residue, which was dissolved in ethyl acetate (15 ml) and 2M solution aqueous hydrochloric acid (2 ml). The aqueous layer was extracted further with ethyl acetate (4 x 40 ml) and the combined organic fractions were washed sequentially with saturated sodium bicarbonate solution saturated sodium chloride (2 x 10 ml), and water (2 x 10 ml). The organic extracts were dried over sodium sulphate, filtered and evaporated to dryness to furnish the title compound. The crude product was purified by flash column chromatography using a 95: 5 mixture of DCM and methanol as the eluent to afford the formed amide as yellow oil.

Thioctic carbamate analogue of 2',3',5'-O-(*tert*-Butyldimethylsilyl) adenosine (**120**) (62% yield); ν_{max} / cm^{-1} (neat) 3382, 2954, 1747, 1695, 1257, 1092, 838, 735, 667; ^{1}H-NMR (400 MHz, $CDCl_3$) 0.10 (s, 6H, Si-CH_3), 0.16 (s, 6H, Si-CH_3), 0.22 (s, 6H, Si-CH_3), 0.65 (s, 9H, C-CH_3), 0.81 (s, 9H, C-CH_3), 0.94 (s, 9H, C-CH_3), 1.32-1.52 (m, 2H, CH_2 aliphatic), 1.54-1.62 (m, 4H, $(CH_2)_2$ aliphatic), 1.65-1.85 (m, 2H, CH_2 cycle), 2.23-2.45 (m, 2H, O=C-C**H**$_2$), 3.0-3.22 (m, 2H, CH_2-S), 3.35-3.52 (m, 1H, CH-S), 3.78-3.95 (m, 1H, $H_{2'}$), 4.14-4.25 (m, 2H, $H_{5'}$), 4.51-4.72 (m, 1H, $H_{4'}$), 4.78-4.94 (m, 1H, $H_{3'}$), 5.65 (t, $J_{apparent}$ = 7Hz, 1H, $H_{1'}$), 7.90 (br s, 1H, HNCO), 8.13 (s, 1H, H_2), 8.16 (s, 1H, H_8); ^{13}C -NMR (100 MHz, $CDCl_3$) -6.2 (6C-silyl), 14.7 (3C), 20.5 (9C), 26.5, 28.5, 34.4, 36.7, 39.8, 43.6, 54.0 (7C, thioctic), 62.3, 72.4, 74.0, 87.0, 88.6, 128,5, 144.5, 148.5, 151.7, 155.1 177.0 (O=**C**-CH_2 quart). Anal. Calcd. for $C_{36}H_{67}N_5O_5S_2Si_3$: C, 54.16; H, 8.46; N, 8.77; Found C, 54.55; H, 8.18; N, 8.59; HRFABMS calcd for $C_{36}H_{67}N_5O_5S_2Si_3$: 797.38914 found 797.3869.

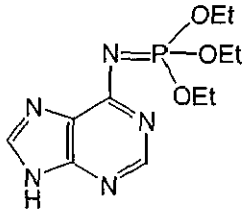
(E) Modification at the base of the nucleosides

6-Azido-9*H*-purine 123

6-chloropurine (3 mmol, 1eq), which slowly dissolved in anhydrous dimethylformamide (10 ml) with stirring, was treated with sodium azide (15 mmol, 5eq) and the reaction mixture was stirred at room temperature overnight (15h). The solvent was evaporated under high vacuum and the residue was dissolved in water (10 ml). The off-white insoluble solid was collected by suction filtration, washed with water (2 x 10 ml) and dried in vacuo to give the title compound as an off white solid.

Azide of purine base (**123**) 85% yield, ν_{max} / cm^{-1} (nujol) 2154, 1652, 1045, 845, 742; ^{1}H-NMR (250 MHz, (DMSO-d_6) 8.17 (m, 2H, HN-C**H**=N+N-C**H**=N), 9.54 (br s, 1H, NH); ^{13}C -NMR (100 MHz, (DMSO-d_6) 128.4, 144.8, 146.5, 152.1, 154.9; Anal. Calcd. For $C_5H_3N_7$: C, 37.27, H, 1.88, N, 60.85; Found C, 37.08; H, 2.02; N, 60.63; HRFABMS calcd for $C_5H_3N_7$: 161.04499 found 161.0419.

9*H*-Purin-6-yl-phosphorimidic acid triethyl ester 124.

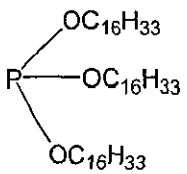


In a solution of the azide **123** (2 mmol, 1eq) in anhydrous dimethylformamide (10 ml), triethyl phosphite (2 mmol, 1eq) was added dropwise and the reaction mixture was stirred at room temperature under nitrogen, until TLC showed that consumption of the azide was complete (18h). The solvent was evaporated under high vacuum to give a light yellow solid. Recrystallization

from a mixture of dichloromethane and petroleum ether (4: 1) afforded the triethyl ester phosphoramidite as an off-yellow solid.

Triethyl ester phosphoramidate of purine base (**124**) 82% yield, ν_{max} / cm^{-1} (nujol) 1599, 1685, 1031, 857, 763; ^{1}H-NMR (250 MHz, $CDCl_3$,) 1.28-1.35 (m, 9H, CH_3CH_2), 4.06-4.20 (m, 6H, CH_3CH_2), 7.98 (m, 2H, HN-CH=N+N-CH=N), 9.37 (br s, 1H, NH); ^{13}C -NMR (100 MHz, $CDCl_3$) 16.4 [3C, $(CH_3)_3$], 47.2 [3C, $(CH_2)_3$], 127.4, 143.7, 145.6, 151.1, 153.7; ^{31}P-NMR (100 MHz, $CDCl_3$,) 1.93; Anal. Calcd. For $C_{11}H_{18}N_4O_3P$: C, 44.15, H, 6.06, N, 23.40, O, 16.04; Found C, 43.92; H, 6.34; N, 23.72; HRFABMS calcd for $C_{11}H_{18}N_5O_3P$; 299.11473 found 299.1117.

Phosphorous acid trihexadecyl ester 127

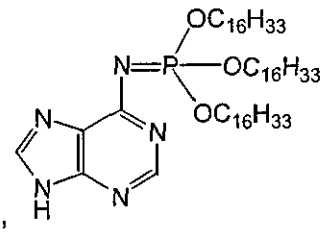


Cetyl alcohol (30 mmol, 3eq) was dissolved in 50ml of dichloromethane and in the solution which was kept at 0 ^{0}C and under nitrogen, phosphorous trichloride (10 mmol, 1eq) was added dropwise followed by the addition of triethylamine (30 mmol, 3eq). The reaction mixture was stirred at ambient temperature for 12h. After removing the triethyl-ammonium salts through suction filtration, the solution was transferred into an extraction funnel in which saturated hydrogen carbonate solution was added (30 ml). The aqueous layer was extracted further with dichloromethane (3 x 30 ml) and the combined organic fractions were washed with brine (2 x 20 ml). The organic extracts were dried over magnesium sulphate, filtered and evaporated to dryness, to give an off-white solid. Recrystallization from dichloromethane and petroleum ether furnished the tricetyl phosphite as white solid.

Tricetyl phosphite (**127**) 63%yield, ν_{max} / cm^{-1} (nujol) 1463, 1474, 1040, 955, 730; ^{1}H-NMR (250 MHz, $CDCl_3$,) 0.88 (t, J = 7.2, 9H, $(CH_3CH_2)_3$], 1.52-1.75

(m, 84H, $(CH_2)_{42}$], 3.63 (t, J = 7.6, 6H, $(OCH_2)_3$]; ^{13}C -NMR (100 MHz, $CDCl_3$) 14.2 [3C, $(CH_3)_3$], 23.4 [3C, $(CH_2)_3CH_3$], 25.6 (3C), 29.5 (3C), 30.3 (27C), 32.4 (3C), 32.8 (3C), 59.4 (3C); ^{31}P-NMR (100 MHz, $CDCl_3$,) 0.48; Anal. Calcd. For $C_{48}H_{99}O_3P$: C, 76.33, H, 13.21, O, 6.36; Found C, 76.59, H, 13.05; HRFABMS calcd for $C_{48}H_{99}O_3P$: 754.73318 found 754.7299.

9*H*-Purin-6-yl-phosphorimidic acid trihexadecyl ester 128



In a solution of the azide **123** (2 mmol, 1eq) in anhydrous DMF (10 ml), the formed tricetyl phosphite **127** (2 mmol, 1eq) dissolved in anhydrous dimethylformamide (10 ml), was added. The reaction mixture was stirred at room temperature under nitrogen, until TLC showed that consumption of the azide was complete (15h). The solvent was evaporated under high vacuum to give an off-yellow solid. Recrystallization from a mixture of ethyl acetate and hexane (3: 1) afforded the triethyl ester phosphoramidite as an off-yellow solid.

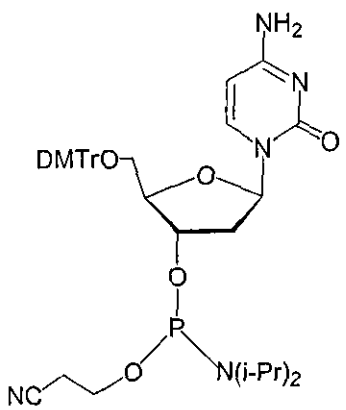
Tricetyl ester phosphoramidite of purine base (**128**) 71%yield, ν_{max} / cm^{-1} (nujol) 1565, 1472, 1045, 932, 732; ^{1}H-NMR (250 MHz, $CDCl_3$,) 0.92 (t, J = 7.4, 9H, $(CH_3CH_2)_3$], 1.56-1.79 (m, 84H, $(CH_2)_{42}$], 3.68 (t, J = 7.4, 6H, $(OCH_2)_3$]; 7.92 (m, 2H, HN-CH=N+N-CH=N), 9.27 (br s, 1H, NH); ^{13}C -NMR (100 MHz, $CDCl_3$) 15.7 [3C, $CH_3)_3$], 24.6 [3C, $(CH_2)_3CH_3$], 26.5 (3C), 29.8 (3C), 31.3 (27C), 33.1 (3C), 33.8 (3C), 60.1 (3C), 127.2, 143.7, 145.4, 151.3, 153.7; ^{31}P-NMR (100 MHz, $CDCl_3$,) 0.95; Anal. Calcd. For $C_{53}H_{102}N_5O_3P$: C, 71.65, H, 11.57, N, 7.88, O, 5.40; Found C, 71.91, H, 11.32, N, 7.53; HRFABMS calcd for $C_{53}H_{102}N_5O_3P$: 887.77203 found 887.7699.

4. FORMATION OF CPG DINUCLEOTIDES

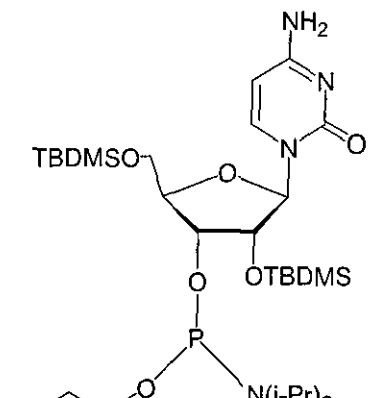
(A) Synthesis of N-free-3'-phosphoramidites of cytidine

Procedure for the preparation of N-free 3'-cyanoethyl phosphoramidites of 2'-deoxycytidine 27 and 129

A solution of 5'-O-(Dimethoxytrityl)-2-deoxycytidine **26** and 2',5'-O-TBDMS cytidine **73** (5 mmol, 1eq) in anhydrous THF (10 ml) was put under nitrogen and cooled at -78 ^{0}C. In the stirred solution ethyldiisopropylamine (7.5 mmol, 1.5eq) was added, followed by dropwise addition of (2-cyanoethyl)(*N,N*-diisopropylamino)chlorophosphine (5.5 mmol, 1.1eq). After the addition concluded, the reaction mixture was allowed to warm and after stirring at ambient temperature for 1h, TLC showed consumption of the starting material. The solvent was evaporated to dryness under high vacuum and the residue obtained was dissolved in dichloromethane (25 ml) and saturated sodium bicarbonate solution (25 ml). The aqueous layer fraction was extracted further with further dichloromethane (3 x 30ml) and the organic fractions were washed with saturated sodium bicarbonate solution (2 x 10ml) and brine (2 x 10ml). The combined organic extracts were dried over magnesium sulphate, filtered and evaporated to dryness, to give viscous oil. The oil was dissolved in dichloromethane (10 ml) and was poured into a vigorously stirred petroleum ether (100 ml) to give colourless precipitates in both cases. Suction filtration of the solid material afforded the 3'-phosphoramidites of 2'-deoxycytidine as white solid in both cases. No further purification of the material was required.



27



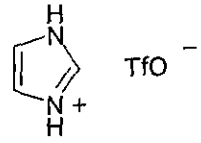
129

4,4'-Dimethoxy-biphenyl-diisopropyl-phosphoramidous acid-5-(4-amino-2-oxo-2*H*-pyrimidin-1-yl)-2-(4methoxy-benzyloxymethyl)-tetrahydro-furan-yl ester 2 isocyano-ethyl ester (**27**) 85% yield, ν_{max} / cm^{-1} (nujol) 1757, 1612, 1510, 1362, 1179, 831; ^{1}H-NMR (250 MHz, $CDCl_3$) 1.02-1.15 (m, 12H, $CH_3)_4$], 1.94-2.12 (m, 2H, $H_{2'2''}$), 2.48-2.61 (m, 2H, $(CH)_2$], 3.24-3.37 (m, 2H, $H_{5'5''}$), 3.44-3.51 (m, 2H), 3.80 [s, 6H, O-$(CH_3)_2$], 3.88-3.94 (m, 2H), 4.12-4.23 (m, 1H, $H_{4'}$), 4.42-4.54 (m, 1H, $H_{3'}$), 5.52 (d, $J_{5\text{-}6}$ = 8 Hz, 1H, H_5), 6.17 (t, $J_{apparent}$ = 6.9 Hz, 1H, $H_{1'}$), 6.34 (br s, 2H, NH_2), 6.65-6.71 (m, 4H, trityl), 7.05-7.24 (m, 9H, trityl), 7.82 (d, $J_{5\text{-}6}$ = 8.1 Hz, 1H, H_6); ^{13}P-NMR (250 MHz, $CDCl_3$) 148.07, 148.3; Anal. Calcd for $C_{39}H_{48}N_5O_7P$: C, 64.01; H, 6.89; N, 9.57; Found: C, 63.58; H, 6.64, N, 9.72; HRFABMS Calcd for: $C_{39}H_{48}N_5O_7P$: 729.32913 found 729.3266.

Diisopropyl-phosphoramidous acid-5-(4-amino-2-oxo-2*H*-pyrimidin-1-yl)-4-(*tert*-butyl-dimethyl-silanyloxy)-2-(*tert*-butyl-dimethyl-silanyloxy)-tetrahydro-furan-3-yl ester 2 isocyano-ethyl ester (**129**) 83% yield, ν_{max} / cm^{-1} (nujol) 1725, 1644, 1462, 1265, 1035, 842; ^{1}H-NMR (250 MHz, $CDCl_3$) 0.20 (s, 6H, Si-CH_3), 0.66 (s, 6H, Si-CH_3), 0.82 (s, 9H, C-CH_3), 1.0 (s, 9H, C-CH_3), 1.05-1.18 (m, 12H, $(CH_3)_4$], 1.98-2.16 (m, 1H, $H_{2'}$), 2.53-2.65 (m, 2H, $(CH)_2$], 3.28-3.41 (m, 1H, $H_{5'5''}$), 3.47-3.54 (m, 2H), 3.92-3.97 (m, 2H), 4.17-4.28 (m, 1H, $H_{4'}$), 4.45-4.57 (m, 1H, $H_{3'}$), 5.56 (d, $J_{5\text{-}6}$ = 7.4 Hz, 1H, H_5), 6.21 (t, $J_{apparent}$ = 6.5 Hz, 1H, $H_{1'}$), 6.39 (br s, 2H, NH_2), 7.87 (d, $J_{5\text{-}6}$ = 7.2 Hz, 1H, H_6); ^{13}P-NMR (250 MHz, $CDCl_3$) 150.5; Anal. Calcd for $C_{30}H_{58}N_5O_6PSi_2$: C, 53.62, H, 8.70, N, 10.42; Found: C, 53.24, H, 8.42, N, 10.84; HRFABMS Calcd for: $C_{30}H_{57}N_5O_6PSi_2$: 670.36633 found 670.3638.

