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Chemical Synthesis of DNA & RNA Containing Thio-substituted Bases and Their Post-Synthetic Modifications

YANG WANG

A thesis submitted for the degree of Doctor of Philosophy

Aston University

June 2003

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Summary

Modified oligonucleotides containing sulphur group have been useful tools for studies of carcinogenesis, protein or nucleic acid structures and functions, protein-nucleic acid interactions, and for antisense modulation of gene expression. One successful example has been the synthesis and study of oligodeoxynucleotides containing 6-thio-2'-deoxyguanine. 6-Thio-2'-deoxyguanosine was first discovered as metabolic compound of 6-mercaptopurine (6-MP). Later, it was applied as drug to cure leukaemia. During the research of its toxicity, a method was developed to use the sulphur group as a versatile position for post-synthetic modification.

The advantage of application of post-synthetic modification lies in its convenience. Synthesis of oligomers with normal sequences has become routine work in most laboratories. However, design and synthesis of a proper phosphoramidite monomer for a new modified nucleoside are always difficult tasks even for a skilful chemist. Thus an alternative method (post-synthetic method) has been invented to overcome the difficulties. This was achieved by incorporation of versatile nucleotides into oligomers which contain a leaving group, that is sufficiently stable to withstand the conditions of synthesis but can be substituted by nucleophiles after synthesis, to produce, a series of oligomers each containing a different modified base. In the current project, a phosphoramidite monomer with 6-thioguanine has been successfully synthesised and incorporated into RNA. A deprotection procedure, which is specific for RNA was designed for oligomers containing 6-thioguanosine. The results were validated by various methods (UV, HPLC, enzymatic digestion). Pioneer work in utilization of the versatile sulphur group for post-synthetic modification was also tested.

Post-synthetic modification was also carried out on DNA with 6-deoxythioguanosine. Electrophilic reagents with various functional groups (aliphatic, aromatic, fluorescent) and bi-functional groups have been attached with the oligomers.

Keyword: DNA, RNA, thioguanosine, post-synthetic, modification

To my parents and sister

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Abbreviations

A	adenine, adenosine
AP	alkaline phosphatase
Bz	benZoyl
C	cytosine, cytidine
CD	circular dichroism
CPG	controlled pore glass
DBA	diaminobenzidine
DBU	1,8-diazabicyclo[5,4,0]undec-7-ene
DCA	dichloroacetic acid
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethylsulphoxide
DMT(r)	dimethoxytrityl-
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dA	2'-deoxyadenosine
dC	2'-deoxycytidine
dG	2'-deoxyguanosine
dsRNA	double stranded RNA
EDTA	ethylenediamine tetraacetic acid
EtOAc	ethylacetate
G	guanine, guanosine
G ^{OMe}	O ⁶ -methylguanine
G ^S	6-thioguanine
G ^{Sφ}	6-(2,4-dinitrophenyl)thioguanine

HPLC	high performance liquid chromatography
ibu	isobutyryl-
MMT	monomethoxytrityl
mRNA	messenger RNA
Pac	phenoxyacetyl
RNA	ribonucleic acid
SAM	S-adenosyl-L-methionine
SDS	sodium dodecylsulphate
T	thymine, thymidine
TBAF	tetrabutylammonium fluoride
TBDMS	<i>tert</i> -butyldimethylsilyl-
TCA	trichloroacetic acid
TEA	triethylamine
TEAA	tetraethylammonium acetate
TFA	trifluoroacetyl
THF	tetrahydrofuran
TLC	thin layer chromatography
tRNA	transfer RNA
UV	ultraviolet light

Chapter 1 Introduction

1.1 History of nucleic acid

Nucleic acids were first found as a phosphorus containing substance called nuclein by Friedrich Miescher (Blackburn & Gait 1995). The pure nuclein was a strongly acidic substance, which existed in a salt-like combination with a nitrogenous base. Thus it got a name nucleic acid.

Molecular studies on nucleic acids started as early as the beginning of the 20th century, when Leven and Jacobs began an investigation of the structure of nucleotides at the Rockefeller Institute (Blackburn & Gait 1995). Those nucleotides were cleaved by alkaline hydrolysis to give the phosphate and the corresponding nucleosides, inosine and guanosine respectively. Since then, all nucleosides have been characterized as the condensation products of a pentose and a nitrogenous base while nucleotides as the phosphate esters of one of the hydroxyl groups of the pentose in a nucleoside. It was also observed that hydrolysis of nucleic acids gave the appropriate equal proportions of four bases. This observation led to the general acceptance of a tetranucleotide hypothesis for the structure of both thymus and yeast nucleic acids. However, there was a controversy in the tetranucleotide hypothesis, i.e. the molecular mass of nucleic acids was greatly in excess of that calculated for a tetranucleotide. Further work was carried out in search of a real structure of DNA. One important finding was that using enzyme dipped with arsenate DNA could be cut into mononucleotides. By this mean, Klein and Thannhauser (Blackburn & Gait 1995) obtained the four crystalline deoxyribonucleotides whose structures were later proved by chemical synthesis by

Alexander Todd. At this point, all the facts were available to establish the primary structure of DNA as a linear polynucleotides in which each deoxyribonucleoside is linked to the next by means of a 3'- to 5'- phosphodiester. The presence of only diester linkages is essential to explain the stability of DNA to chemical hydrolysis, since phosphate triester and monoester are more labile. In 1953, Crick and Watson established the famous double α -helix structure for DNA (Crick & Watson 1953).

During the 1960s, the chemical synthesis of oligonucleotides became possible. This has greatly accelerated the research in nucleic acids. During 1970s, the invention of recombination DNA technology opened a whole new research field for the scientists. Recombination DNA technology can help to locate individual gene in the human chromosome. Thus, the Human Genome Project was launched in 1998 in search of the whole human genes. Scientists' interests went even a bit further with human genes. It has now been widely accepted that some cancers are generated because of mutation of specific genes. This has turned the interests of scientists from identify human gene into assigning the mutated gene for specific diseases like cancer. It is hypothesised that mending of the mutation might cure the diseases eventually. Although, it is still long way to go, lots of research groups have already dedicated their efforts in this area.