(B) Solution Phase synthesis of CpG dinucleotides

Imidazolium triflate 132



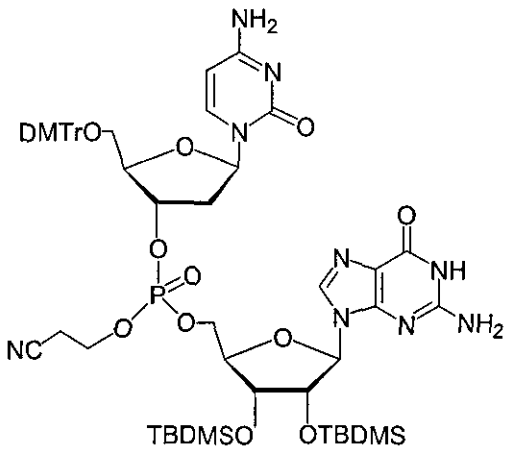
In a stirred solution of imidazole (20 mmol, 1eq) in dichloromethane (5 ml) trifluoromethanesulfonic acid (20 mmol, 1eq) was added dropwise. After the addition concluded the reaction mixture was stirred at ambient temperature for 30 min. TLC showed the reaction to be complete. The reaction mixture was diluted with diethyl ether and the title compound was collected through suction filtration as crystals. The product did not require further purification

Imidazolium Triflate **132**, 97% yield, m.p. 194-196 ^{0}C (lit[315] 197-198 ^{0}C); ν_{max} / cm^{-1} (nujol) 3148, 1594, 1452, 1259, ^{1}H-NMR (250 MHz, CD_3OD) 8.12 (s, 2H), 9.42 (s, 1H), ^{13}C-NMR (100 MHz, CD_3OD) 121, 125, 138, Anal. Calcd for $C_4H_5F_3N_2O_3S$: C, 22.02; H, 2.31; N, 12.84; Found: C, 21.95; H, 2.34; N, 12.77;

Stepwise synthesis of dinucleotide CpG phosphates using stoichiometric amounts of imidazolium triflate 132, N-free-3'-phosphoramidite of cytidine 27 and 2',3'-O-TBDMS protected guanosine 79 or 2',3'-O-TBDMS-N-carbamate cholesteryl 104 and tocopheryl 105 conjugates of guanosine.

Procedure for the synthesis of 2'-cyanoethyl-5'-DMTr-2'deoxycytidine-2',3'-TBDMS guanosine or 2'-cyanoethyl-5'-DMTr-2'deoxycytidine-2',3'-O-TBDMS-N-carbamate cholesteryl and tocopheryl conjugates of guanosine

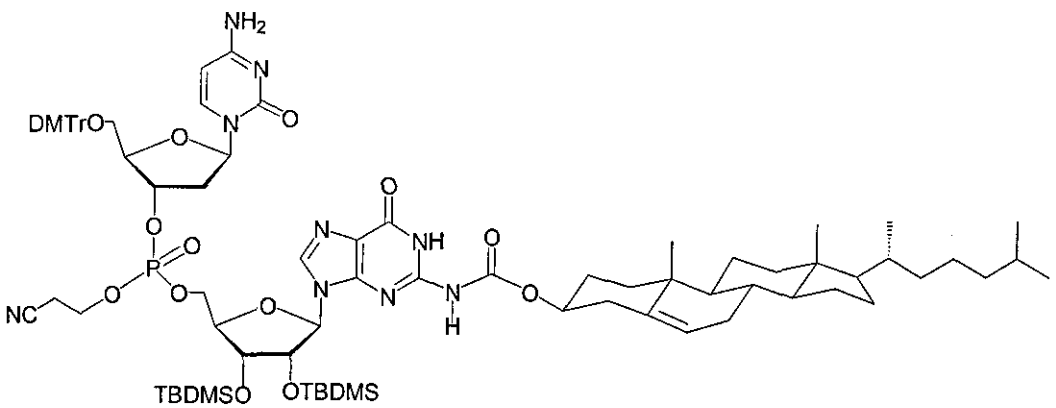
In a stirred solution of N-free-3'-phosphoramidite of cytidine **27** (4 mmol, 1eq) in 8 ml dry of acetonitrile, a solution of 2',3'-O-TBDMS protected guanosine **79** or 2',3'-O-TBDMS-N-carbamate cholesteryl **104** or tocopheryl **105** conjugates of guanosine (4 mmol, 1eq) was added along with a solution of imidazolium triflate **132** (4 mmol, 1eq) in 15 ml of dry acetonitrile. After the reaction mixture was stirred at room temperature for 10 min, 1.0 M of toluene solution of *tert*-butyl hydroperoxide (TBHP) (1.5 mmol, 1.5eq) was added and the stirring continued for 10 min. Evaporation of the reaction mixture gave an oil which was dissolved in dichloromethane and subjected to chromatography on a silica gel pad eluting with a 10:1 mixture of dichloromethane and methanol. The organic filtrate was evaporated to dryness to afford purified **133** as a colorless amorphous solid in 76% yield, **139** as a off-white solid in 72% yield and **140** as an off-yellow solid in 75% yield



133

4,4'-dimethoxy-biphenyl-phosphoric acid-5-(2-amino-6-oxo-1,6-dihydro-purin-9-yl) 3,4-bis-(*tert*-butyl-dimethyl-silanyloxy)-tetrahydro-furan-2-ylmethyl ester 5-(4-amino-2-oxo-2H-pyrimidin-1-yl)-2-benzyloxymethyl)-tetrahydro-furan-3-ylmethyl ester 2 isocyano-ethyl ester (**133**) 76% yield, ν_{max} / cm^{-1} (nujol) 1727, 1695, 1548, 1477, 1265, 1045, 874; ^{1}H-NMR (250 MHz, $CDCl_3$) 0.16 (s, 6H, Si-CH_3), 0.20 (s, 6H, Si-CH_3), 0.84 (s, 9H, C-CH_3), 0.96 (s, 9H, C-CH_3), 1.97-2.19 (m, 2H, $H_{2'2''}$), 3.11-3.22 (m, 2H), 3.28-3.42 (m, 2H, $H_{5'5''}$), 3.46-3.54 (m, 2H), 3.69-3.78 (m, 1H), 3.84 [s, 6H, O-$(CH_3)_2$], 3.91-

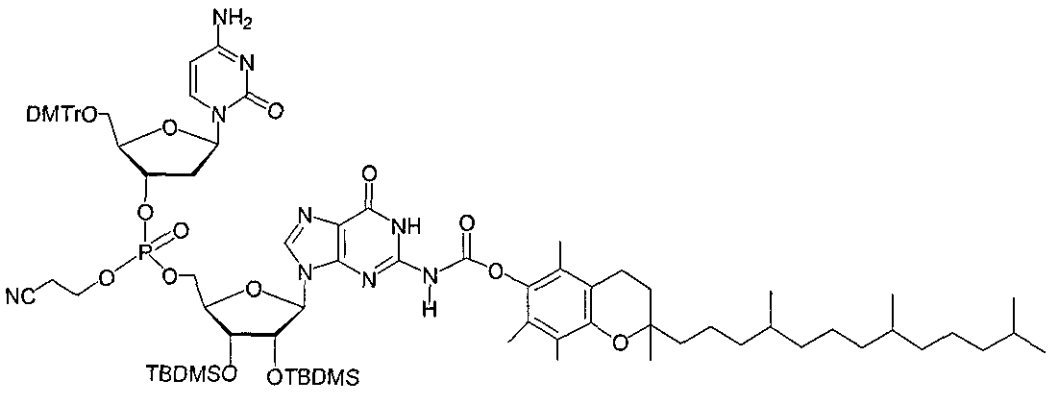
3.95 (m, 2H), 4.15-4.25 (m, 1H, $H_{4'}$), 4.45-4.58 (m, 1H, $H_{3'}$), 4.91 (t, $J_{apparent}$ = 12.1 Hz, 1H), 5.36 (t, $J_{apparent}$ = 11.5 Hz, 1H), 5.56 (d, $J_{5\text{-}6}$ = 7.3 Hz, 1H, H_5), 5.98 (d, $J_{apparent}$ = 6.3Hz, 1H), 6.17 (t, $J_{apparent}$ = 7.1 Hz, 1H, $H_{1'}$), 6.34 (br s, 2H, NH_2), 6.68-6.74 (m, 4H, trityl), 6.91 (br s, 2H, NH_2), 7.07-7.27 (m, 9H, trityl), 7.72 (s, 1H), 7.84 (d, $J_{5\text{-}6}$ = 7.8 Hz, 1H, H_6), 7.93 (s, 1H, NH).; ^{13}C-NMR (100 MHz, $CDCl_3$) -6.6 (4C-silyl), 15.5 (2C), 18.8, 21.3 (6C), 33.2, 56.9 (2C), 61.1, 61.8, 65.1, 68.1, 68.9, 72.5, 74.6, 75.3, 76.9, 77.4, 77.9, 99.1, 114.9 (4C), 117.2, 118.1, 127.8 (3C), 128.8 (6C), 130.1 (2C), 131.8, 137.6, 138.7, 140.6, 148.8, 158.7 (C=O, cytidine), 160.7 (C=O, guanosine), 161.8 (2C), 163.7, 164.5; ^{13}P-NMR (250 MHz, $CDCl_3$) 1.95, -2.01; Anal. Calcd for $C_{55}H_{76}N_9O_{13}PSi_2$: C, 57.03, H, 6.61, N, 10.88, O, 17.96; Found: C, 56.82, H, 6.84, N, 10.53; HRFABMS Calcd for: $C_{55}H_{74}N_9O_{13}PSi_2$: 1155.48388 found 1155.4809.



139

4,4'-dimethoxy-biphenyl-phosphoric acid-5-(4-amino-2-oxo-2*H*-purimidin-1-yl) {9-[3,4-Bis-(*tert*-butyl-dimethyl-silanyloxy)-tetrahydro-furan-2-yl]-6-oxo-6,9-dihydro-1*H* -purin-2-yl}-carbamic acid 17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H* cyclopenta[a]phenanthren-tetrahydro-furan-3-ylmethyl ester 2 isocyano-ethyl ester (139) 72% yield, ν_{max} / cm^{-1} (nujol) 3265, 3114, 1741, 1685, 1548, 1486, 1268, 1048, 855, 767; ^{1}H-NMR (250 MHz, $CDCl_3$) 0.15 (s, 6H, Si-CH_3), 0.18 (s, 6H, Si-CH_3), 0.81 (s, 9H, C-CH_3), 0.92 (s, 9H, C-CH_3), 0.69-2.38 (m, 45H, aliphatic),1.94-2.13 (m, 2H, $H_{2'2''}$),

3.06-3.17 (m, 2H), 3.23-3.35 (m, 2H, $H_{5'5''}$), 3.42-3.50 (m, 2H), 3.65-3.75 (m, 1H), 3.81 [s, 6H, O-$(CH_3)_2$], 3.86-3.90 (m, 2H), 4.11-4.21 (m, 1H, $H_{4'}$), 4.41-4.52 (m, 1H, $H_{3'}$), 4.56-4.77 (m, 1H, O-CH), 4.87 (t, $J_{apparent}$ = 11.6 Hz, 1H), 5.32 (t, $J_{apparent}$ = 12.0 Hz, 1H), 5.38 (d, 1H, J = 2.6 Hz, HC=C), 5.52 (d, J_{5-6} = 8.2 Hz, 1H, H_5), 5.93 (d, $J_{apparent}$ = 6.7 Hz, 1H), 6.12 (t, $J_{apparent}$ = 7 Hz, 1H, $H_{1'}$), 6.30 (br s, 2H, NH_2), 6.64-6.70 (m, 4H, trityl), 7.02-7.23 (m, 9H, trityl), 7.55 (br s, 1H, CONH), 7.67 (s, 1H), 7.80 (d, J_{5-6} = 8 Hz, 1H, H_6), 7.89 (s, 1H, NH).; ^{13}C-NMR (100 MHz, $CDCl_3$) –5.6 (4C-silyl), 15.2 (2C), 17.3, 18.3, 18.8, 19.4, 19.7, 21.3 (6C), 23.4 (2C), 26.5, 29.9, 31.5, 32.5, 33.2, 34.2, 35.4, 36.2, 38.5, 39.6, 40.9, 42.5, 43.7, 48.3, 48.8, 51.2, 56.2 (2C), 61.2, 64.8, 67.8, 69.4, 71.8, 72.8, 73.1, 74.5, 74.9, 76.3, 76.9, 77.4, 98.1, 115.1 (4C), 117.7, 122.8 (CH=C), 126.4, 127.6 (3C), 128.2 (6C), 130.3 (2C), 131.5, 137.4, 138.5, 139.6, 145.8, 148.2, 149.5 (CH=C), 155.9, 158.1, 159.2 (C=O, cytidine), 160.8 (C=O, guanosine), 161.9 (2C), 163.2, 164.4, 170.0 (HN-C=O); ^{13}P-NMR (250 MHz, $CDCl_3$) -2.16, -2.24; Anal. Calcd for $C_{83}H_{118}N_9O_{15}PSi_2$: C, 63.56, H, 7.58, N, 8.04, O, 15.28; Found: C, 63.21, H, 7.98, N, 7.86; HRFABMS Calcd for: $C_{83}H_{118}N_9O_{15}PSi_2$: 1567.80235 found 1567.7996.



140

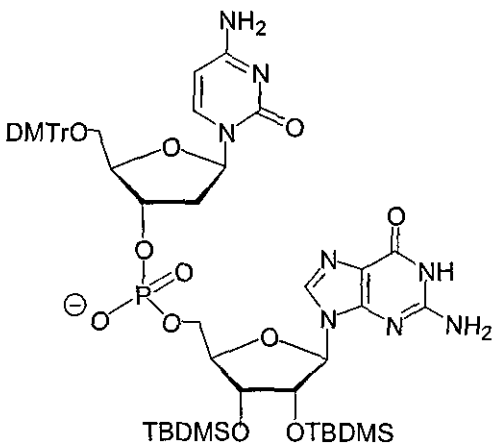
4,4'-dimethoxy-biphenyl-phosphoric acid-5-(4-amino-2-oxo-2*H*-purimidin-1-yl) {9-[3,4-Bis-(*tert*-butyl-dimethyl-silanyloxy)-5-methyl-tetrahydro-furan-2-yl]-6-oxo-6,9-dihydro-1*H*-purin-2-yl}-carbamic acid 2,7,8-trimethyl-2-(4,8,12-trimethyl-tridecylhexyl)-chroman-tetrahydro-furan-3-ylmethyl ester 2 isocyano-ethyl ester (140) 75% yield, ν_{max} / cm^{-1}

(nujol) 3388, 3085, 1749, 1694, 1580, 1492, 1272, 1048, 874, 776; ^{1}H-NMR (250 MHz, $CDCl_3$) 0.16 (s, 6H, Si-CH_3), 0.18 (s, 6H, Si-CH_3), 0.83 (s, 9H, C-CH_3), 0.95 (s, 9H, C-CH_3), 0.75-0.95 [m, 12H, (C**H**$_3$-CH)$_4$], 1.12-1.73 [m, 20H, $(CH_2)_{10}$], 1.62-1.82 [m, 3H, (C**H**-CH_3)$_3$], 1.92 (s, 3H, CH_3-Ar), 1.96-2.12 (m, 2H, $H_{2'2''}$), 2.15 (s, 3H, CH_3-Ar), 2.21 (s, 3H, CH_3-Ar), 2.34 (s, 3H, CH_3-C-O), 2.55-2.68 (m, 2H, CH_2-Ar), 3.10-3.21 (m, 2H), 3.23-3.34 (m, 2H, $H_{5'5''}$), 3.44-3.51 (m, 2H), 3.65-3.75 (m, 1H), 3.83 [s, 6H, O-$(CH_3)_2$], 3.88-3.90 (m, 2H), 4.12-4.22 (m, 1H, $H_{4'}$), 4.43-4.54 (m, 1H, $H_{3'}$), 4.89 (t, $J_{apparent}$ = 11.3 Hz, 1H), 5.35 (t, $J_{apparent}$ = 12.4 Hz, 1H), 5.56 (d, $J_{5\text{-}6}$ = 8.1 Hz, 1H, H_5), 5.97 (d, $J_{apparent}$ = 6.3 Hz, 1H), 6.12 (t, $J_{apparent}$ = 7.5 Hz, 1H, $H_{1'}$), 6.30 (br s, 2H, NH_2), 6.64-6.70 (m, 4H, trityl), 7.02-7.23 (m, 9H, trityl), 7.58 (br s, 1H, CON**H**), 7.71 (s, 1H), 7.82 (d, $J_{5\text{-}6}$ = 8 Hz, 1H, H_6), 7.91 (s, 1H, NH).; ^{13}C-NMR (100 MHz, $CDCl_3$) -5.6 (4C-silyl), 4.8, 5.0, 5.3, 15.2 (2C), 16.9, 18.6, 19.4, 20.1 (2C), 21.3 (6C), 22.3 (2C), 25.5 (2C), 28.5, 31.7 (2C), 32.8, 34.2, 35.4, 37.4 (3C), 38.8, 41.2, 45.6, 56.2 (2C), 61.2, 64.7, 67.7, 68.2, 71.8, 72.8, 73.7, 75.2, 75.8, 76.3, 78.2, 98.8, 114.6 (4C), 117.7, 121.3, 123.1, 127.6 (3C), 127.8, 128.4 (6C), 129.5 (2C), 131.3, 136.6, 138.3, 139.2, 144.5, 147.7, 155.3, 158.5, 159.3 (C=O, cytidine), 160.5 (C=O, guanosine), 161.2 (2C), 162.7, 164.8, 170.05 (HN-C=O); ^{13}P-NMR (250 MHz, $CDCl_3$) -2.19, -2.3; Anal. Calcd for $C_{84}H_{122}N_9O_{16}PSi_2$: C, 63.01, H, 7.68, N, 7.87, O, 15.28; Found: C, 62.75, H, 7.94, N, 7.57; HRFABMS Calcd for: $C_{84}H_{122}N_9O_{16}PSi_2$: 1599.82857 found 1599.8253.

Procedure for the removal of 2'-cyanoethyl protecting group.

In a stirred solution of **133** or **139** or **140** (2 mmol, 1eq) in dry acetonitrile (4 ml) anhydrous *tert*-butylamine (5 ml) was added and the reaction mixture was stirred at room temperature for 2 days. After completion of the reaction the solvent was evaporated to dryness and the residue was dissolved in 50 ml of dichloromethane and 50 ml of saturated sodium bicarbonate solution. After separation, the aqueous phase was further extracted with dichloromethane (5 x 30 ml) and the combined organic layers were washed with brine (4 x 30 ml).

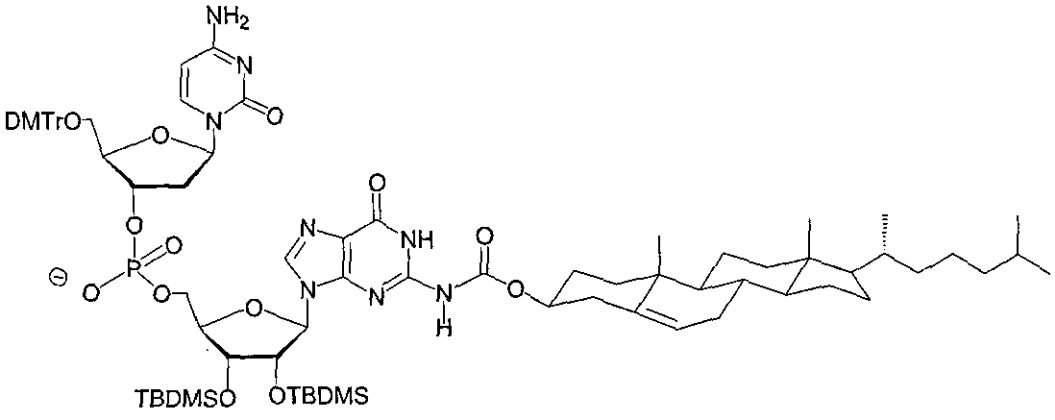
The organic fractions were dried over sodium sulphate, filtered and evaporated to dryness. The crude products were purified on a silica gel column eluting with a 10:1 mixture of dichloromethane and methanol containing a trace amount of triethylamine to afford **134** as a colorless foam in 73% yield while **141** and **142** were furnished as an off-white foam and off-yellow foam in 70 and 72% yield, respectively.



134

4,4'-dimethoxy-biphenyl-phosphoric acid-5-(2-amino-6-oxo-1,6-dihydro-purin-9-yl) 3,4-bis-(*tert*-butyl-dimethyl-silanyloxy)-tetrahydro-furan-2-ylmethyl ester 5-(4-amino-2-oxo-2*H*-pyrimidin-1-yl)-2-benzyloxymethyl-tetrahydro-furan-3-yl ester (134) 73% yield, ν_{max} / cm^{-1} (*nujol*) 1735, 1686, 1520, 1465, 1275, 1042, 797; ^{1}H-NMR (250 MHz, $CDCl_3$) 0.14 (s, 6H, Si-CH_3), 0.19 (s, 6H, Si-CH_3), 0.82 (s, 9H, C-CH_3), 0.94 (s, 9H, C-CH_3), 1.94-2.16 (m, 2H, $H_{2'2''}$), 3.25-3.38 (m, 2H, $H_{5'5''}$), 3.44-3.51 (m, 2H), 3.67-3.76 (m, 1H), 3.82 [s, 6H, O-$(CH_3)_2$], 4.13-4.22 (m, 1H, $H_{4'}$), 4.42-4.55 (m, 1H, $H_{3'}$), 4.88 (t, $J_{apparent}$ = 11.5 Hz, 1H), 5.33 (t, $J_{apparent}$ = 12.2 Hz, 1H), 5.54 (d, J_{5-6} = 8.0 Hz, 1H, H_5), 5.95 (d, $J_{apparent}$ = 6.3 Hz, 1H), 6.14 (t, $J_{apparent}$ = 7.2 Hz, 1H, $H_{1'}$), 6.33 (br s, 2H, NH_2), 6.66-6.72 (m, 4H, trityl), 6.90 (br s, 2H, NH_2), 7.05-7.24 (m, 9H, trityl), 7.70 (s, 1H), 7.82 (d, J_{5-6} = 8 Hz, 1H, H_6), 7.91 (s, 1H, NH).; ^{13}C-NMR (100 MHz, $CDCl_3$) -6.4 (4C-silyl), 15.2 (2C), 20.8 (6C), 32.8, 56.2 (2C), 61.4, 64.8, 67.7, 68.2, 71.8, 74.4, 74.8, 76.3, 76.8, 77.4, 98.3, 114.6 (4C), 127.5 (3C), 128.4 (6C), 129.8 (2C), 131.5, 137.3, 138.2, 139.5, 148.5, 158.4 (C=O, cytidine), 160.3 (2C), 161.5 (C=O), 163.3, 164.5; Anal.

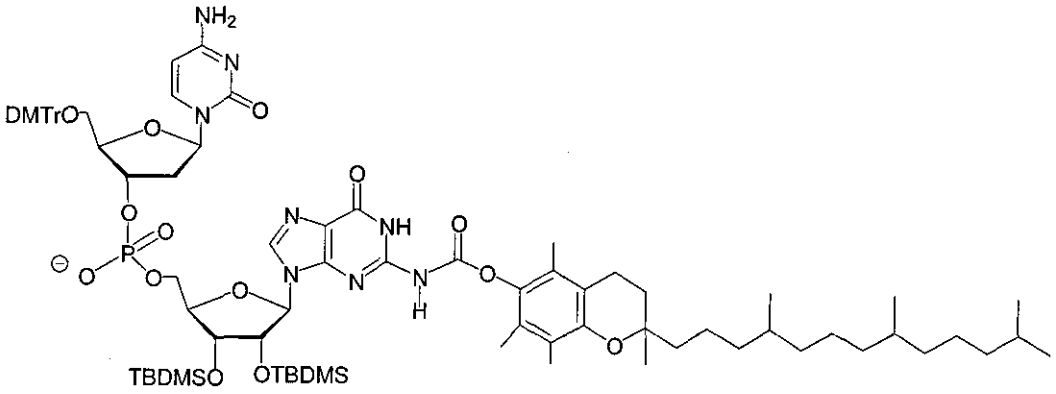
Calcd for $C_{52}H_{70}N_8O_{13}PSi_2$: C, 56.56, H 6.57, N 10.15, O 18.83; Found: C, 56.24, H 6.96, N 9.84; HRFABMS Calcd for: $C_{52}H_{70}N_8O_{13}PSi_2$: 1101.43385 found 1101.4106.



141

4'-dimethoxy-biphenyl-phosphoric acid [5-(4-amino-2-oxo-2*H*-purimidin-1-yl)] {9-[3,4-Bis-(*tert*-butyl-dimethyl-silanyloxy)-tetrahydro-furan-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-carbamic acid 17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren- tetrahydro-furan-3-ylmethyl ester (**141**) 70% yield, ν_{max} / cm^{-1} (nujol) 3451, 3080, 1741, 1685, 1575, 1492, 1274, 1036, 855, 792; ^{1}H-NMR (250 MHz, $CDCl_3$) 0.13 (s, 6H, Si-CH_3), 0.17 (s, 6H, Si-CH_3), 0.80 (s, 9H, C-CH_3), 0.94 (s, 9H, C-CH_3), 0.67-2.36 (m, 45H, aliphatic),1.92-2.11 (m, 2H, $H_{2'2''}$), 3.22-3.34 (m, 2H, $H_{5'5''}$), 3.62-3.73 (m, 1H), 3.79 [s, 6H, O-$(CH_3)_2$], 3.85-3.89 (m, 2H), 4.10-4.20 (m, 1H, $H_{4'}$), 4.39-4.50 (m, 1H, $H_{3'}$), 4.53-4.74 (m, 1H, O-CH), 4.85 (t, $J_{apparent}$ = 11.3 Hz, 1H), 5.32 (t, $J_{apparent}$ = 12.4 Hz, 1H), 5.36 (d, 1H, J = 2.5 Hz, HC=C), 5.51 (d, $J_{5\text{-}6}$ = 8.7 Hz, 1H, H_5), 5.91 (d, $J_{apparent}$ = 6.4 Hz, 1H), 6.10 (t, $J_{apparent}$ = 7 Hz, 1H, $H_{1'}$), 6.32 (br s, 2H, NH_2), 6.62-6.69 (m, 4H, trityl), 7.01-7.21 (m, 9H, trityl), 7.54 (br s, 1H, CONH), 7.65 (s, 1H), 7.79 (d, $J_{5\text{-}6}$ = 8.1 Hz, 1H, H_6), 7.87 (s, 1H, NH).; ^{13}C-NMR (100 MHz, $CDCl_3$) -5.2 (4C-silyl), 15.0 (2C), 17.1, 18.2, 19.5, 19.9, 21.7 (6C), 23.8 (2C), 26.9, 29.7, 31.8, 32.6, 33.5, 34.7, 35.6, 36.7, 38.6, 39.8, 41.3, 42.6, 43.8, 48.5, 49.1, 51.5, 56.6 (2C), 64.9, 68.2, 69.5, 72.0, 72.7, 73.1, 74.6, 75.3, 76.5, 77.0, 78.1, 99.3, 115.4 (4C), 123.1 (CH=C), 126.5 127.8 (3C), 128.4

(6C), 130.3 (2C), 131.5, 137.6, 138.7, 140.0, 146.3, 148.5, 149.5 (CH=**C**), 156.2, 158.4 (C=O, cytidine), 159.6 (C=O, guanosine), 161.0 (2C), 163.2, 164.5, 170.1 (HN-C=O); Anal. Calcd for $C_{80}H_{114}N_8O_{15}PSi_2$: C, 63.45, H, 7.59, N, 7.40, O, 15.82 Found: C, 63.12, H, 7.98, N, 7.07; HRFABMS Calcd for: $C_{80}H_{114}N_8O_{15}PSi_2$: 1513.76798 found 1513.7651.



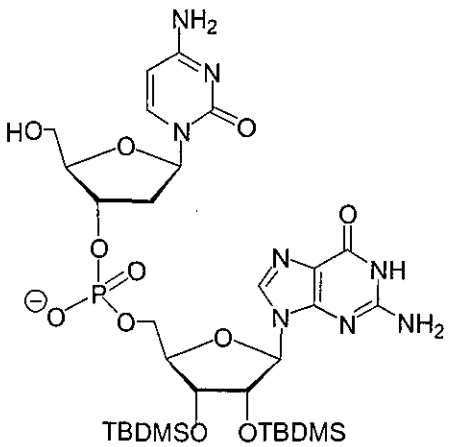
142

4,4'-dimethoxy-biphenyl-phosphoric acid 5-(4-amino-2-oxo-2*H*-purimidin-1-yl) {9-[3,4-Bis-(*tert*-butyl-dimethyl-silanyloxy)-5-methyl-tetrahydro-furan-2-yl]-6-oxo-6,9-dihydro-1*H*-purin-2-yl}-carbamic acid 2,7,8-trimethyl-2-(4,8,12-trimethyl-tridecylhexyl)-chroman-tetrahydro-furan-3-ylmethyl ester (142) 72% yield, ν_{max} / cm^{-1} (nujol) 3420, 3080, 1755, 1675, 1582, 1492, 1265, 1033, 882, 785; ^{1}H-NMR (250 MHz, $CDCl_3$) 0.15 (s, 6H, Si-CH_3), 0.17 (s, 6H, Si-CH_3), 0.81 (s, 9H, C-CH_3), 0.93 (s, 9H, C-CH_3), 0.73-0.94 [m, 12H, (CH_3-CH)$_4$], 1.10-1.71 [m, 20H, $(CH_2)_{10}$], 1.60-1.80 [m, 3H, (C**H**-CH_3)$_3$], 1.91 (s, 3H, CH_3-Ar), 1.95-2.10 (m, 2H, $H_{2'2''}$), 2.14 (s, 3H, CH_3-Ar), 2.20 (s, 3H, CH_3-Ar), 2.32 (s, 3H, CH_3-C-O), 2.53-2.65 (m, 2H, CH_2-Ar), 3.21-3.32 (m, 2H, $H_{5'5''}$), 3.66-3.77 (m, 1H), 3.82 [s, 6H, O-$(CH_3)_2$], 3.89-3.92 (m, 2H), 4.11-4.20 (m, 1H, $H_{4'}$), 4.44-4.55 (m, 1H, $H_{3'}$), 4.91 (t, $J_{apparent}$ = 11.1 Hz, 1H), 5.34 (t, $J_{apparent}$ = 12.2 Hz, 1H), 5.55 (d, $J_{5\text{-}6}$ = 8.2 Hz, 1H, H_5), 5.95 (d, $J_{apparent}$ = 6.3 Hz, 1H), 6.13 (t, $J_{apparent}$ = 7.5 Hz, 1H, $H_{1'}$), 6.31 (br s, 2H, NH_2), 6.66-6.72 (m, 4H, trityl), 7.05-7.24 (m, 9H, trityl), 7.60 (br s, 1H, CONH), 7.72 (s, 1H), 7.81 (d, $J_{5\text{-}6}$ = 8.2 Hz, 1H, H_6), 7.92 (s, 1H, NH); ^{13}C-NMR (100 MHz, $CDCl_3$) -5.8 (4C-silyl), 4.5, 5.0, 5.2, 15.0 (2C), 16.5, 19.1, 20.2 (2C), 21.6 (6C),

22.1 (2C), 22.7, 25.6 (2C), 29.2, 31.9 (2C), 33.1, 34.5, 35.7, 37.8 (3C), 39.3, 41.6, 46.2, 56.7 (2C), 65.3, 67.9, 68.8, 72.1, 72.9, 74.1, 75.5, 75.9, 76.6, 79.1, 100.2, 115.1 (4C), 121.5, 123.7, 127.8 (3C), 128.1, 128.8 (6C), 129.9 (2C), 131.6, 137.2, 138.5, 139.6, 146.0, 147.4, 155.1, 158.7 (C=O, cytidine), 160.4 (C=O, guanosine), 161.5 (2C), 163.2, 165.4, 170.5 (HN-C=O); Anal. Calcd for $C_{81}H_{118}N_8O_{16}PSi_2$: C, C, 62.89, H, 7.69, N, 7.24, O, 16.55; Found: C, 62.66, H, 8.01, N, 7.12; HRFABMS Calcd for: $C_{81}H_{118}N_8O_{16}PSi_2$: 1545.79420 found 1545.7911.