1.2 Structure of DNA & RNA

Nucleic acids can be divided into two families: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). DNA is the chemical carrier of a cell's genetic information. The sequence of DNA determines the nature of cells, controls cell growth and division, and directs biosynthesis of the enzymes and other proteins

required for all cellular functions. Gene is transcribed into mRNA which in turn assemble different kinds of proteins. This translation is realized with the help of RNA. In those procedures, two kinds of RNA play important roles. They are messenger RNA (mRNA) and transfer RNA (tRNA). A linear sequence of gene is first transcribed to mRNA and is later translated by the ribosome into a specific sequence of amino acids in protein. Amino acids are carried to the ribosome as aminoacyl-tRNA. Each tRNA is aminoacylated by a specific amino acid in a reaction catalysed by a specific aminoacyl-tRNA synthetase. This cycle is carried on until the wanted peptide oligomers have been formed. The peptides are then released from the ribosome to form proteins.

The secondary structure of DNA based on X-ray diffraction patterns of DNA fibres is shown in Figure 1-1. The common structure is the so-called double-helix. The essential features of this structure are: (a) two polynucleotides chains run in opposite directions and coil around a common axis to form right-handed double helix which are held mainly by hydrogen bonds between G:C and A:T base pairs (Figure 1-2); (b) purine and pyrimidine bases are on the inside of the helix (major groove), whereas the phosphate and deoxyribose unit are on the outside of the helix (minor groove); (c) the diameter of the helix is 20\AA , the distance of adjacent bases is 3.4\AA along the helix axis and related by rotation of 36° .



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Figure 1-1. The secondary structure of B-DNA in (a) ball and stick representation; (b) space filling representation; (c) structure of Z-DNA in space filling representation [Taken from Sanger (1984)]

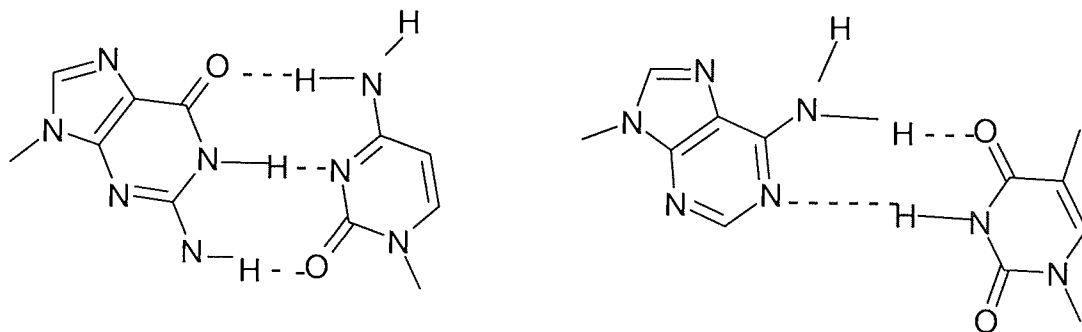


Figure 1-2. Watson-Crick base pairing for C:G (left) and T:A (right)

The structure of DNA was far more variable than had first been thought (Dickerson & Drew 1981) when the structures of crystals of short stretches of DNA were solved by X-ray crystallography. An extreme example was a completely new form of DNA that was a left-handed helix (Wang *et al.* 1979; Wang *et al.* 1981) now known as Z-DNA (Figure 1-1 c). Even oligonucleotides that have overall B-DNA structure show considerable deviations from classical uniform B-DNA at the local level (Blackburn & Gait 1995).

1.3 Modified oligonucleotides

1.3.1 Discovery of modified oligonucleotides

Normal oligonucleotides (DNA/RNA) are usually composed of four basic nucleosides. They are adenosine, guanosine, cytidine, uridine in RNA; deoxyadenosine, deoxyguanosine, deoxycytidine, thymidine for DNA. Besides normal nucleosides, modified nucleosides also exist. Modified nucleosides can be seen as normal nucleosides with changes at specific positions by the addition, deletion or substitution of one or more functional groups. These modified nucleosides have played important roles in many research fields, for example, cancer therapy, anti-HIV therapy, DNA/RNA protein interaction, structure study of DNA/RNA, etc (Broom *et al.* 1995; Brian S.S. 1995; Blackburn & Gait 1995).

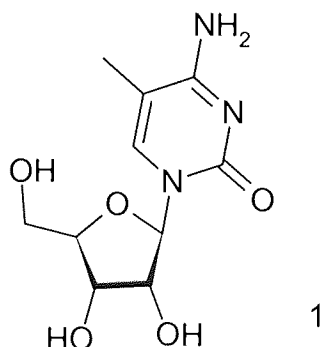


Figure 1-3. Structure of 5-methylcytosine

The presence of modified nucleosides in nucleic acids was first realized by Hotchkiss (1948) who had detected a rare component of a nucleic acid, 5-methylcytosine (Figure 1-3, compound 1), in a sample of calf thymus RNA. Since then, more naturally occurring modified nucleosides have been identified. Nowadays, scientists have turned their interests from wild-type modified

nucleosides to chemically modified nucleosides, which are more useful and give wider choices.