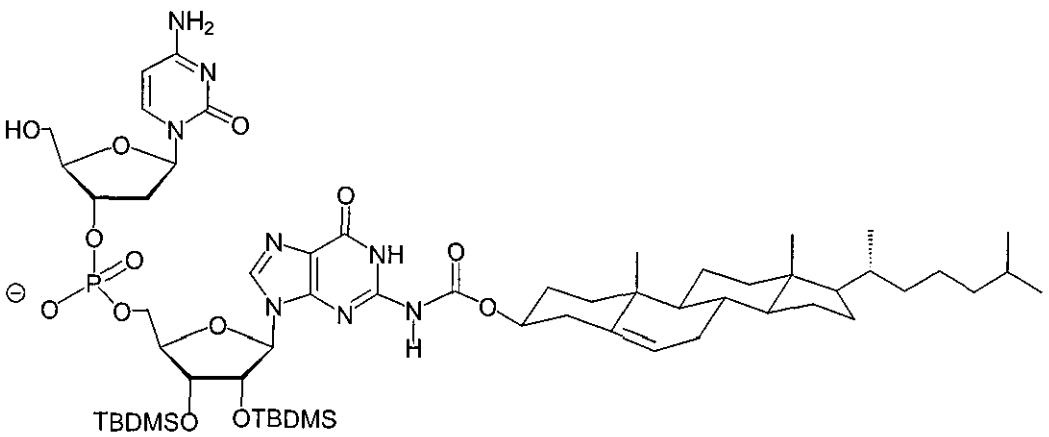
Detritylation of 134, 141 and 142.

In a solution of **134** or **141** or **142** (1.5 mmol, 1eq) in 10 ml of dichloromethane, 3% dichloroacetic acid in dichloromethane (4.8 mmol, 3.2eq) was added and the reaction mixture was stirred at room temperature for 20 min (30 min in the case of **139** or **140**). After completion the reaction was quenched with by the addition 50 ml of saturated sodium bicarbonate solution and the solution was extracted with dichloromethane (2 x 50 ml). The organic extracts were washed with brine (4 x 30 ml) and saturated sodium bicarbonate solution (4 x 40 ml) and then dried over sodium sulphate, filtered and evaporated. Evaporation gave a residual oil in all cases, which was chromatographed using a 5:1 mixture of dichloromethane and methanol to afford **135, 143** and **144** in 68, 63 and 66% yield respectively.



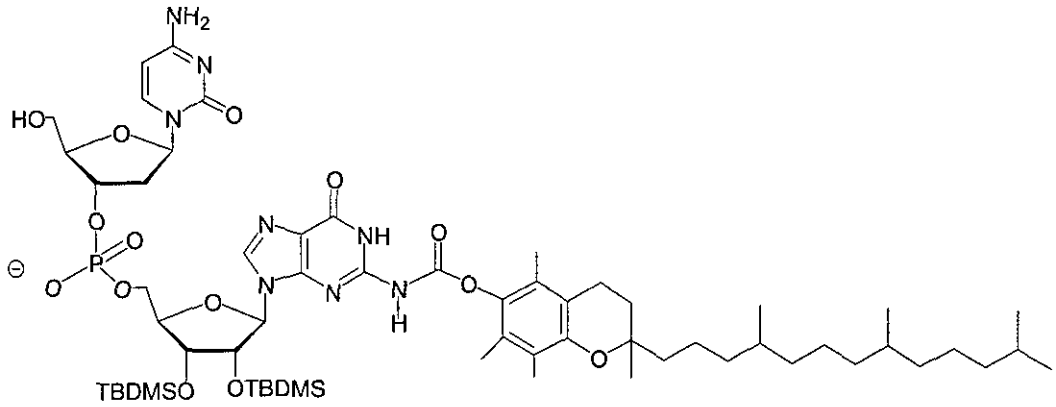
135

Phosphoric acid-5-(2-amino-6-oxo-1,6-dihydro-purin-9-yl) 3,4-bis-(*tert*-butyl-dimethyl-silanyloxy)-tetrahydro-furan-2-ylmethyl ester 5-(4-amino-2-oxo-2*H*-pyrimidin-1-yl)-2-(4-methoxy-benzyloxymethyl)- tetrahydro-furan-2-yl ester (135) 68% yield, ν_{max} / cm^{-1} (nujol) 3454, 1757, 1695, 1410, 1265, 1135, 1048, 852, 694; ^{1}H-NMR (250 MHz, 20% DMSO-d_6 in $CDCl_3$) 0.17 (s, 6H, Si-CH_3), 0.21 (s, 6H, Si-CH_3), 0.85 (s, 9H, C-CH_3), 0.97 (s, 9H, C-CH_3), 1.98-2.19 (m, 2H, $H_{2'2''}$), 3.29-3.42 (m, 2H, $H_{5'5''}$), 3.48-3.54 (m, 2H), 3.70-3.79 (m, 1H), 4.16-4.25 (m, 1H, $H_{4'}$), 4.45-4.57 (m, 1H, $H_{3'}$), 4.91 (t, $J_{apparent}$ = 11.4 Hz, 1H), 4.99 (br s, 1H, OH), 5.36 (t, $J_{apparent}$ = 12.1 Hz, 1H), 5.57 (d, $J_{5\text{-}6}$ = 8.2 Hz, 1H, H_5), 5.98 (d, $J_{apparent}$ = 6.5 Hz, 1H), 6.18 (t, $J_{apparent}$ = 7 Hz, 1H, $H_{1'}$), 6.37 (br s, 2H, NH_2), 6.93 (br s, 2H, NH_2), 7.74 (s, 1H), 7.85 (d, $J_{5\text{-}6}$ = 8.2 Hz, 1H, H_6), 7.94 (s, 1H, NH).; ^{13}C-NMR (100 MHz, 20% DMSO-d_6 in $CDCl_3$) -6.1 (4C-silyl), 15.8 (2C), 21.5 (6C), 33.4, 61.9, 65.2, 67.9, 68.8, 72.4, 74.9, 75.6, 77.0, 77.6, 98.8, 132.1, 138.6, 140.2, 148.9, 158.8 (C=O, cytidine), 161.8 (C=O, guanosine), 163.7, 165.1; Anal. Calcd for $C_{31}H_{52}N_8O_{11}PSi_2$: C, 46.55, H, 6.55, N, 14.01; Found: C, 46.24, H 6.84, N 13.72; HRFABMS Calcd for: $C_{31}H_{52}N_8O_{11}PSi_2$: 799.30317 found 799.3004.



143

Phosphoric acid mono-[5-(4-amino-2-oxo-2*H*-purimidin-1-yl)] {9-[3,4-Bis-(*tert*-butyl-dimethyl-silanyloxy)-tetrahydro-furan-2-yl]-6-oxo-6,9-dihydro-1*H*-purin-2-yl}-carbamic acid 17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-yl ester (143) 63% yield, ν_{max} / cm^{-1} (nujol) 3445, 3386, 3074, 1712, 1695, 1367, 1265, 1132, 1042, 874, 763; ^{1}H-NMR (250 MHz, 20% DMSO-d_6 in $CDCl_3$) 0.16 (s, 6H, Si-CH_3), 0.20 (s, 6H, Si-CH_3), 0.84 (s, 9H, C-CH_3), 0.97 (s, 9H, C-CH_3), 0.70-2.39 (m, 45H, aliphatic),1.95-2.14 (m, 2H, $H_{2'2''}$), 3.26-3.38 (m, 2H, $H_{5'5''}$), 3.66-3.77 (m, 1H), 3.88-3.92 (m, 2H), 4.12-4.22 (m, 1H, $H_{4'}$), 4.43-4.53 (m, 1H, $H_{3'}$), 4.57-4.76 (m, 1H, O-CH), 4.87 (t, $J_{apparent}$ = 11.2 Hz, 1H), 4.98 (br s, 1H, OH), 5.35 (t, $J_{apparent}$ = 12.2 Hz, 1H), 5.38 (d, 1H, J = 2.4 Hz, HC=C), 5.53 (d, $J_{5\text{-}6}$ = 8.8 Hz, 1H, H_5), 5.94 (d, $J_{apparent}$ = 6.3 Hz, 1H), 6.12 (t, $J_{apparent}$ = 7.1 Hz, 1H, $H_{1'}$), 6.35 (br s, 2H, NH_2), 7.57 (br s, 1H, CONH), 7.68 (s, 1H), 7.82 (d, $J_{5\text{-}6}$ = 8.2 Hz, 1H, H_6), 8.0 (s, 1H, NH).; ^{13}C-NMR (100 MHz, 20% DMSO-d_6 in $CDCl_3$) -4.9 (4C-silyl), 15.6 (2C), 17.7, 19.9 20.2, 21.9 (6C), 24.3 (2C), 27.4, 29.9, 32.2, 32.8, 33.8, 35.1, 35.7, 36.9, 39.3, 40.2, 41.8, 42.9, 44.3, 48.8, 49.5, 51.8, 65.32, 68.7, 69.8, 72.7, 73.3, 73.9, 74.7, 75.6, 77.5, 78.6, 99.8, 123.7 (CH=C), 126.9, 130.8 (2C), 132.2, 139.4, 140.5, 145.0, 149.1, 149.9 (CH=C), 159.5 (C=O, cytidine), 160.8 (C=O, guanosine), 163.8, 164.6, 170.2 (HN-C=O); Anal. Calcd for $C_{59}H_{96}N_8O_{13}PSi_2$: C, 58.44, H, 7.98, N, 9.24, Found: C, 58.77, H, 8.24, N, 8.96; HRFABMS Calcd for: $C_{59}H_{96}N_8O_{13}PSi_2$: 1211.63730 found 1211.6333.

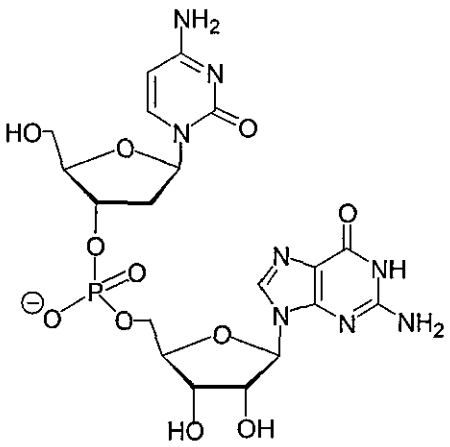


144

Phosphoric acid mono-[5-(4-amino-2-oxo-2*H*-purimidin-1-yl) {9-[3,4-Bis-(*tert*-butyl-dimethyl-silanyloxy)-5-methyl-tetrahydro-furan-2-yl]-6-oxo-6,9-dihydro-1*H*-purin-2-yl}-carbamic acid 2,7,8-trimethyl-2-(4,8,12-trimethyl-tridecyl)-chroman-6-yl ester **(144)** 66% yield, ν_{max} / cm^{-1} (nujol) 3414, 3337, 3091, 1736, 1692, 1352, 1269, 1114, 1044, 845, 773; ^{1}H-NMR (250 MHz, 20% DMSO-d_6 in $CDCl_3$) 0.18 (s, 6H, Si-CH_3), 0.21 (s, 6H, Si-CH_3), 0.85 (s, 9H, C-CH_3), 0.97 (s, 9H, C-CH_3), 0.78-0.98 [m, 12H, (CH_3-CH)$_4$], 1.14-1.74 [m, 20H, $(CH_2)_{10}$], 1.64-1.84 [m, 3H, (CH-CH_3)$_3$], 1.94 (s, 3H, CH_3-Ar), 1.98-2.12 (m, 2H, $H_{2'2''}$), 2.17 (s, 3H, CH_3-Ar), 2.25 (s, 3H, CH_3-Ar), 2.36 (s, 3H, CH_3-C-O), 2.57-2.68 (m, 2H, CH_2-Ar), 3.24-3.35 (m, 2H, $H_{5'5''}$), 3.69-3.80 (m, 1H), 3.92-3.96 (m, 2H), 4.14-4.23 (m, 1H, $H_{4'}$), 4.47-4.57 (m, 1H, $H_{3'}$), 4.92 (t, $J_{apparent}$ = 11.3 Hz, 1H), 4.99 (br s, 1H, OH). 5.37 (t, $J_{apparent}$ = 12.2 Hz, 1H), 5.58 (d, J_{5-6} = 8.4 Hz, 1H, H_5), 5.97 (d, $J_{apparent}$ = 6.5 Hz, 1H), 6.15 (t, $J_{apparent}$ = 7.7 Hz, 1H, $H_{1'}$), 6.34 (br s, 2H, NH_2), 7.62 (br s, 1H, CONH), 7.75 (s, 1H), 7.83 (d, J_{5-6} = 8 Hz, 1H, H_6), 7.95 (s, 1H, NH); ^{13}C-NMR (100 MHz, 20% DMSO-d_6 in $CDCl_3$) -5.2 (4C-silyl), 4.9, 5.5, 5.8, 15.7 (2C), 16.9, 19.6, 20.7 (2C), 22.2 (6C), 22.8 (2C), 23.1, 26.1 (2C), 29.8, 32.4 (2C), 33.6, 35.0, 36.3, 38.4 (3C), 39.6, 41.8, 46.5, 65.8, 68.3, 69.5, 72.3, 73.2, 74.6, 75.8, 76.5, 79.6, 100.7, 122.1, 124.0, 128.4, 131.9, 139.2, 139.9, 146.4, 147.8, 155.7, 159.2 (C=O, cytidine), 160.6 (C=O, guanosine), 163.7, 165.9, 169.5 (HN-C=O); Anal. Calcd for $C_{60}H_{100}N_8O_{14}PSi_2$: C, 57.92, H, 8.10, N, 9.01; Found: C, 58.25, H, 8.38, N, 8.76; HRFABMS Calcd for: $C_{60}H_{100}N_8O_{14}PSi_2$: 1243.66354 found 1243.6603.

Desilylation of 135, 143 and 144.

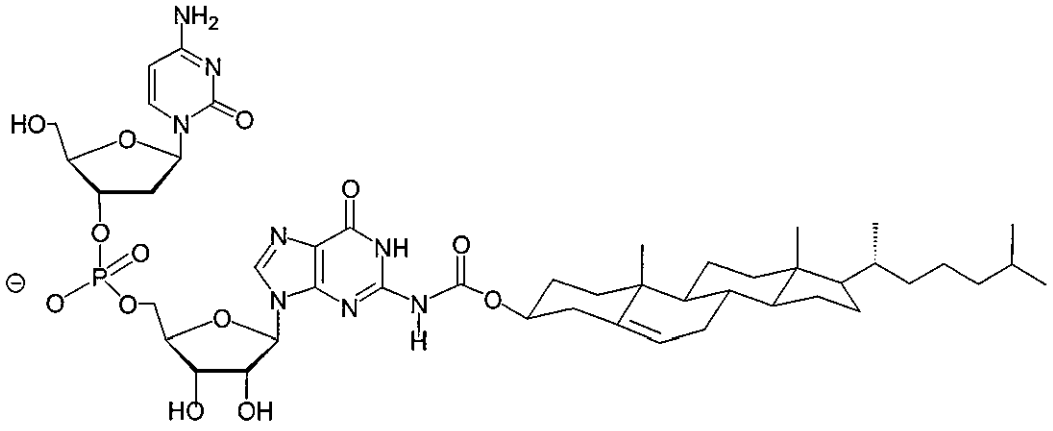
To a stirred solution of **135** or **143** or **144** (1 mmol, 1eq) in 15ml of THF a 1.0 M THF solution of tetrabutylammonium fluoride (TBAF) (2.4 mmol, 2.4eq) was added and the reaction mixture was stirred at ambient temperature for 2h. After completion of the reaction, the solvent was evaporated under high vacuum to give a residue which was dissolved in 60 ml of ethyl acetate and 50 ml of saturated ammonium chloride solution. The aqueous layer was extracted further with ethyl acetate (5 x 40 ml) and the combined organic fractions were washed with saturated sodium bicarbonate solution (3 x 30 ml) and brine (3 x 30 ml). The organic extracts were dried over sodium sulphate, filtered and evaporated to dryness to afford the deprotected CpG **136** as colorless foam in 61% yield, while **145** and **146** were furnished as an off-white foam in 57% yield and an off-yellow foam in 60% yield, respectively.



136

Phosphoric acid-5-(2-amino-6-oxo-1,6-dihydro-purin-9-yl) 3,4-dihydroxy-tetrahydro-furan-2-ylmethyl ester 5-(4-amino-2-oxo-2*H*-pyrimidin-1-yl)-2-hydroxymethyl-tetrahydro-furan-3-yl ester (136) 61% yield, ν_{max} / cm^{-1} (nujol) 3352, 3274, 3155, 1683, 1345, 1286, 1146, 1050, 716; ^{1}H-NMR (250 MHz, DMSO-d_6) 2.02-2.24 (m, 2H, $H_{2'2''}$), 3.33-3.46 (m, 2H, $H_{5'5''}$), 3.53-3.59 (m, 2H), 3.75-3.84 (m, 1H), 4.20-4.29 (m, 1H, $H_{4'}$), 4.49-4.61 (m, 1H, $H_{3'}$), 4.96 (t, $J_{apparent}$ = 11.6 Hz, 1H), 5.03 (br s, 1H, OH), 5.13 (br s, 1H, OH), 5.32 (br s, 1H, OH), 5.40 (t, $J_{apparent}$ = 12.4 Hz, 1H), 5.61 (d, J_{5-6} = 8.2 Hz, 1H, H_5), 6.03

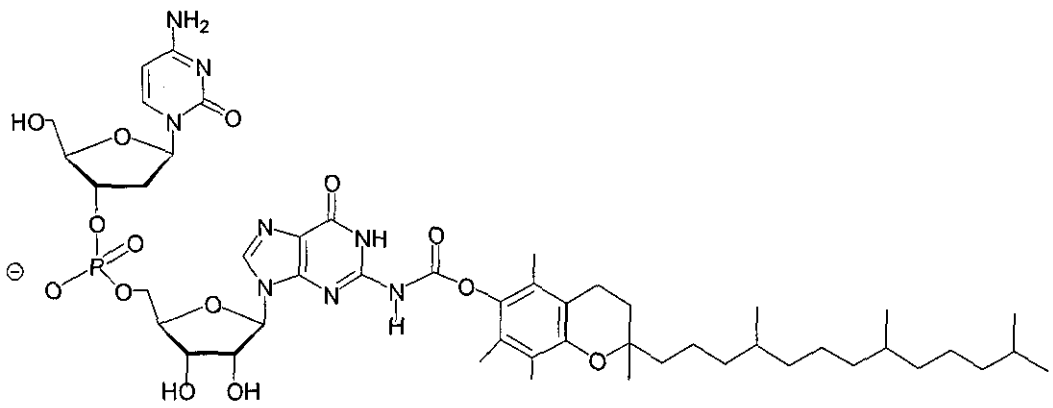
(d, $J_{apparent}$ = 6.6 Hz, 1H), 6.22 (t, $J_{apparent}$ = 7.1 Hz, 1H, $H_{1'}$), 6.43 (br s, 2H, NH_2), 6.97 (br s, 2H, NH_2), 7.78 (s, 1H), 7.89 (d, $J_{5\text{-}6}$ = 8.2 Hz, 1H, H_6), 7.97 (s, 1H, NH).; ^{13}C-NMR (100 MHz, DMSO-d_6) 33.9, 62.4, 65.7, 68.5, 69.3, 72.9, 75.3, 75.9, 77.7, 78.2, 99.3, 132.6, 139.0, 140.8, 149.4, 159.3 (C=O, cytidine), 162.4 (C=O, guanosine), 167.5, 165.7; Anal. Calcd for $C_{19}H_{24}N_8O_{11}P$: C, 39.94, H, 4.23, N, 19.61; Found: C, 40.23, H 4.61, N 19.29; HRFABMS Calcd for: $C_{19}H_{24}N_8O_{11}P$: 571.13022 found 571.1275.



145

Phosphoric acid mono-[5-(4-amino-2-oxo-2*H*-purimidin-1-yl)] [9-(3,4-Dihydroxy-tetrahydro-furan-2-yl]-6-oxo-6,9-dihydro-1*H*-purin-2-yl}-carbamic acid 10,13-dimethyl-17-(1-methyl-hexyl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-yl ester (145) 57% yield, ν_{max} / cm^{-1} (nujol) 3458, 3386, 3244, 3085, 1734, 1684, 1385, 1268, 1124, 1080, 886; ^{1}H-NMR (250 MHz, DMSO-d_6) 0.75-2.42 (m, 45H, aliphatic), 2.0-2.19 (m, 2H, $H_{2'2''}$), 3.31-3.43 (m, 2H, $H_{5'5''}$), 3.71-3.82 (m, 1H), 3.93-3.97 (m, 2H), 4.16-4.26 (m, 1H, $H_{4'}$), 4.47-4.58 (m, 1H, $H_{3'}$), 4.62-4.81 (m, 1H, O-CH), 4.92 (t, $J_{apparent}$ = 11.4 Hz, 1H), 5.01 (br s, 1H, OH), 5.12 (br s, 1H, OH), 5.30 (br s, 1H, OH), 5.37 (t, $J_{apparent}$ = 12.2 Hz, 1H), 5.40 (d, 1H, J = 2.3 Hz, HC=C), 5.57 (d, $J_{5\text{-}6}$ = 9 Hz, 1H, H_5), 5.98 (d, $J_{apparent}$ = 6.4 Hz, 1H), 6.16 (t, $J_{apparent}$ = 7.4 Hz, 1H, $H_{1'}$), 6.39 (br s, 2H, NH_2), 7.61 (br s, 1H, CONH), 7.72 (s, 1H), 7.86 (d, $J_{5\text{-}6}$ =

8.4 Hz, 1H, H_6), 8.4 (s, 1H, NH).; ^{13}C-NMR (100 MHz, DMSO-d_6) 18.2, 20.4, 20.9, 24.9 (2C), 27.9, 30.5, 32.8, 33.5, 34.2, 35.7, 36.4, 37.3, 39.8, 40.7, 42.4, 43.3, 44.7, 49.2, 49.9, 52.1, 65.7, 68.9, 70.2, 73.4, 73.8, 74.3, 74.9, 75.8, 77.9, 79.0, 100.1, 124.2 (**C**H=C), 127.4, 131.3 (2C), 132.8, 139.7, 140.9, 145.6, 149.8, 150.2 (CH=**C**), 159.8 (C=O, cytidine), 161.3 (C=O, guanosine), 164.3, 164.9, 170.6 (HN-C=O); Anal. Calcd for $C_{47}H_{68}N_8O_{13}P$: C, 57.36, H, 6.96, N, 11.39; Found: C, 57.72, H, 7.26, N, 11.07; HRFABMS Calcd for: $C_{47}H_{68}N_8O_{13}P$: 983.46435 found 983.4612.



146

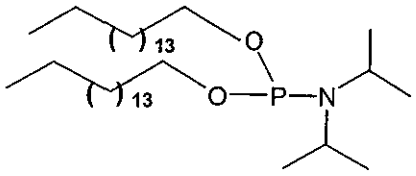
Phosphoric acid mono-[5-(4-amino-2-oxo-2*H*-purimidin-1-yl) [9-(3,4-Dihydroxy-5-methyl-tetrahydro-furan-2-yl]-6-oxo-6,9-dihydro-1*H*-purin-2-yl}-carbamic acid 2,7,8-trimethyl-2-(4,8,12-trimethyl-tridecyl)-chroman-6-yl ester (146) 60% yield, ν_{max} / cm^{-1} (nujol) 3427, 3356, 3392, 3234, 3096, 1745, 1678, 1352, 1284, 1135, 867, 756; ^{1}H-NMR (250 MHz, DMSO-d_6) 0.82-1.02 [m, 12H, (C**H**$_3$-CH)$_4$], 1.18-1.77 [m, 20H, $(CH_2)_{10}$], 1.69-1.88 [m, 3H, (C**H**-$CH_3)_3$], 1.98 (s, 3H, CH_3-Ar), 2.04-2.18 (m, 2H, $H_{2'2''}$), 2.22 (s, 3H, CH_3-Ar), 2.3 (s, 3H, CH_3-Ar), 2.41 (s, 3H, CH_3-C-O), 2.62-2.73 (m, 2H, CH_2-Ar), 3.29-3.40 (m, 2H, $H_{5'5''}$), 3.74-3.85 (m, 1H), 3.96-4.01 (m, 2H), 4.18-4.27 (m, 1H, $H_{4'}$), 4.51-4.61 (m, 1H, $H_{3'}$), 4.97 (t, $J_{apparent}$ = 11.2 Hz, 1H), 5.02 (br s, 1H, OH), 5.12 (br s, 1H, OH), 5.30 (br s, 1H, OH), 5.40 (t, $J_{apparent}$ = 12.4 Hz, 1H), 5.62 (d, $J_{5\text{-}6}$ = 8.4 Hz, 1H, H_5), 6.02 (d, $J_{apparent}$ = 6.5 Hz, 1H), 6.19 (t, $J_{apparent}$ = 7.7 Hz, 1H, $H_{1'}$), 6.38 (br s, 2H, NH_2), 7.67 (br s, 1H, CON**H**), 7.79 (s, 1H), 7.83

(d, $J_{5\text{-}6}$ = 8 Hz, 1H, H_6), 8.01 (s, 1H, NH); ^{13}C-NMR (100 MHz, DMSO-d_6) 5.4, 5.9, 7.2, 17.3, 19.9, 21.3 (2C), 23.4 (2C), 23.7, 26.8 (2C), 30.4, 32.9 (2C), 34.2, 35.8, 37.4, 38.9 (3C), 40.2, 42.3, 46.9, 66.4, 68.7, 69.9, 72.7, 73.6, 75.0, 75.9, 77.1, 80.3, 101.4, 122.7, 124.6, 128.9, 132.5, 139.7, 140.4, 146.9, 148.5, 156.2, 159.8 (C=O, cytidine), 161.2 (C=O, guanosine), 164.3, 166.4, 170.2 (HN-C=O); Anal. Calcd for $C_{48}H_{72}N_8O_{14}P$: C, 56.80, H, 7.15, N, 11.04; Found: C, 57.09, H, 7.41, N, 10.77; HRFABMS Calcd for: $C_{48}H_{72}N_8O_{14}P$: 1015.49056 found 1015.4873.

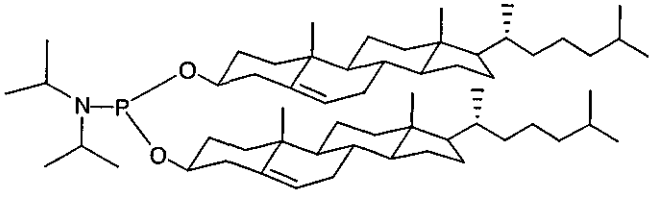
(C) Formation of novel phosphoramidites of lipophilic compounds

Procedure for the preparation of the *N,N*-diisopropyl-phosphoramidites of cetyl alcohol cholesterol and tocopherol

In a three necked flask fitted with addition funnel, mechanical stirrer and argon delivery system, phosphorous trichloride (0.01 mol, 1eq) was placed and dissolved in dry diethyl ether (10 ml). In the solution, which was cooled at -10°C, *N,N*-diisopropylamine (0.02 mol, 2eq) dissolved in 20 ml of dry diethyl ether, was added dropwise by means of the dropwise funnel. When the addition concluded the solution was stirred at -10 ^{0}C for 30 min. While temperature was kept constant at -10 ^{0}C, tocopherol, cholesterol or cetyl alcohol (0.014 mol, 2eq, assuming 70% yield of the diichloro-diisopropylamino phosphine intermediate formed) was dissolved in dry THF, in which triethylamine (0.014 mol, 2eq) was added. The solution of the each of the alcohols was added dropwise to the above reaction mixture through the means of a dropwise funnel. After addition concluded, the mixture was stirred at room temperature for 3h. Diisopropyl-amine hydrochloride salts were removed by suction filtration while washed with diethyl ether. The filtrate and washings were evaporated to complete dryness to afford the phosphoramidites of cholesterol and cetyl alcohol as white solid while; the phosphoramidite of tocopherol was furnished as yellow oil. Any remaining unreacted cholesterol or cetyl alcohol was removed by recrystallization using dichloromethane and petrol to furnish the purified *N,N*-diisopropyl-phosphoramidites of cetyl alcohol and cholesterol in a 72 and 66% yield respectively. The purified *N,N*-diisopropyl-phosphoramidite of tocopherol was obtained in a 69% yield, after trituration with hexane.

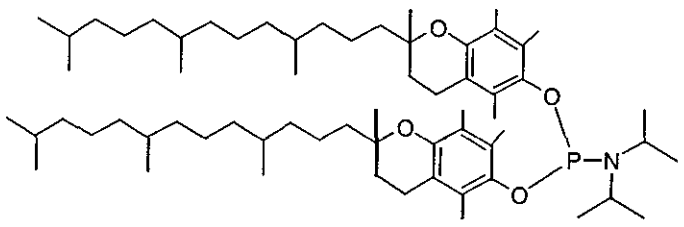


Diisopropyl-phosphoramidous acid dihexadecyl ester (37) 72% yield, ν_{max} / cm^{-1} (nujol) 2354, 1466, 1378, 1242 1035, 985, 720; ^{1}H-NMR (250 MHz, $CDCl_3$,) 0.85 (t, *J* = 6.7, 6H, (CH_3CH_2)$_2$], 1.19 (s, 12H, (CH_3CH)$_4$], 1.38-1.58 (m, 56H, (CH_2)$_{28}$], 2.64-2.75 (m, 2H, (N-CH)$_2$], 3.58 (t, *J* = 6.8, 4H, (OCH_2)$_2$]; ^{13}C-NMR (100 MHz, $CDCl_3$) 14.6 [2C, CH_2(CH_3)$_2$], 23.9 [2C, (CH_2)$_2$$CH_3$], 24.2 [4C, (HC-$CH_3$)$_4$], 26.4 (2C), 29.3 (2C), 30.7 (18C), 32.1 (2C), 32.5 (2C), 36.5 [2C, HN(CH)$_2$], 60.6 [2C, (OCH_2)$_2$]; ^{31}P-NMR (100 MHz, $CDCl_3$,) 178.82; Anal. Calcd. For $C_{38}H_{80}NO_2P$: C, 74.33, H, 13.13, N, 2.28; Found C, 74.02, H, 12.84, N, 2.58; HRFABMS calcd $C_{38}H_{80}NO_2P$: 613.59267 found 613.5897.



Diisopropyl-phosphoramidous acid bis-[17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl] ester (38) 66% yield, ν_{max} / cm^{-1} (nujol) 2455, 1462, 1343, 1045, 1023, 958, 862; ^{1}H-NMR (400 MHz, $CDCl_3$), 0.79 (s, 12H, (CH_3CH)$_4$], 0.85-2.23 (m, 90H, aliphatic), 2.42-2.51 (m, 2H, (N-CH)$_2$], 5.39 (d, 1H, *J* = 3.5 Hz, HC=C), 5.51 (d, 2H, *J* = 305 Hz, HC=C); ^{13}C-NMR (100 MHz, $CDCl_3$) 16.9 (2C), 17.5 (2C), 18.5 (2C), 19. 4 (2C), 20.8 (2C), 21.9 (2C), 22.7 (4C), 23.9 (2C), 25.1 [4C, 2 x CH(CH_3)$_2$], 26.1 (2C), 28.5 (2C), 29.8 (2C), 30.5 (2C), 33.8 (2C), 34.5 (2C), 35.0 (2C), 35.8 (2C), 37.2 (2C), 38.8 (2C, N(CH_2)$_2$], 41.7 (2C), 42.5 (2C), 43.9 (2C), 47.4 (2C), 48.8 (2C), 52.7 (2C), 70.2 (2C), 123.5 (2C, C=CH), 149.2 (2C, CH=C), ^{31}P-NMR (100 MHz, $CDCl_3$,)

182.54; Anal. Calcd for $C_{60}H_{104}NO_2P$: C, 79.85, H, 11.62, N, 1.55; Found: C, 80.18; H, 11.91, N, 1.23; HRFABMS Calcd for $C_{60}H_{104}NO_2P$: 901.78047 found 901.7776.