1.3.2 Chemical modification of oligonucleotides

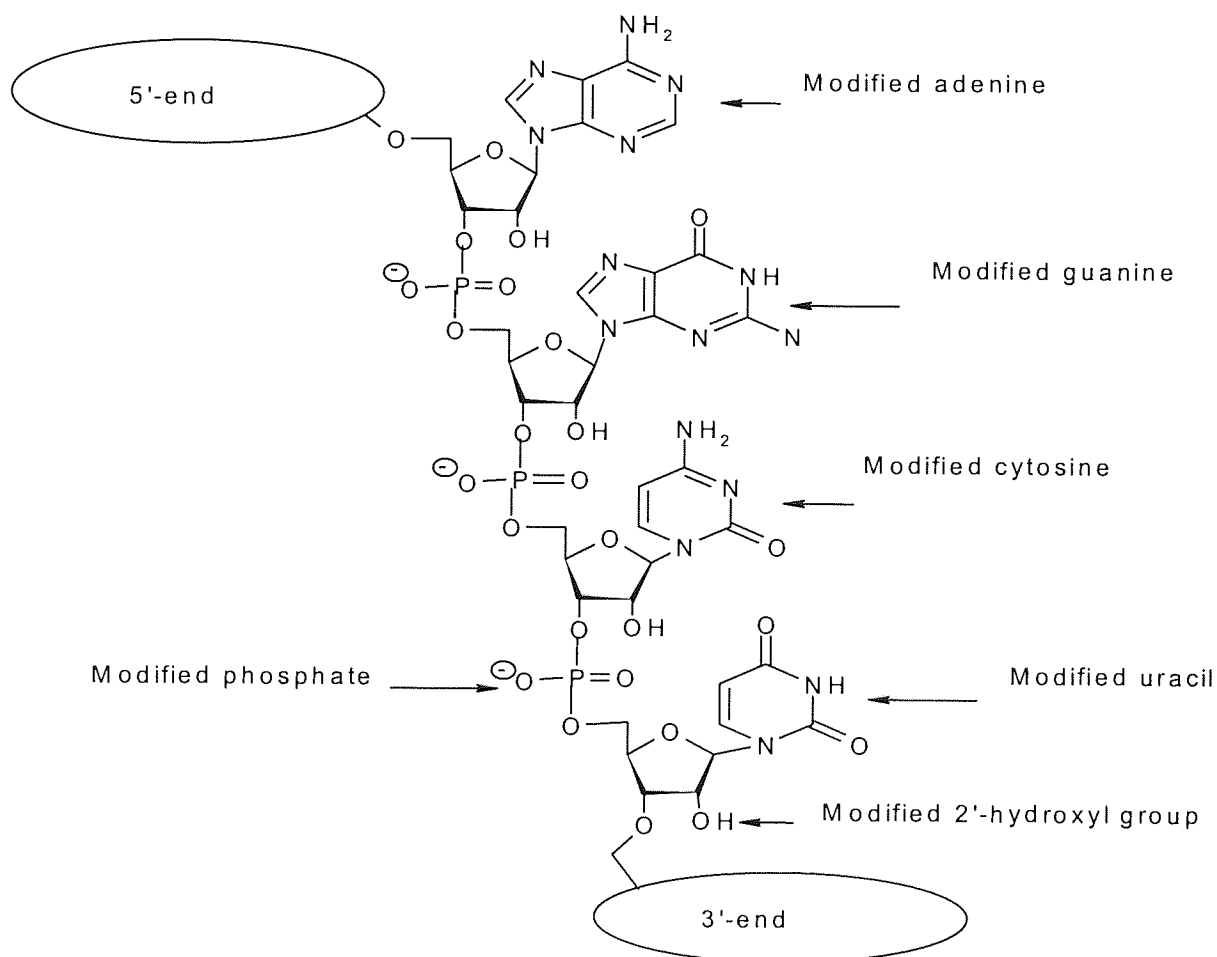


Figure 1-4. Illustration of modification of nucleic acids

Chemical modified nucleosides have more diversities than their natural-occurring counterparts. The basic strategy to modify a natural nucleotide includes adding, deleting functional groups of active sites of oligomers (Figure 1-4). These include sugars, bases and phosphate backbone of these molecules and additionally, the 3'-

and 5'- ends. The increasing utilization of synthetic oligonucleotides in genetic analysis and diagnostics has stimulated the development of a variety of reagents for the purpose of modification of oligonucleotides. Details are discussed below in the following sections.

1.3.2.1 Phosphorothioate

Phosphorothioate analogues of DNA and RNA have sulphur in place of oxygen as one of the non-bridging ligands bound to the phosphorus (Figure 1-5 left). Phosphorothioates have been shown to be more resistant to nuclease degradation than the natural DNA and RNA and still to bind to complementary nucleic acid sequences. Phosphorothioate oligodeoxynucleotides have demonstrated their usefulness as antisense molecules which can inhibit gene expression and as potential chemotherapeutic agents.

Furthermore, phosphorothioate can be introduced into an oligonucleotide at single site or multiple sites. Phosphate linkage is one of the favourite potential positions for modifications. The reason is that modifications at phosphorus are positioned on the outside of DNA or RNA duplexes so that they are easily accessible to reagents and other macromolecules such as nucleic acid binding proteins, cell receptors, enzymes. Thus introduction of phosphorothioate can affect the interaction of DNA with proteins.

The benefits of these modified DNA and RNA are (a) resistance of the internucleoside phosphorothioate linkage to enzymatic cleavage; (b) chirality at phosphorus, which is designated as R_P and S_P ; (c) greater nucleophilicity for sulphur, thus further modification can be carried out with sulphur-alkyl linkage.

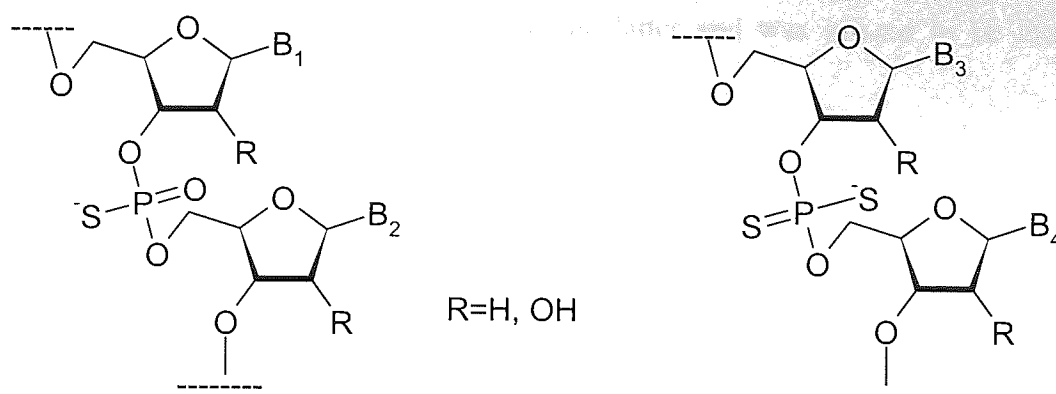


Figure 1-5. Illustration of phosphothioate (left) & phosphodithioates (right)

The first two published studies of antisense phosphorothioate oligonucleotides dealt with inhibition of expression of chloramphenicol acetyl transferase, which was encoded by a plasmid (Marcus-Sekura *et al.* 1987). This affect in turn inhibits the cytopathic effect of HIV (Matsukura *et al.* 1987). The mechanism of the inhibition is that RNase H 'recognizes' DNA-RNA heteroduplexes and selectively cuts only the RNA strand, thus blocks the process of translation. The phenomenon that phosphorothioate-modified DNA duplexed with RNA serves as a substrate for cleavage of RNA by RNase H has been experimentally demonstrated by Cazenave *et al.* (1989).

1.3.2.2 Phosphorodithioates

Phosphorodithioate (Figure 1-5 right) has been one of the focuses for antisense technology because of its achiral properties. Phosphorothioate linkages, among the others (methylphosphonate, phosphoramidite), are all chiral. Synthesis of these inter-linkages inevitably leads to a large number of non-resolvable diastereomeric oligomers. These oligomers have variable biophysical, biochemical, and biological properties. To overcome this problem, two methods can be chosen (a) stereo-

selective approaches for synthesizing these derivatives or (b) to develop an achiral analogue. Phosphorodithioates belong to the latter and was proved to be more economic than the former method.

1.3.2.3 Site-specific attachment of labels to the DNA backbone

Site-specific attachment of labels to the DNA backbone is more exciting since this method provides new materials for structural study or as therapeutic and diagnostic agents. However, some consideration should be given to the structural effects that the label agents will have on the nucleic acids, because insert of large groups might affect the stability of DNA duplex. Nevertheless, the addition of an agent or label to a terminal phosphomonoester would be unlikely to alter the general structure or stability of a double-stranded DNA.

Two approaches were employed in the first instance to introduce labels into the backbone of DNA sequences. Letsinger (1989) has described the oxidation of an H-phosphonate to the *N*-substituted phosphoramidate with the desired agent tethered via the nitrogen. Reaction with the substituted amine occurs, instead of other forms of oxidation, immediately after the introduction of the H-phosphonate linkage. The phosphoramidite is stable to the chemical DNA synthesis and deprotection. The second method involved the use of phosphorothioate as a site for alkylation by the label of interests (Cosstick *et al.* 1984).

1.3.2.4 2'-*O*-Methyloligoribonucleotides

2'-*O*-Methyloligoribonucleotide-RNA duplex is thermally more stable than the corresponding oligodeoxyribonucleotide-RNA one and the former duplex is not a

substrate for RNase H. Thus, 2'-*O*-methyloligonucleotides are proved to be useful reagents for various biological experiments.

The synthesis of 2'-*O*-methyloligoribonucleotide monomer (4) is similar to that of normal nucleosides, except for protection of 2'-hydroxyl group with a methyl group. To achieve this aim, 3'-, 5'-hydroxyl group and other active groups on the bases needed to be transiently protected (Figure 1-6). Then, methyl iodide was used with silver oxide in 2-butanone to attach the methyl group to 2'-hydroxyl group of compound (2) (Figure 1-6). The acetyl-protecting group was then removed by treatment of aqueous ammonia. A nucleoside with 2'-methoxyl group was then obtained (Cramer & Pfeleiderer 1999). Compound (3) was converted to nucleoside phosphoramidite monomer by conventional methods. With an automatic synthesizer, 2'-*O*-methyl nucleotide phosphoramidite can be incorporated into oligomers and ready for the biological tests.

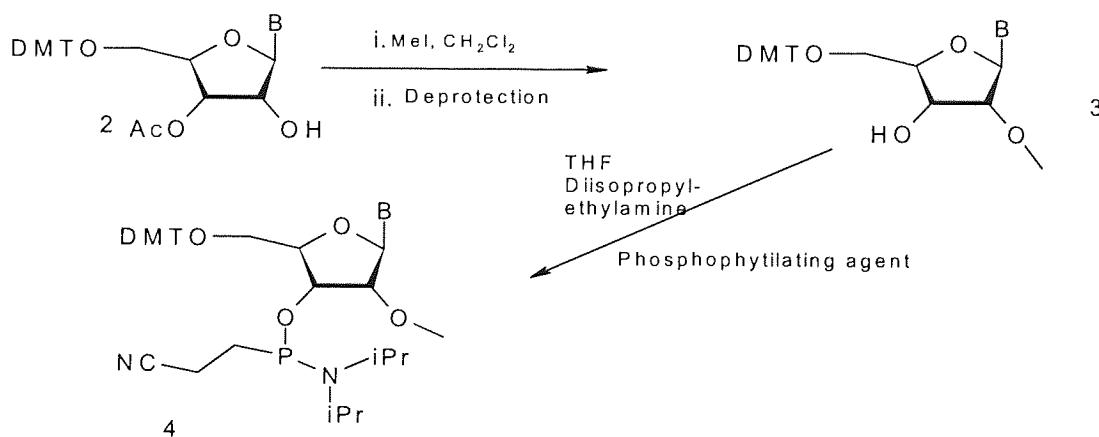


Figure 1-6. Synthesis of 2'-*O*-methylphosphoramidite; 2'-OH was methylated by iodomethane, the monomer was phosphilylated to form phosphoramidite

1.4 Application of modified oligonucleotides

1.4.1 Oligonucleotides for study of DNA-damage produced by alkylating agents

Genotoxic agents such as chemical carcinogens can cause cancer via covalent chemical-DNA adducts and photoproducts formed in DNA of cells. During the past three decades, much effort has been directed toward the identification of those adducts. However, few results have been obtained because genotoxic agents are problematic and can generate more than one DNA modifications (Singer & Kasmierek 1983). It is very difficult to assess the impact of individual adduct on DNA structure and the contribution of each adduct to the spectrum of mutations induced by chemical or radiation treatment. Thus, it is useful to introduce modification at specific site of the oligomers. Methods of synthesis of oligonucleotides containing DNA adducts at defined sites have been developed to explore the contribution of individual DNA adducts to these biological effects.

DNA damages are caused by various reasons, for example, alkylating agents like *N*-nitroso compounds. The mechanism was thought to be the alkylation of DNA, in particular, alkylation of the O^6 -position of guanine and O^4 -position of thymine (Swann *et al.* 1996). Alkylated DNA consequently loses an imine proton at *N1* position of guanine or *N3* position of thymine therefore leads to a complete change in the pattern of hydrogen bonding in base-pairing and results in the production of GC-AT and TA-CG transition mutations, which may lead to cancer (Swann 1990; Swann *et al.* 1996). To test this hypothesis, alkylated analogues monomers have been synthesised and incorporated into oligonucleotides. The biological

investigation proved that DNA with alkyl groups, such as O^6 -alkylguanine or O^4 -alkylthymine could cause mismatch in DNA duplex, which may cause cancer.