Diisopropyl-phosphoramidous acid bis-[2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-tridecyl)-choman-6-yl] ester (39) 69% yield ν_{max} / cm^{-1} (neat) 2364, 1575, 1476, 1346, 1159, 1204, 1044, 918, 853; ^1H NMR (400 MHz, $CDCl_3$) 0.82 (s, 12H, $(CH_3CH)_4$], 0.85-1.05 [m, 24H, $(CH_3\text{-}CH)_8$], 1.21-1.83 [m, 40H, $(CH_2)_{20}$], 1.91-2.12 [m, 6H, $(CH\text{-}CH_3)_6$], 2.24 (s, 6H, $(CH_3)_2$-Ar], 2.30 (s, 6H, $(CH_3)_2$-Ar], 2.34 (s, 6H, $(CH_3)_2$-Ar], 2.55 (s, 6H, O-C-$(CH_3)_2$), 2.62-2.71 (m, 2H, $(N\text{-}CH)_2$], 2.83-2.94 (m, 4H, $(CH_2)_2$-Ar]; ^{13}C-NMR (100 MHz, $CDCl_3$), 16.8, 17.6, 19.7 (2C), 20.1 (2C), 20.9 (4C), 21.2 (2C), 22.5 (2C), 23.7 (4C), 24.8 (4C), 25.6 [4C, HC-$(CH_3)_4$], 29.5 (2C), 32.8 (4C), 35.5 (2C), 36.2 (2C), 37.0 (4C), 37.9 (2C), 39.5 (2C, $N(CH)_2$], 41.7 (2C), 46.5 (2C), 73.9 (2C), 121.8 (2C), 122.5 (2C), 123.3 (2C), 124.5 (2C), 125.7 (2C), 151.1 (2C), 152.7 (2C); ^{31}P-NMR (100 MHz, $CDCl_{3,}$) 184.67; Anal. Calcd for $C_{64}H_{112}NO_4P$: C, 77.60, H, 11.40, N, 1.41; Found C, 7.94; H, 11.14; N, 1.72; HRFABMS Calcd for $C_{64}H_{112}NO_4P$: 989.83290 found 989.8298.

(D) Solid Phase synthesis of CpG dinucleotides

Procedure for the preparation of the 5'-dicetyl, 5'-ditocopherol and 5'-dicholesterol phospate of the CpG dinucleotide via the phosphitylation of the 5'OH group

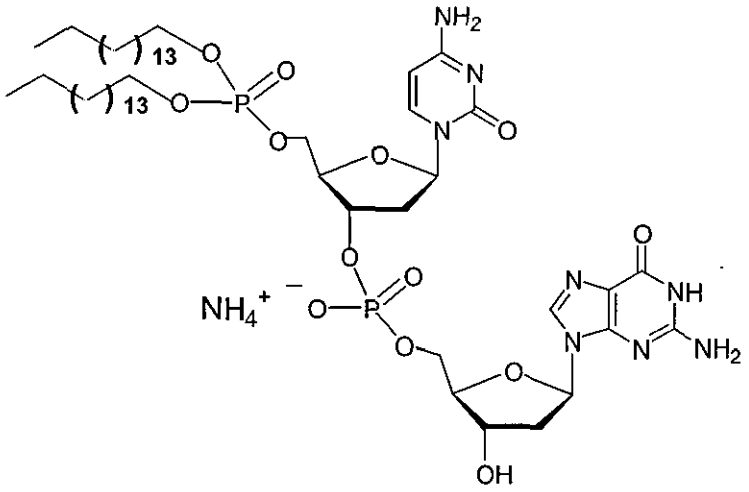
24 μmol (1g) of the controlled glass beads (CPG) containing 5'-O-dimethoxytritylated-deoxyguanosine were placed in a column type reactor which was maintained airtight throughout the process with a septum cap. The 5'-O-dimethoxytritylated-deoxyguanosine was detritylated using 3% CCl_2COOH in dichloromethane (5 x 2 ml). The suspension was shaken for 2 min and after washing with acetonitrile (3 x 5 ml for 2 min), the beads were dried in *vacuo* for 5 min. 0.1 M solution of the solid active 3'-cyanoethyl phosphoramidite of 2'-deoxycytidine (600 μmol, 25eq) in CH_3CN and 0.1 M solution of imidazolium triflate (600 μmol, 25eq) also in CH_3CN were added to the column. The column was capped with a septum cap and flushed with nitrogen. The suspension of the CPG beads was shaken gently for 25 min. The excess of the reagents were removed by washing with CH_3CN (10 x 3 ml for 2 min).

Oxidation was then performed using 4ml (2 x 2 ml) of 0.1 M iodine in 80:40:20 solution of THF: pyridine: H_2O. After shaking gently for 5 min, the excess of oxidizing agent was removed by washing with acetonitrile (10 x 3 ml for 2 min). Deprotection of cytidine 2-cyanoethyl phosphoramidite (removal of dimethoxytrityl group) and washing with acetonitrile was performed as above. After drying in vacuo for 5 min 0.1 M solution of the solid dicetyl, dicholesterol or ditocopherol phosphoramidite (600 μmol, 25eq) in THF and 0.1 M solution of imidazolium triflate (600 μmol, 25eq) also in CH_3CN were added to the column. The suspension was again shaken gently for 25 min. The excess of the reagents were removed by washing with acetonitrile (10 x 3 ml for 2 min). The oxidation and washing of excess, steps, were repeated as above.

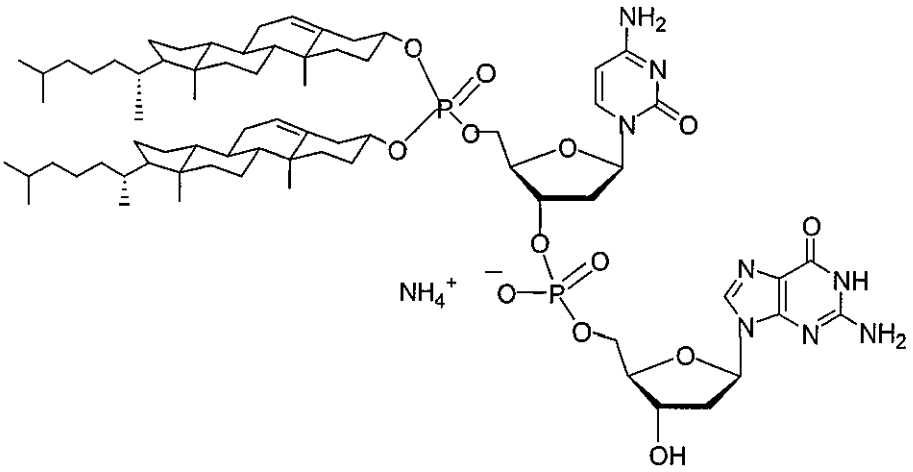
After the final elongation step of the desired sequence, the CPG beads were thoroughly washed and dried. Then they were transferred to a flask and treated with a 3:1 solution of concentrated aqueous ammonia: ethanol for 30

min at room temperature and then heating at 55 ^{0}C for 16h to remove the protecting groups and detach the product from the solid support.

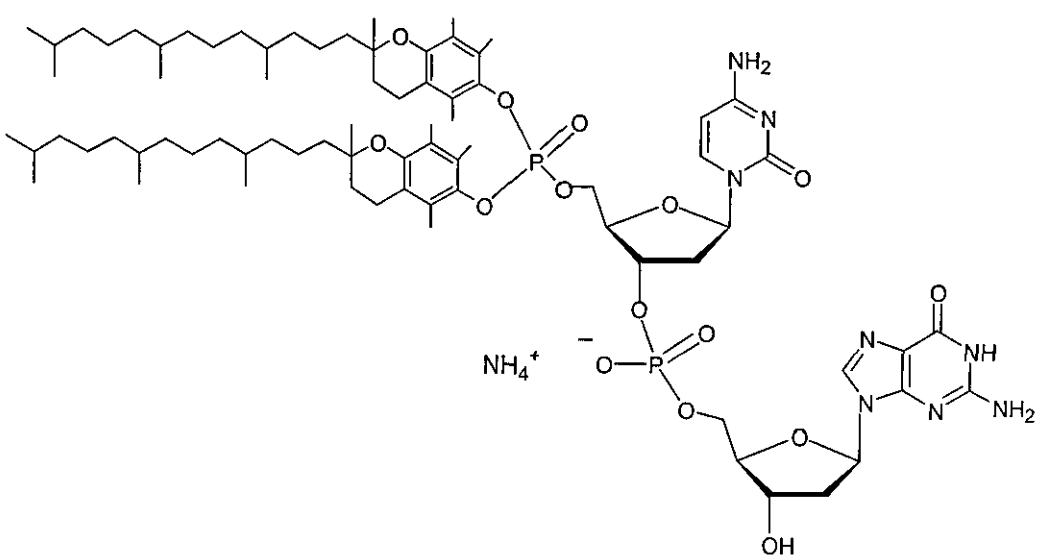
After cooling the supernatant liquid was separated from the polymer by suction filtration and washed with methanol. The combined supernatant and washing were concentrated to a small volume and the insoluble material resulting from the β-elimination and polymerisation of the CH_2=CH-CN was removed by filtering through millipore filter. The filtrate was evaporated to dryness to give the ammonium salt of the desired 5'-dicetyl, 5'-dicholesterol and 5'-ditocopherol CpG dinucleotide as off-white solid in all three cases.



Ammonium salt of phosphoric acid-5-(2-amino-6-oxo-1,6-dihydro-purin-9-yl) 3-hydroxy-tetrahydro-furan-2-ylmethyl ester 5-(4-amino-2-oxo-2*H*-pyrimidin-1-yl)-2-(bis-hexadecyloxy-phosphoryloxymethyl-tetrahydro-furan-3-yl ester (136) 50% yield, Anal. Calcd for $C_{51}H_{89}N_8O_{13}P_2$: C, 56.50, H 8.27, N 10.33; Found C, 56.96; H, 8.65; N, 10.72; HRFABMS Calcd for: $C_{51}H_{89}N_8O_{13}P_2$: 1083.60244 found 1083.5997.

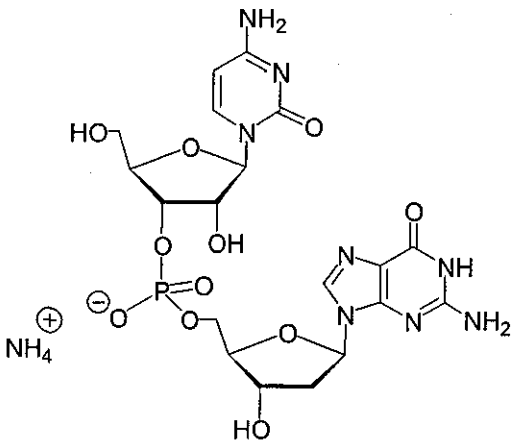


Ammonium salt of phosphoric acid 5-(4-amino-2-oxo-2*H*-purimidin-1-yl)-4- hydroxy-tetrahydro-furan-2-yl-1,9-dihydro-purin-6-one-2-(bis-[17-(1,5-dimethyl-hexyl]10,13-dimethyl-17-(1-methyl-hexyl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-yl methyl ester (148) 52% yield, Anal. Calcd for $C_{73}H_{113}N_8O_{13}P_2$: C, 63.87, H, 8.30, N, 8.16; Found C, 64.46; H, 8.76; N, 8.68; HRFABMS Calcd for: $C_{73}H_{113}N_8O_{13}P_2$: 1371.79024 found 1371.7875.



Ammonium salt of phosphoric acid 5-(4-amino-2-oxo-2*H*-purimidin-1-yl)-4- hydroxy-tetrahydro-furan-2-yl-1,9-dihydro-purin-6-one-2-(bis-[2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-tridecyl)-chroman-6-yl ester (149)

55% yield, Anal. Calcd for $C_{77}H_{121}N_8O_{15}P_2$: C, 63.31, H 8.35, N 7.67; Found C, 63.87; H, 8.89; N, 8.12; HRFABMS Calcd for: $C_{77}H_{121}N_8O_{15}P_2$: 1459.84267 found 1459.8397.



150

Phosphoric acid-5-(2-amino-6-oxo-1,6-dihydro-purin-9-yl)-3-methyl-tetrahydro-furan-2-ylmethyl ester 5-(4-amino-2-oxo-2*H*-pyrimidin-1-yl)-4-hydroxymethyl-tetrahydro-furan-3-yl ester (150) 61% yield, ν_{max} / cm^{-1} (nujol) 3364, 3263, 3157, 1689, 1348, 1291, 1165, 1054, 757; ^{1}H-NMR (250 MHz, DMSO-d_6) 2.03-2.25 (m, 1H, $H_{2'2''}$), 3.34-3.45 (m, 2H, $H_{5'5''}$), 3.55-3.60 (m, 2H), 3.77-3.85 (m, 1H), 4.21-4.30 (m, 1H, $H_{4'}$), 4.50-4.62 (m, 1H, $H_{3'}$), 4.98 (t, $J_{apparent}$ = 10.5 Hz, 1H), 5.04 (br s, 1H, OH), 5.14 (br s, 1H, OH), 5.40 (br s, 1H, OH), 5.44 (t, $J_{apparent}$ = 12.2 Hz, 1H), 5.63 (d, $J_{5\text{-}6}$ = 8.1 Hz, 1H, H_5), 6.08 (d, $J_{apparent}$ = 6.3 Hz, 1H), 6.25 (t, $J_{apparent}$ = 7.7 Hz, 1H, $H_{1'}$), 6.45 (br s, 2H, NH_2), 6.99 (br s, 2H, NH_2), 7.80 (s, 1H), 7.92 (d, $J_{5\text{-}6}$ = 8.4 Hz, 1H, H_6), 7.98 (s, 1H, NH).; ^{13}C-NMR (100 MHz, DMSO-d_6) 33.9, 62.4, 65.7, 68.5, 69.3, 72.9, 75.3, 75.9, 77.7, 78.2, 99.3, 132.6, 139.0, 140.8, 149.4, 159.3 (C=O, cytidine), 162.4 (C=O, guanosine), 167.5, 165.7; Anal. Calcd for $C_{19}H_{24}N_8O_{11}P$: C, 39.94, H, 4.23, N, 19.61; Found: C, 40.19, H 4.58, N 19.26; HRFABMS Calcd for: $C_{19}H_{24}N_8O_{11}P$: 571.13022 found 571.1275.

CONCLUSIONS

The recent widespread use of modified oligonucleotides as diagnostic biochemical probes and as tools for the control of gene expression by antisense and antigene strategies has fostered the development of synthetic methods for the preparation of nucleic acids containing a wide variety of modified bases and backbone structures.

In order to block the synthesis of virus components in a cell it is reasonable to use the antisense inhibition of this process by short complementary oligonucleotides, which are regarded as one of the most promising tools for the treatment of a variety of diseases, including viral infections and cancer. However, their application is considerably limited by their inadequate stability in the presence of nucleases and their low efficiency of their penetration into intact cells. In recent years, chemical modifications of antisence oligonucleotides have resulted in improved solubility, stability towards nucleases, cellular uptake and appropriate hybridization to target genes.

For this purpose in this research we aimed at synthesizing nucleotide constructs modified with lipophilic substituents as they can enhance their of the nucleotides to penetrate the cell membrane. Originally a series of novel acetal, carbonate, carbamate and ester linkers of tocopherol and cholesterol have been successfully prepared in high yield. They were used for the incorporation of the lipophilic compounds to the nucleotide derivatives. An X-ray structure of the first example of a carbonylimidazolide derivative of cholesterol was determined.

These linker groups were intended to be easily cleaved off once the DNA construct has entered the cell, stable enough to stand up the conditions of DNA synthesis but also be easily hydrolyzed in the cell. They also acted as good leaving groups for the subsequent coupling reactions. This reduced the potential for side reactions and simplified purification. The reagents were soluble in organic solvent suitable for the coupling procedure and could withstand the deblocking procedure.

Another linker was also synthesized to link tumour targeting GRE1, GRE4 and G34 rabbit antibodies to different DNA constructs containing antisense insert. The antibodies retained at the binding sites were eluted and showed biological activity, which unfortunately could not be maintained. Future plans involve establishing whether the tumour targeting rabbit antibodies can be linked to a biotinylated oligonucleotide through synthesized linker in a manner that appears to maintain increased biological activity. Also investigation into determining whether immuno-reactivity could be gained with further purification of the eluted antibodies retained at the binding sites is underway.

New improved methods were developed for the global and partial protection of nucleosides employing *tert*-butyldimethylsilyl chloride (TBDMSCl). It has proved to be an exceedingly useful group that provided a rapid and sufficient protection of the hydroxyl moieties of the nucleosides without the need for base protection. The method developed averted any silylation of the amino bases, and provided increased rate of reactions resulting in high yields of the protected nucleosides. Selective 5'-desilylation was also achieved and the silylated derivatives were employed in the formation of lipophilic nucleoside conjugates and subsequent synthesis of CpG dinucleotides.