1.4.2 Non-isotopic oligonucleotides as probes

Oligonucleotides are widely used as probes for detection of specific genes and as primers for sequencing purposes. The most common method for the labelling of oligonucleotides is the incorporation of isotope ^{32}P . Although its sensitivity is high, this isotope has a short half-life, and the technique has intrinsic hazards such as radioactivity. Other methods, like fluorophores, chromophores and biotins have been developed to overcome this problem. Beaucage & Iyer (1993) has shown that these markers can be attached to 3'- or 5'- terminus, even modified phosphate groups or heterocyclic bases.

To incorporate a non-isotopic reporter groups to a base, a linker arm with a length of 3-12 atoms is usually used. The linker arm is attached to a primary reactive group such as an amino, hydroxyl, or sulphhydryl function on the base. The non-isotopic reporter group can then be attached to the linker. An example on pyrimidines is the C_5 position, which faces outward into the groove of double-stranded DNA helix and therefore modification causes least disruption of the double helix structure of the DNA. For purine bases, location like C_8 is usually chosen for reporter group attachment, since this position has less steric effects in a double helix and is not involved in hydrogen bonding.

1.4.3 Recent research in RNAi

RNAi (RNA interference) refers to the introduction of homologous double stranded RNA (dsRNA) to specifically target a gene's product. The use of antisense RNA to interfere with a gene's activity was first utilised by Guo & Kemphues (1995). Subsequently, it was observed by Fire *et al.* (1998) that it is the presence of dsRNA (double stranded RNA), formed from the annealing of sense and antisense strands present in the *in vitro* RNA preparations, that is responsible for producing the interfering activity. Introduction of dsRNA into an adult worm results in the loss of the targeted endogenous mRNA from both the adult and its progeny. The most interesting aspects of RNAi are the following:

- dsRNA, rather than single-stranded antisense RNA, is the interfering agent
- it is highly specific and remarkably potent (only a few dsRNA molecules per cell are required for effective interference)
- the interfering activity (and presumably the dsRNA) can cause interference in cells and tissues far removed from the site of introduction

The mechanism of how dsRNA results in the loss of the targeted homologous mRNA is still not fully understood. A number of observations indicated that the primary interference effects were post-transcriptional. First it was observed by Fire *et al.* (1998) that only dsRNA targeting exon sequences was effective (promoter and intron sequences could not produce an RNAi effect). Additional evidence supporting mature messages as the most likely target of RNA-mediated interference is summarised by Montgomery *et al.* (1998).

Because of its interference effects in degradation of mRNA, RNAi might be a potential drug for gene diseases. Lots of research work have been undertaken to exploit the full scale of application of RNAi. McCaffrey *et al.* (2002) showed the therapeutic potential of this technique by demonstrating effective targeting of a sequence from hepatitis C virus by RNA interference in vivo. Application of RNAi has also been investigated by Zhang *et al.* (1999). They demonstrated that the mRNA technology can be married to RNAi. They demonstrated model systems to introduce targeting vectors into cells to cause RNAi - thus again pointing the way for therapeutic use of this new technology.

1.4.4 Modified nucleosides and gene therapy

It is estimated that over 4000 diseases are caused by single gene defective and even much more diseases are related to the gene-controlled protein expression. Thus, cure of diseases at gene level become a very attractive and promising direction for new therapeutic methods. This is assisted by the developments of new techniques such as gene engineering, DNA recombination, chemical synthesis of oligonucleotides, etc.

The basic idea of gene therapy includes discovering the defected gene, delivering normal gene (wild type), replacing the defected gene and curing the disease. Usually, DNA recombination technology will be used to detect the mutant. Then either viral vector or non-viral vector will be used to deliver the wild-type gene (non-mutant gene) into cells. DNA was then released from the vehicle and is transferred into the nucleus. Therapeutic DNA (wild type) is transcribed and translated. After that, either normal gene or the desired protein will be formed to cure the disease. Gene therapies now have been tried in many diseases, including

severe combined immunodeficiencies (SCIDs), cancer, cardiovascular diseases, AIDS and other infectious diseases, rheumatoid arthritis and neurological diseases. For many diseases, gene therapy was just in infant stage. However, the greatest clinical experience with gene therapy is in use of murine retroviral vectors for the treatment of SCID, particularly for patients with adenosine deaminase deficiency (ADA) (Hoogerbrugge *et al.* 1996).

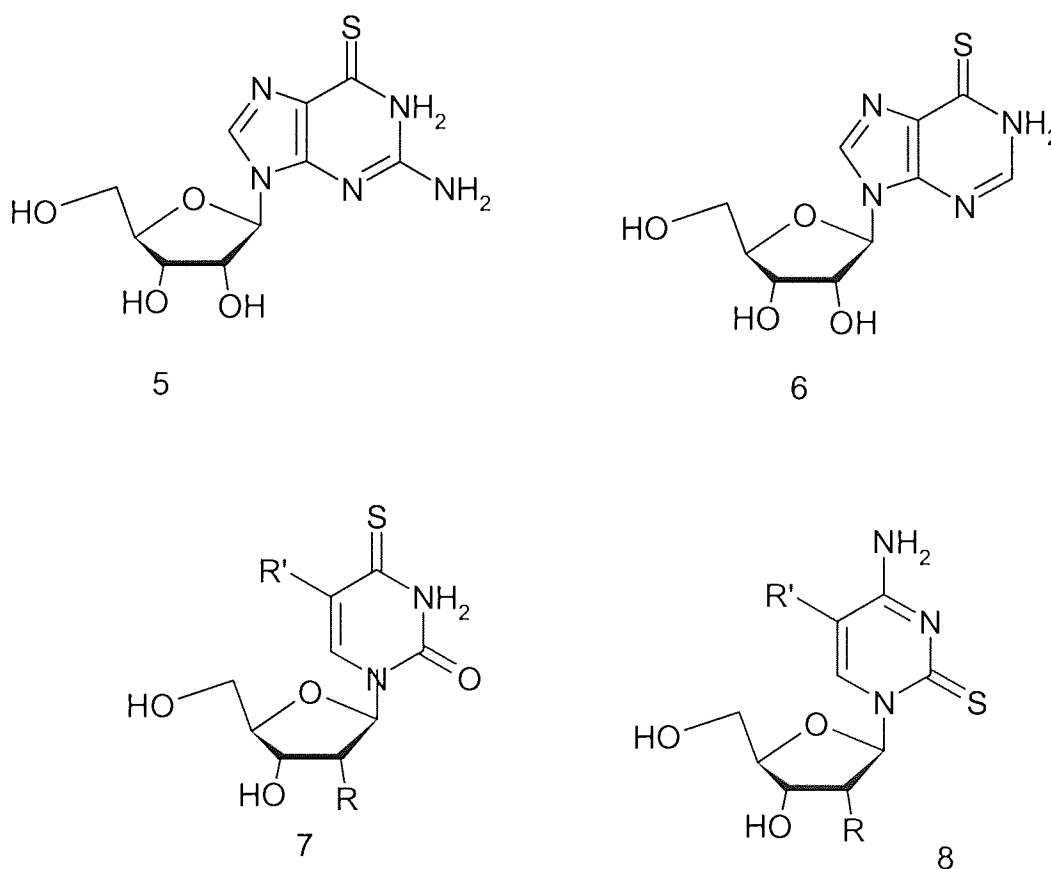
Although the application of gene therapy is still in its infant and the main idea of gene therapy now is concentrated on the replacement of abnormal gene with a normal gene, modified oligonucleotides will get their parts in gene therapy because they provide various choices other than normal sequences.

1.5 Recent developments in the synthesis, chemical modification of sulfur modified nucleosides

Because of the particular interests of our group in sulfur-modified oligonucleotides, the current project has concentrated in these oligomers. Former research work in this area is to be discussed in this section. In search for new bioactive agents and tools, many sulfur nucleosides, nucleotides and oligonucleotides have been synthesized over the last 40 years (Chambert *et al.* 2002). Some of them have been found with interesting therapeutic activities and a great many of them have been efficient tools in studies of protein or nucleic acid structures and functions, protein-nucleic acid interactions and for antisense modulation of gene expression.

1.5.1 Nucleosides and nucleotides containing thiol group

A large number of nucleosides and nucleotides containing thiol function at different positions of the bases have been prepared. To synthesis these modified nucleosides or nucleotides, oxygen atom of base ring is usually replaced by a sulfur group. The method is to transform the oxygen atom into a leaving group which is then substituted with a thiol function, obtaining the corresponding thionucleoside or its protected form. One-step substitution of oxygen for a sulfur atom is also possible using phosphorous pentasulfide, for example in the synthesis of 4-thiothymidine (Roget *et al.* 1989). Some of thiol-substituted nucleosides are listed below (Figure 1-7; compounds 5, 6, 7a, 7b, 7c, 8a, 8b, 8c):



a: R=OH, R'=H; b: R=H, R'=H; c: R=H, R'=CH₃

Figure 1-7. Some examples of thiol-containing nucleosides

Thiol functions are often found in the form of the corresponding stable thione. Introduction of a thione function at the 8 position of purine nucleosides or nucleotides (Figure 1-8) was achieved by displacement of a bromine atom introduced by bromination of the natural adenine and guanine nucleosides or nucleotides (Scheit 1980; Ikehara *et al.* 1973; Come *et al.* 1991).

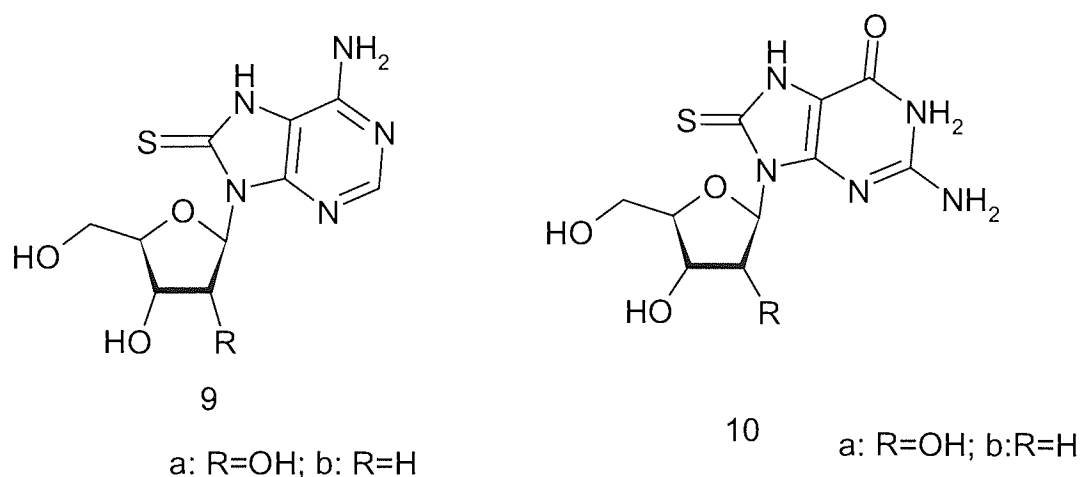


Figure 1-8. 8-Thione nucleosides

Sulfur modification of thymidine on the 5-methyl group was achieved via the 5-chloro derivatives. The 5-thiomethyl derivatives were prepared from this intermediate and revealed efficient anti-herpes activities (Schmidt *et al.* 2000). Photochemical bromination of 3',5'-diacetylthymidine (11) on the 5-methyl group led to 2'-deoxy-3',5'-diacetyl-5-bromoethyluridine which was transformed into an easily oxidizable 2'-deoxy-5-thiomethyluridine (12) or into the corresponding thioacetate (Barwolff & Langen 1974) (Figure 1-9).