Nucleotides were covalently linked to the lipophilic molecules through sites present naturally in nucleic acids such as the exocyclic amino base or the 3'-hydroxyl group. Lipophilic N-carbamate and N-acetamide conjugates of globally protected guanosine and adenosine were synthesized in very good yields, forming novel carbonyl-type links. Conjugation at the 3'-terminus of the nucleosides, which can potentiate their immunostimulatory activity, gave lipophilic O-carbonate analogues in good yields.
These modifications can enhance the uptake and cellular efficacy of the nucleotides, increase their nuclease resistance, thus increasing their efficiency in antisense experiments, compared to non-derivatized oligonucleotides. Modifications at the 2'-position of the sugar or the nucleobases and lipophilic conjugation using other nucleosides can also be investigated.

In order to test whether the synthesized modified lipophilic nucleotides are taken up into the cell membrane more efficiently they were released of their protecting groups, to give the deprotected analogues for comparison. Deprotection was achieved in good yields and the lipophilic nucleotide conjugates were sent for testing. Results are not known as investigation is still underway.

Thioctic acid was also employed as a lipophilic compound for the formation of N-carbamate analogues. Different attempts were made firstly through the formation of the acid chloride of thioctic acid and then by converting the thioctic acid to the amide of carboxylic acid to an amide in a one-pot reaction using oxalyl chloride. These methods failed but the formation of the thioctic N-carbamate analogue of adenosine was finally achieved through employment of coupling reagents.

CpG dinucleotides were successfully prepared in good yields with or without the attachment of lipophilic molecules using both solution and solid phase synthesis. Accessibility at the 5'-end of the CpG dinucleotide is critical for the immunostimulatory activity and in order to examine if terminal modifications with alkylic or lipophilic chains can enhance cellular uptake and increase immunostimulatory activity, the 5'-dicetyl, dicholesterol and ditocopherol phosphates of the CpG dinucleotides were synthesized. For that purpose the *N,N*-diisopropyl-phosphoramidites of cetyl alcohol, cholesterol and tocopherol were synthesized in very good yields and they were subsequently coupled to CpG through its 5'-end, in order to form the lipophilic phosphodiester CpG dinucleotides.

Efficient synthesis of 5'-di-lipophilic phosphates of CpG dinucleotides was attained by the phosphoramidite approach without cytidine base protection. The crude products were generally of very good purity and were obtained in satisfactory yields.

Solid-phase synthesis was achieved with good chemoselectivity and phosphitylation was performed using a 1:1 mixture of the promoter and the cyanoethyl- phosphoramidite or the synthesized di-lipophilic phosphoramidites. Imidazolium triflate proved to be an excellent promoter allowing rapid and highly chemoselective condensation of the phosphoramidite and the corresponding nucleoside.

The immunostimulatory activity of these dinucleotides is currently being studied *in vitro* using BALB/c mice spleen lympocytes, measured in a lympocyte proliferation assay. The secretion of cytokines IL-12, IL-6 and IL-10 known to be induced by the activation of immune cells by CpG is also being studied *in vitro*. The formed N-carbamate lipophilic analogues of guanosine are also being investigated to determine whether the coupling of the lipophilic groups had any effect in the improvement of nucleotide uptake by the cells.

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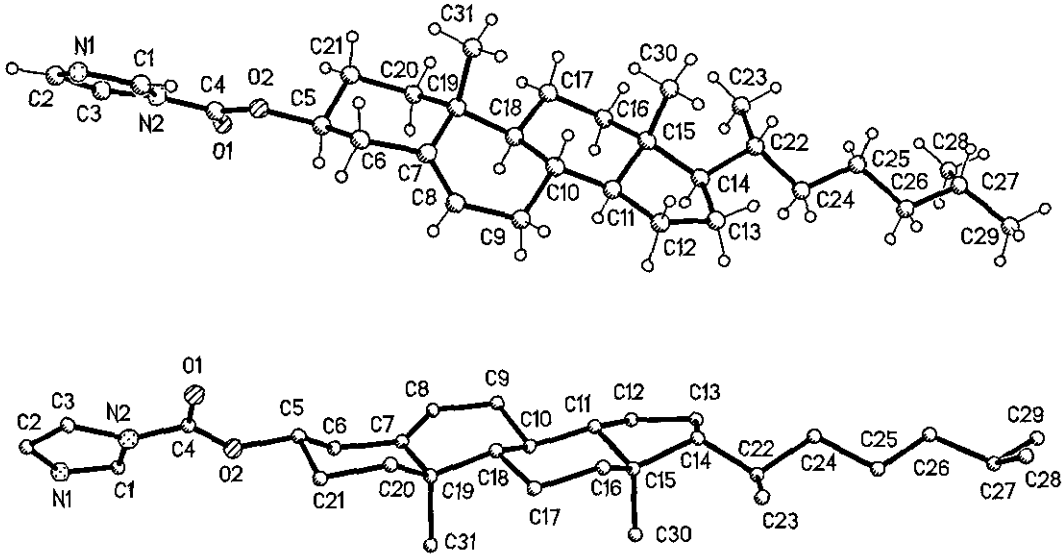
APPENDIX



Data collected on a Bruker SMART 1000 diffractometer, conditions as shown in Table 1. The structure was solved by direct methods and refined by full-matrix least-squares on F^2 using SHELXTL[1]. All non-hydrogen atoms were refined with anisotropic atomic displacement parameters and hydrogen atoms were inserted at calculated.

1. G.M. Sheldrick, SHELXTL version 5.1, Bruker-AXS, Madison WI, 1998.

Table 1. Crystal data and structure refinement for gw3.

Identification code	gw3	
Empirical formula	$C_{31} H_{48} N_2 O_2$	
Formula weight	480.71	
Temperature	150(2) K	
Wavelength	0.71073 Å	
Crystal system	Monoclinic	
Space group	P2(1)	
Unit cell dimensions	a = 5.7921(5) Å	α= 90°.
	b = 9.7965(9) Å	β= 94.634(2)°.
	c = 24.932(2) Å	γ = 90°.
Volume	1410.1(2) Å^3	
Z	2	
Density (calculated)	1.132 Mg/m^3	
Absorption coefficient	0.070 mm^{-1}	
F(000)	528	
Crystal size	0.05 x 0.14 x 0.30 mm^3	
Theta range for data collection	1.64 to 25.00°.	
Index ranges	-6<=h<=6, -11<=k<=11, -29<=l<=29	
Reflections collected	10189	
Independent reflections	4767 [R(int) = 0.0359]	
Completeness to theta = 25.00°	99.9 %	
Absorption correction	Multiscan	
Max. and min. transmission	1.00000 and 0.622497	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	4767 / 1 / 316	
Goodness-of-fit on F^2	0.963	
Final R indices [I>2sigma(I)]	R1 = 0.0442, wR2 = 0.0992	
R indices (all data)	R1 = 0.0614, wR2 = 0.1075	
Absolute structure parameter	-0.5(15)	
Largest diff. peak and hole	0.187 and -0.234 e.Å^{-3}	

Table 2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$x 10^3) for gw3. U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor.

	x	y	z	U(eq)
C(1)	4597(4)	2560(3)	6817(1)	28(1)
N(1)	5109(3)	2493(3)	7331(1)	33(1)
C(2)	2989(4)	2371(3)	7554(1)	34(1)
C(3)	1227(4)	2367(3)	7172(1)	30(1)
N(2)	2242(3)	2483(2)	6687(1)	26(1)
C(4)	1074(4)	2523(3)	6173(1)	24(1)
O(1)	-992(3)	2547(2)	6097(1)	31(1)
O(2)	2591(2)	2549(2)	5802(1)	27(1)
C(5)	1630(4)	2645(3)	5241(1)	25(1)
C(6)	3611(4)	3101(3)	4924(1)	28(1)
C(7)	2912(4)	3156(3)	4326(1)	26(1)
C(8)	3420(5)	4241(3)	4040(1)	31(1)
C(9)	2903(5)	4377(3)	3446(1)	33(1)
C(10)	2188(4)	3035(2)	3172(1)	25(1)
C(11)	1066(4)	3273(3)	2607(1)	27(1)
C(12)	2440(5)	4051(3)	2211(1)	35(1)
C(13)	1144(5)	3720(3)	1660(1)	39(1)
C(14)	-630(4)	2580(3)	1748(1)	29(1)
C(15)	235(4)	1962(2)	2303(1)	24(1)
C(16)	-1569(4)	1319(3)	2638(1)	26(1)
C(17)	-562(4)	1016(3)	3213(1)	26(1)
C(18)	531(4)	2257(2)	3511(1)	24(1)
C(19)	1700(4)	1900(2)	4080(1)	22(1)
C(20)	-189(4)	1461(3)	4445(1)	25(1)
C(21)	668(4)	1301(2)	5038(1)	27(1)
C(22)	-976(4)	1636(3)	1255(1)	31(1)
C(23)	-2697(5)	487(3)	1323(1)	39(1)
C(24)	-1718(5)	2467(3)	750(1)	41(1)
C(25)	-1991(5)	1652(3)	226(1)	43(1)
C(26)	-2238(5)	2552(4)	-272(1)	45(1)
C(27)	-2645(5)	1788(3)	-799(1)	41(1)

C(28)	-5032(6)	1152(4)	-873(1)	63(1)
C(29)	-2229(5)	2690(4)	-1276(1)	50(1)
C(30)	2231(5)	964(3)	2236(1)	30(1)
C(31)	3481(4)	746(3)	4048(1)	28(1)

Table 3. Bond lengths [Å] and angles [°] for gw3.

C(1)-N(1)	1.295(3)	C(12)-C(13)	1.545(3)
C(1)-N(2)	1.378(3)	C(13)-C(14)	1.545(4)
N(1)-C(2)	1.393(3)	C(14)-C(22)	1.538(3)
C(2)-C(3)	1.338(3)	C(14)-C(15)	1.557(3)
C(3)-N(2)	1.391(3)	C(15)-C(16)	1.526(3)
N(2)-C(4)	1.401(3)	C(15)-C(30)	1.534(3)
C(4)-O(1)	1.196(2)	C(16)-C(17)	1.533(3)
C(4)-O(2)	1.326(2)	C(17)-C(18)	1.534(3)
O(2)-C(5)	1.466(2)	C(18)-C(19)	1.561(3)
C(5)-C(21)	1.502(4)	C(19)-C(31)	1.537(3)
C(5)-C(6)	1.512(3)	C(19)-C(20)	1.539(3)
C(6)-C(7)	1.514(3)	C(20)-C(21)	1.529(3)
C(7)-C(8)	1.326(3)	C(22)-C(23)	1.523(4)
C(7)-C(19)	1.521(3)	C(22)-C(24)	1.532(3)
C(8)-C(9)	1.494(3)	C(24)-C(25)	1.528(4)
C(9)-C(10)	1.524(3)	C(25)-C(26)	1.519(4)
C(10)-C(11)	1.520(3)	C(26)-C(27)	1.514(4)
C(10)-C(18)	1.534(3)	C(27)-C(28)	1.514(4)
C(11)-C(12)	1.521(3)	C(27)-C(29)	1.516(4)
C(11)-C(15)	1.548(3)		
N(1)-C(1)-N(2)	111.9(2)	C(21)-C(5)-C(6)	111.0(2)
C(1)-N(1)-C(2)	105.10(19)	C(5)-C(6)-C(7)	111.79(19)
C(3)-C(2)-N(1)	111.3(2)	C(8)-C(7)-C(6)	120.3(2)
C(2)-C(3)-N(2)	105.5(2)	C(8)-C(7)-C(19)	123.2(2)
C(1)-N(2)-C(3)	106.25(18)	C(6)-C(7)-C(19)	116.5(2)
C(1)-N(2)-C(4)	127.50(18)	C(7)-C(8)-C(9)	124.7(2)
C(3)-N(2)-C(4)	126.25(18)	C(8)-C(9)-C(10)	113.2(2)
O(1)-C(4)-O(2)	126.93(19)	C(11)-C(10)-C(9)	111.3(2)
O(1)-C(4)-N(2)	123.2(2)	C(11)-C(10)-C(18)	110.37(18)
O(2)-C(4)-N(2)	109.91(18)	C(9)-C(10)-C(18)	109.88(19)
C(4)-O(2)-C(5)	116.38(16)	C(10)-C(11)-C(12)	118.1(2)
O(2)-C(5)-C(21)	111.6(2)	C(10)-C(11)-C(15)	114.7(2)
O(2)-C(5)-C(6)	105.77(17)	C(12)-C(11)-C(15)	104.84(19)

C(11)-C(12)-C(13)	103.3(2)	C(7)-C(19)-C(20)	108.34(19)
C(14)-C(13)-C(12)	107.9(2)	C(31)-C(19)-C(20)	109.75(19)
C(22)-C(14)-C(13)	111.6(2)	C(7)-C(19)-C(18)	109.70(18)
C(22)-C(14)-C(15)	119.2(2)	C(31)-C(19)-C(18)	111.17(19)
C(13)-C(14)-C(15)	103.68(19)	C(20)-C(19)-C(18)	108.78(18)
C(16)-C(15)-C(30)	110.6(2)	C(21)-C(20)-C(19)	114.11(19)
C(16)-C(15)-C(11)	105.90(19)	C(5)-C(21)-C(20)	108.64(19)
C(30)-C(15)-C(11)	112.02(19)	C(23)-C(22)-C(24)	110.1(2)
C(16)-C(15)-C(14)	117.56(19)	C(23)-C(22)-C(14)	113.5(2)
C(30)-C(15)-C(14)	110.0(2)	C(24)-C(22)-C(14)	110.3(2)
C(11)-C(15)-C(14)	100.25(19)	C(25)-C(24)-C(22)	115.3(2)
C(15)-C(16)-C(17)	111.51(19)	C(26)-C(25)-C(24)	113.0(2)
C(16)-C(17)-C(18)	114.2(2)	C(27)-C(26)-C(25)	114.8(3)
C(10)-C(18)-C(17)	112.23(18)	C(26)-C(27)-C(28)	112.8(2)
C(10)-C(18)-C(19)	111.61(17)	C(26)-C(27)-C(29)	111.7(2)
C(17)-C(18)-C(19)	113.20(19)	C(28)-C(27)-C(29)	110.2(2)
C(7)-C(19)-C(31)	109.05(18)		

Table 4. Anisotropic displacement parameters ($Å^2 x\ 10^3$) for gw3. The anisotropic displacement factor exponent takes the form: $-2\pi^2[\ h^2\ a^{*2}U^{11} + ... + 2\ h\ k\ a^*\ b^*\ U^{12}\ ]$

	U^{11}	U^{22}	U^{33}	U^{23}	U^{13}	U^{12}
C(1)	30(1)	30(1)	25(1)	-1(1)	4(1)	1(1)
N(1)	38(1)	39(1)	23(1)	-2(1)	0(1)	5(1)
C(2)	43(1)	38(2)	22(1)	1(1)	6(1)	5(1)
C(3)	33(1)	31(2)	27(1)	1(1)	11(1)	-3(1)
N(2)	27(1)	27(1)	23(1)	0(1)	2(1)	0(1)
C(4)	30(1)	19(1)	25(1)	-1(1)	4(1)	3(1)
O(1)	29(1)	39(1)	25(1)	1(1)	3(1)	2(1)
O(2)	29(1)	37(1)	15(1)	2(1)	5(1)	-2(1)
C(5)	31(1)	29(1)	16(1)	2(1)	1(1)	1(1)
C(6)	35(1)	31(1)	19(1)	-4(1)	3(1)	-9(1)
C(7)	27(1)	29(1)	21(1)	-1(1)	4(1)	-2(1)
C(8)	44(2)	25(2)	23(2)	-4(1)	2(1)	-8(1)
C(9)	50(2)	25(1)	25(1)	1(1)	6(1)	-9(1)
C(10)	29(1)	25(1)	22(1)	-3(1)	5(1)	-3(1)
C(11)	34(1)	27(2)	22(1)	0(1)	6(1)	-1(1)
C(12)	53(2)	28(2)	24(2)	-1(1)	6(1)	-13(1)
C(13)	63(2)	34(2)	22(1)	0(1)	7(1)	-8(1)
C(14)	36(1)	30(1)	22(1)	1(1)	5(1)	2(1)
C(15)	26(1)	24(1)	21(1)	1(1)	3(1)	0(1)
C(16)	28(1)	30(2)	21(1)	-3(1)	2(1)	0(1)
C(17)	25(1)	29(1)	23(1)	1(1)	5(1)	-5(1)
C(18)	25(1)	27(2)	19(1)	0(1)	3(1)	3(1)
C(19)	25(1)	24(1)	18(1)	0(1)	4(1)	-1(1)
C(20)	26(1)	24(1)	24(1)	2(1)	2(1)	-2(1)
C(21)	29(1)	30(2)	23(1)	4(1)	4(1)	-1(1)
C(22)	36(1)	34(2)	22(1)	-1(1)	3(1)	1(1)
C(23)	46(2)	43(2)	27(2)	-3(1)	3(1)	-6(1)
C(24)	61(2)	37(2)	25(1)	0(2)	0(1)	2(2)
C(25)	68(2)	40(2)	21(1)	1(1)	-1(1)	1(2)
C(26)	68(2)	42(2)	26(1)	0(2)	2(1)	-7(2)
C(27)	58(2)	37(2)	27(2)	-3(1)	-2(1)	3(2)