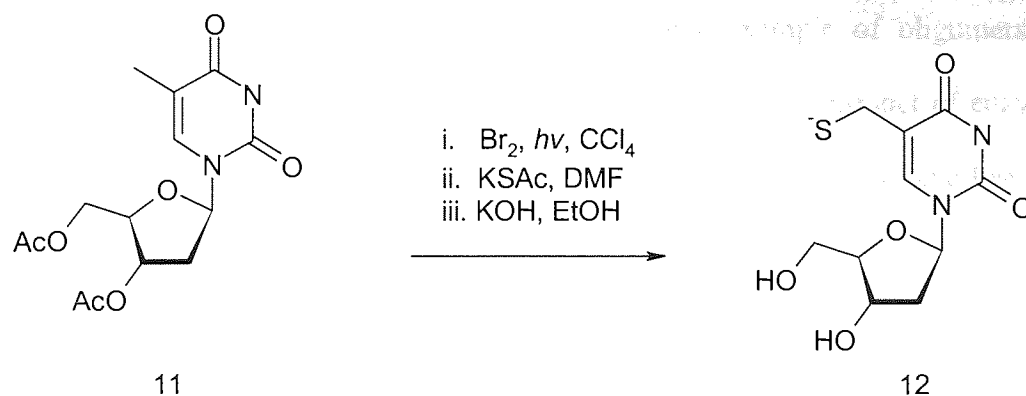


Figure 1-9. Formation of 5-thiomethyl-2'-deoxy-3',5'-diacetyl thymidine

The first direct aminolysis of 2-thiouracil nucleosides to 2-thiocytosine nucleosides has been reported (Strekowski *et al.* 2000). In the thiopurine series, heating at elevated temperature of peracetylated 6-thioguanosine induces a transglycosylation to form a stable 9- S^6 -bis(ribose) derivative and N^2 -acetyl-6-thioguanine (Manikowski & Boryski 1999). A synthesis of 9-2'-deoxy- β -D-ribofuranosyl)purine-2-thione from 2'-deoxy-6-thioguanosine has been also recently reported (Rappaport 2000).

Other modifications have been introduced in thiobase containing nucleosides and nucleotides, for example, 2'-deoxy-5-fluoro and 2'-azido-2'-deoxythiopyrimidine nucleosides and/or nucleotides were prepared (Palomin *et al.* 1990; Bretner *et al.* 1993, Lin *et al.* 1995; Bretner *et al.* 1995). In the search for new anti-HIV agents, 2',3'-dideoxy-2-thiouridine and its 2'-deoxy analogues have also been prepared (Joshi *et al.* 1995).

1.5.2 Oligonucleotides containing thiobases

Oligonucleotides with thiocarbonyl have also been proved to be useful tools. Similar to the normal bases, thiobases can also be incorporated into oligomers to

produce oligonucleotides with thiobases. An early example of oligomers with thiobases is the DNA with 6-mercaptopguanine, which is the product of enzymatic reaction of 6-mercaptopurine. 6-Mercaptopurine and its analogues have been used as drugs since 1950's for the treatment of acute leukaemia (Elion 1989).

Modification of oligonucleotides containing 4-thio-2'-deoxyuridine after synthesis of oligonucleotides have been reported for incorporating a wide range of functional groups at any base position with a DNA (Nikiforov & Connolly 1991). 4-Thiouridine residues can also be incorporated enzymatically into RNA fragments from the corresponding triphosphates (Osswald & Brimacombe 1999). 2'-Deoxy-2-thiouridine and 2-thiothymidine were also incorporated as probes in oligodeoxynucleotides giving stable hybrids with unmodified oligonucleotides (Connolly & Newman 1998; Kuimelis & Nambiar 1994; Kuimelis & McLaughlin 1996). In one of these syntheses (Kuimelis & Nambiar 1994) N^3 or O^4 acylation with toluoyl chloride was used as protection during the synthesis. This protection prevents the oxidation of the unprotected thiobase induced by the aqueous oxidation reagent used in the phosphoramidite DNA synthesis.

More complex modification can be introduced into oligomers. 5-Methylaminomethyl-2-thiouridine is a hypermodified nucleoside present in the anticodon domain of tRNA. For biophysical investigation, this nucleoside was recently incorporated with other modified nucleotides in the 17 nucleotide anticodon stem-loop of *E. coli* tRNA using the corresponding *O*-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (Sundaram *et al.* 2000). Oligodeoxynucleotides containing a bis-thiouracil nucleoside probe were also synthesized and showed

moderate irreversible photo-binding activity with complementary DNA and RNA targets (Saintome *et al.* 1997; Saintome *et al.* 2000). Peptide nucleic acid (PNA) dimer duplexes incorporating 4-thiothymidine were not able to fully mimic the photochemical behaviour observed in the dinucleotides series (Clivio *et al.* 1998). A study of the self-association properties of 4-thiouridine showed a strong decrease in self-association constant compared to uridine (Dunger *et al.* 1998).

Oligodeoxynucleotides with modified purine were also synthesized and the stability of the corresponding duplexes was studied (Xu *et al.* 1992a; Xu *et al.* 1992b). The synthesis and study of 2'-deoxy-6-thiomethylguanosine containing oligonucleotides revealed that the cytotoxicity of 6-mercaptopguanine depends upon S-adenosylmethionine after incorporation into DNA.

However, less work has been reported with thioribonucleosides. This is largely due to the problem of finding suitable protecting groups for the 2'-hydroxyl and the thione functions. Oligoribonucleotides containing 6-thioinosine or 4-thiouridine were synthesised (Adams *et al.* 1995b Adams *et al.* 1994a; Becker *et al.* 1998). Another report is the study of incorporating 2-thiopyrimidine ribonucleotides into oligoribonucleotides for the intra-molecular hydrogen bonding in RNA structures research (Houssier *et al.* 1988). The results showed possible direction for the drugs to cure the HIV disease. Furthermore, methods of synthesis and incorporation of this modified nucleoside were developed from the corresponding phosphoramidite approach without base protection (Kumar & Davids 1995).

In the solid-phase synthesis of such oligodeoxyribonucleotides or oligoribonucleotides using phosphoramidite or H-phosphonate chemistry, different protective groups for the thione function were used, the methylsulfenyl group (Connelly & Newman 1989), the 2-cyanoethyl (Rao *et al.* 1995; Christopherson & Broom 1991; Rao *et al.* 1992; Coleman & Siedlecki 1992; Coleman & Kesicki 1994; Rao *et al.* 2000; Adams *et al.* 1992a; Adams *et al.* 1995b), the phenyl or the 4-nitrophenyl (Nikiforov & Connolly 1991), the 2,4-dinitrophenyl (Elion 1989), the mesitylene sulfonyl (Nikiforov & Connolly 1992; Coleman & Siedlecki 1992; Xu *et al.* 1992a; Xu *et al.* 1992b) and the pivaloyloxymethyl group (Clivio *et al.* 1992a; Clivio *et al.* 1992b; Clivio *et al.* 1993; Becker *et al.* 1998).