C(28)	83(2)	60(2)	44(2)	-2(2)	-4(2)	-22(2)
C(29)	67(2)	54(2)	29(2)	0(2)	2(1)	-3(2)
C(30)	33(1)	32(2)	24(1)	-2(1)	4(1)	-1(1)
C(31)	30(1)	29(2)	25(1)	1(1)	0(1)	2(1)

Table 5. Hydrogen coordinates (x 10^4) and isotropic displacement parameters ($Å^2$x 10^3) for gw3.

	x	y	z	U(eq)
H(1)	5711	2652	6560	34
H(2)	2817	2300	7928	41
H(3)	-378	2299	7222	36
H(5)	377	3351	5211	30
H(6A)	4925	2461	4991	34
H(6B)	4137	4017	5049	34
H(8)	4167	4985	4227	37
H(9A)	4295	4733	3287	40
H(9B)	1643	5051	3374	40
H(10)	3608	2466	3144	30
H(11)	-356	3828	2651	33
H(12A)	4064	3729	2227	42
H(12B)	2427	5044	2284	42
H(13A)	339	4544	1511	47
H(13B)	2254	3411	1404	47
H(14)	-2150	3033	1792	35
H(16A)	-2138	459	2465	32
H(16B)	-2904	1946	2650	32
H(17A)	628	294	3200	31
H(17B)	-1812	654	3422	31
H(18)	-769	2897	3571	28
H(20A)	-1448	2147	4415	30
H(20B)	-853	581	4312	30
H(21A)	-627	1019	5248	32
H(21B)	1885	591	5077	32
H(22)	554	1209	1199	37
H(23A)	-2820	-84	999	58
H(23B)	-4218	875	1380	58
H(23C)	-2162	-71	1635	58
H(24A)	-3213	2917	803	49

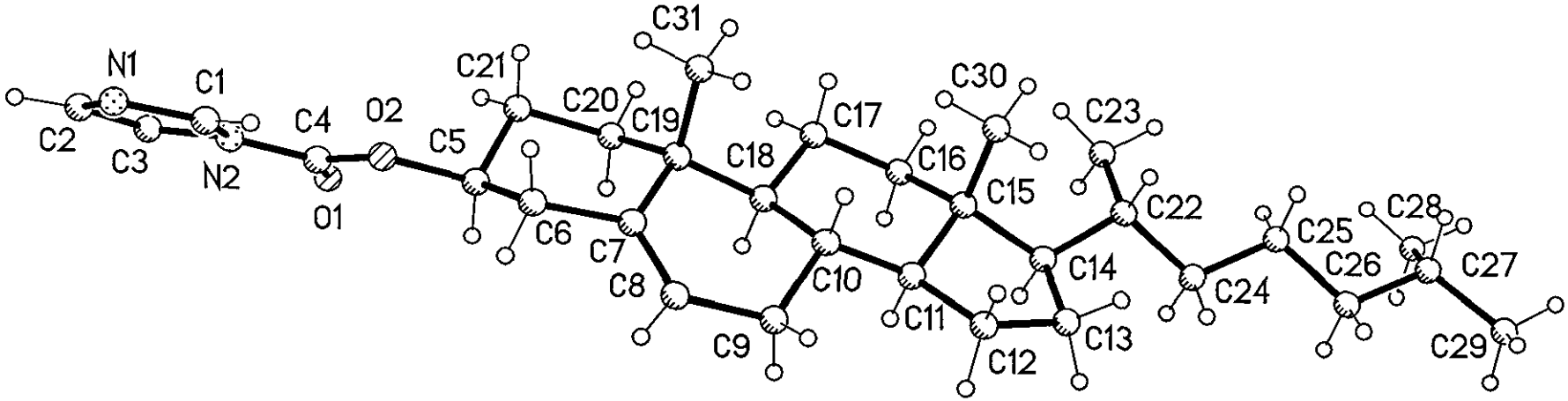
H(24B)	-559	3195	711	49
H(25A)	-623	1054	205	52
H(25B)	-3374	1060	230	52
H(26A)	-3546	3189	-238	54
H(26B)	-814	3107	-283	54
H(27)	-1492	1028	-792	49
H(28A)	-5182	639	-1212	94
H(28B)	-6209	1872	-883	94
H(28C)	-5249	533	-573	94
H(29A)	-661	3070	-1229	75
H(29B)	-3362	3435	-1300	75
H(29C)	-2394	2147	-1607	75
H(30A)	1634	150	2043	45
H(30B)	2937	699	2591	45
H(30C)	3397	1405	2031	45
H(31A)	2717	-61	3883	42
H(31B)	4145	519	4411	42
H(31C)	4717	1045	3829	42

Table 6. Torsion angles [°] for gw3.

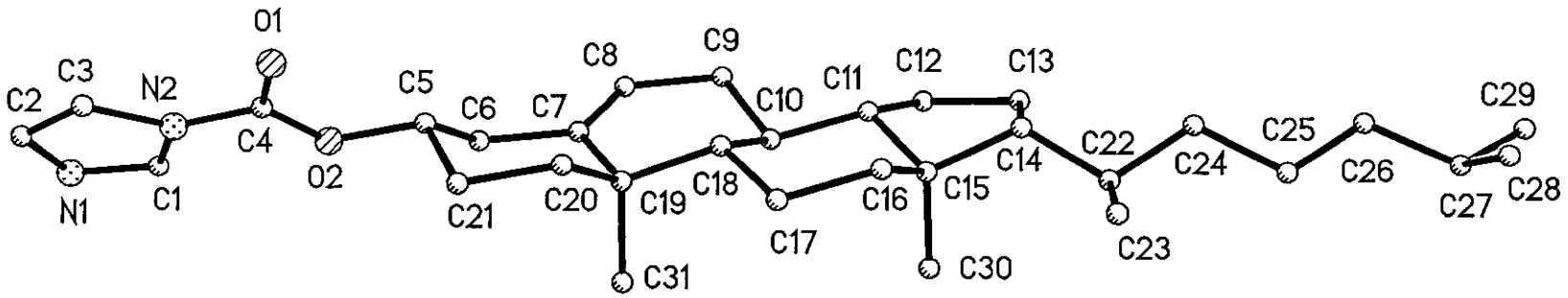
N(2)-C(1)-N(1)-C(2)	-0.3(3)	C(12)-C(11)-C(15)-C(16)	-167.9(2)
C(1)-N(1)-C(2)-C(3)	0.0(3)	C(10)-C(11)-C(15)-C(30)	-59.8(3)
N(1)-C(2)-C(3)-N(2)	0.3(3)	C(12)-C(11)-C(15)-C(30)	71.4(2)
N(1)-C(1)-N(2)-C(3)	0.5(3)	C(10)-C(11)-C(15)-C(14)	-176.42(19)
N(1)-C(1)-N(2)-C(4)	179.9(3)	C(12)-C(11)-C(15)-C(14)	-45.2(2)
C(2)-C(3)-N(2)-C(1)	-0.4(3)	C(22)-C(14)-C(15)-C(16)	-83.5(3)
C(2)-C(3)-N(2)-C(4)	-179.9(3)	C(13)-C(14)-C(15)-C(16)	151.8(2)
C(1)-N(2)-C(4)-O(1)	-174.5(3)	C(22)-C(14)-C(15)-C(30)	44.3(3)
C(3)-N(2)-C(4)-O(1)	4.9(4)	C(13)-C(14)-C(15)-C(30)	-80.5(2)
C(1)-N(2)-C(4)-O(2)	4.6(4)	C(22)-C(14)-C(15)-C(11)	162.4(2)
C(3)-N(2)-C(4)-O(2)	-176.0(2)	C(13)-C(14)-C(15)-C(11)	37.7(2)
O(1)-C(4)-O(2)-C(5)	1.3(4)	C(30)-C(15)-C(16)-C(17)	63.9(3)
N(2)-C(4)-O(2)-C(5)	-177.7(2)	C(11)-C(15)-C(16)-C(17)	-57.7(3)
C(4)-O(2)-C(5)-C(21)	-76.6(3)	C(14)-C(15)-C(16)-C(17)	-168.7(2)
C(4)-O(2)-C(5)-C(6)	162.6(2)	C(15)-C(16)-C(17)-C(18)	55.0(3)
O(2)-C(5)-C(6)-C(7)	176.2(2)	C(11)-C(10)-C(18)-C(17)	47.4(3)
C(21)-C(5)-C(6)-C(7)	55.0(3)	C(9)-C(10)-C(18)-C(17)	170.6(2)
C(5)-C(6)-C(7)-C(8)	132.4(3)	C(11)-C(10)-C(18)-C(19)	175.72(19)
C(5)-C(6)-C(7)-C(19)	-49.6(3)	C(9)-C(10)-C(18)-C(19)	-61.2(3)
C(6)-C(7)-C(8)-C(9)	177.3(2)	C(16)-C(17)-C(18)-C(10)	-48.4(3)
C(19)-C(7)-C(8)-C(9)	-0.5(4)	C(16)-C(17)-C(18)-C(19)	-175.84(19)
C(7)-C(8)-C(9)-C(10)	-12.3(4)	C(8)-C(7)-C(19)-C(31)	104.9(3)
C(8)-C(9)-C(10)-C(11)	164.6(2)	C(6)-C(7)-C(19)-C(31)	-73.0(3)
C(8)-C(9)-C(10)-C(18)	42.0(3)	C(8)-C(7)-C(19)-C(20)	-135.7(3)
C(9)-C(10)-C(11)-C(12)	56.9(3)	C(6)-C(7)-C(19)-C(20)	46.4(3)
C(18)-C(10)-C(11)-C(12)	179.2(2)	C(8)-C(7)-C(19)-C(18)	-17.1(3)
C(9)-C(10)-C(11)-C(15)	-178.7(2)	C(6)-C(7)-C(19)-C(18)	165.0(2)
C(18)-C(10)-C(11)-C(15)	-56.4(3)	C(10)-C(18)-C(19)-C(7)	47.4(2)
C(10)-C(11)-C(12)-C(13)	163.5(2)	C(17)-C(18)-C(19)-C(7)	175.19(19)
C(15)-C(11)-C(12)-C(13)	34.3(3)	C(10)-C(18)-C(19)-C(31)	-73.3(2)
C(11)-C(12)-C(13)-C(14)	-9.8(3)	C(17)-C(18)-C(19)-C(31)	54.5(2)
C(12)-C(13)-C(14)-C(22)	-147.4(2)	C(10)-C(18)-C(19)-C(20)	165.76(19)
C(12)-C(13)-C(14)-C(15)	-17.9(3)	C(17)-C(18)-C(19)-C(20)	-66.5(2)
C(10)-C(11)-C(15)-C(16)	60.9(2)	C(7)-C(19)-C(20)-C(21)	-51.5(3)

C(31)-C(19)-C(20)-C(21)	67.4(3)	C(15)-C(14)-C(22)-C(24)	-177.3(2)
C(18)-C(19)-C(20)-C(21)	-170.74(19)	C(23)-C(22)-C(24)-C(25)	-57.2(3)
O(2)-C(5)-C(21)-C(20)	-177.19(17)	C(14)-C(22)-C(24)-C(25)	176.8(2)
C(6)-C(5)-C(21)-C(20)	-59.5(2)	C(22)-C(24)-C(25)-C(26)	-168.2(2)
C(19)-C(20)-C(21)-C(5)	59.6(3)	C(24)-C(25)-C(26)-C(27)	-176.8(2)
C(13)-C(14)-C(22)-C(23)	179.5(2)	C(25)-C(26)-C(27)-C(28)	70.7(3)
C(15)-C(14)-C(22)-C(23)	58.7(3)	C(25)-C(26)-C(27)-C(29)	-164.5(3)
C(13)-C(14)-C(22)-C(24)	-56.5(3)		

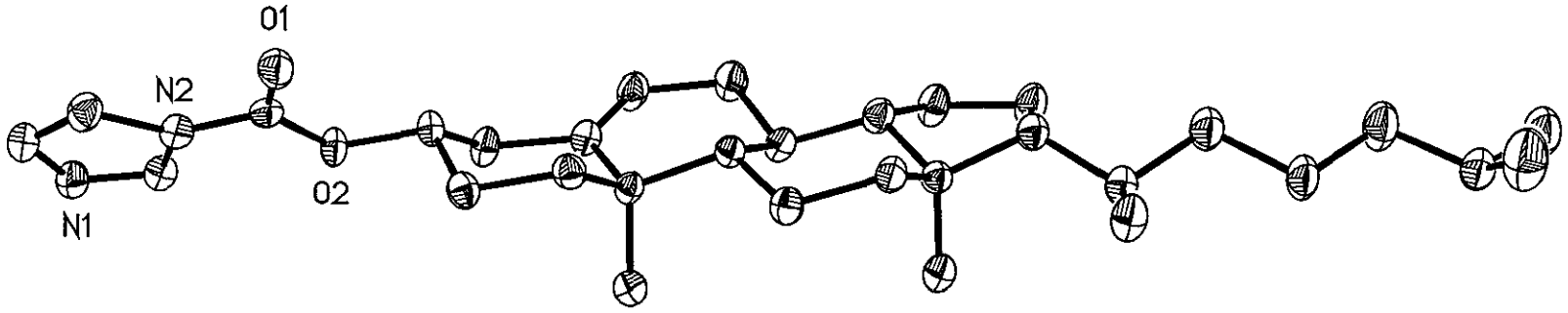
Plan View of Carbonylimidazolide derivative of Cholesterol 13



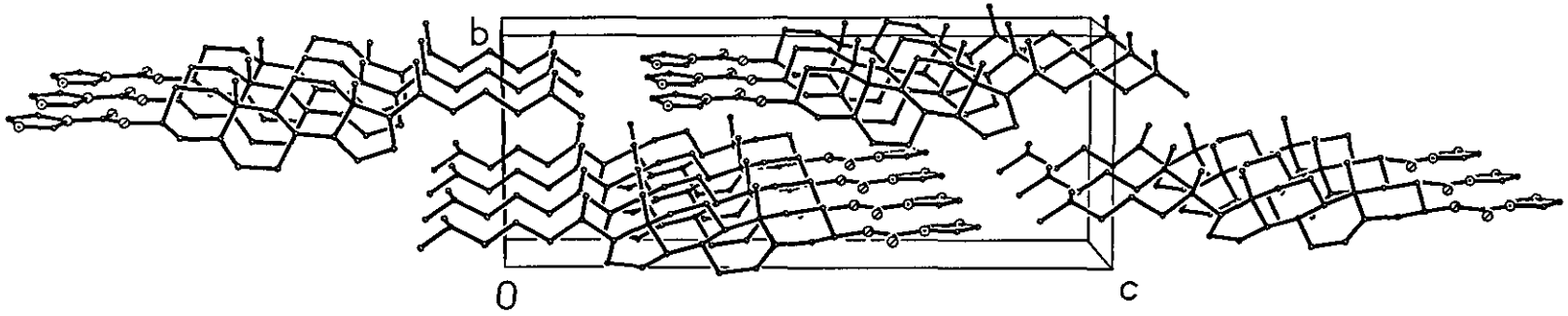
Side View of Carbonylimidazolide derivative of Cholesterol 13



Side View of Carbonylimidazolide derivative of Cholesterol 13 showing thermal ellipsoids



Plan View of Crystal Packing of Carbonylimidazolide derivative of Cholesterol 13



Side View of Crystal Packing of Carbonylimidazolide derivative of Cholesterol 13