Modified nucleosides can also be incorporated into oligonucleotides via enzymatic methods. 6-Thioguanosine, 6-thioinosine, 4-thiouridine were incorporated into oligomers with good yields by enzymatic method via their 3',5'-bisphosphates (Kadokura *et al.* 2000). The 4-thiouridine 3',5'-bisphosphate derivative was found to serve as the most active substrate of T4 RNA ligase with a reaction efficiency of 96%. The synthesis of 4-thiothymidine 5'-triphosphate was reported by Bazin *et al.* in 1999. This nucleotide was found to be an excellent substrate for the DNA polymerase called Klenow fragment and HIV-1 reverse transcriptase, and was used for incorporation of 4-thiothymidine into oligodeoxynucleotides (Rao *et al.* 2000).

1.6 Chemical synthesis of DNA & RNA

1.6.1 Brief history of chemical synthesis of oligonucleotides

During the 1950s, the understanding of the general chemistry and structures of the natural polynucleotides and their enzymatic degradation products developed incredibly rapidly. At the same time, research in DNA/RNA required more details of their structures as well as oligomers with designed sequences. So the targets and the methods (chemical understanding on which synthetic strategies based) were both in place. Eventually, a dinucleoside monophosphate was synthesised in 1950s (Barnes 1989).

Like all other common chemical reactions, this two-nucleotides synthesis was carried out in solution phase. Later, solid-phase synthesis of nucleotides was made possible, stimulated by the solid phase synthesis of peptides in 1950s and 1960s. As peptides and oligonucleotides share some common natures, the technique was tried and successfully applied in the synthesis of oligonucleotides. Since 1980 efficient automated method of solid phase oligonucleotides synthesis has been widely used.

1.6.2 Protecting groups

Nucleosides have multiple active nucleophilic sites. To make the reaction take place according to the design route, all the nucleophilic sites, except those sites for the polymer enlongation, need to be protected. A DNA/RNA biopolymer is synthesized by assembling monomeric or oligomeric blocks. Each block features at least a nucleophilic and an electrophilic function, i.e. the 5'-OH and the 3'-function (phosphate, phosphoramidite, or phosphonate). The nucleophilic and electrophilic sites are linked together at the coupling step. Since on DNA and RNA, there are more than one active nucleophilic sites, the protection of unwanted reaction sites is a necessity.

Protecting groups can be divided into two catalogues: persistent and transient. The persistent protections remain on the biopolymer during all the synthesis. They are cleaved at the very end. They cap the functions of the hydroxyl residue of nucleotides, or of the side chains of amino group on the base or other functional groups like thiocarbonyl group in the present study. They also cap the phosphate oxygen. The transient protection block the functions to be coupled at a given time of the synthesis, like DMT or MMT groups protecting 5'-OH (Chattopadhyaya *et al.* 1978).

It is possible to synthesize DNA or RNA by one of the three classical methods (phosphotriester, phosphoramidite, H-phosphonate strategies) without protecting thymidine or uridine residues. However, cytidine and deoxycytidine are usually protected by acylation on *N*4. The protection of adenosine and deoxyadenosine requires a special comment, because the chemical stability of the nucleoside is altered by the protection of the exocyclic amino function of the nucleic base (Sonveaux 1986). Adenosine and deoxyadenosine can undergo a depurination in acidic environments (Hevesi *et al.* 1972). Thus the reaction condition needs to be carefully selected. When acylation was carried out on the *N*⁶ on the deoxyadenosine, the *N*⁶, *N*⁶, *O*^{3'}, *O*^{5'} tetra-acylated derivative is obtained (Schaller *et al.* 1963). Thus the usual strategy used is to *O*-silylation before *N*-acylation. That means 2',3',5'-triacylation of riboside was carried out first without concomitant *N*-acetylation, to perform the desired *N*-acylation, and finally to rapidly cleave the labile *O*-acetyl functions. Guanosine and deoxyguanosine, just like their counterparts of adenosine, can easily undergo a depurination in acidic or

conditions. The function of the exocyclic amino function is neither very basic, nor very nucleophilic. Like adenosine, guanosine has to be *O*-silylated, followed by *N*-acylation, and finally *O*-desilylation (Mishra *et al.* 1986; Brown *et al.* 1979; Buchi *et al.* 1972).

Protection of the 2'-OH is a persistent protection. This problem only existed in RNA synthesis. The protecting group has to be removed at the ultimate step of the synthesis, because oligoribonucleotides with a free 2'-OH function are easily cleaved, either chemically, under acidic or basic catalysis (Reese & Skone 1985; Griffin *et al.* 1968). The 2'-OH protecting group has to be resistant to the conditions of cleavage of the phosphate and hydroxyl protections. It also has to withstand the conditions used to remove the 5'-OH protection before each coupling. Acyl-type groups are not suitable for the protection of 5'-OH because they can easily migrate from 2'-OH to 3'-OH (Reese & Trentham 1965), would be prematurely cleaved. To overcome this problem, three types of protections have been investigated:

1. The acetal/ketal type protection, cleaved by 0.01 *M* HCl
2. The *O*-nitrobenzyl group, cleaved by photolysis
3. The *t*-butyldimethylsilyl group, cleaved by the fluoride anion.

These protections increase the steric crowding of the nucleotides. The coupling is thus slower in the RNA series than in the DNA series. Now the popular choice (also the one we used) is *t*-butyldimethylsilyl group (Wu *et al.* 1989). When 5'-*O*-DMTr protected nucleosides are treated with *t*-butyldimethylsilyl chloride in

pyridine, or in DMF/imidazole, a mixture, where 2'-*O* and 3'-*O* monosilylated derivatives dominate, is obtained. They can be separated carefully by silica gel column chromatography.

The protection of the 5'-OH function is transient one. This protection is removed before each coupling. The removal has thus to be not only quantitative, but also very rapid. The protecting groups of the trityl family are the best of the routine synthesis of oligoribonucleotides containing the four natural bases only. Trityl chloride, being sterically crowded, alkylates the primary 5'-OH far more readily than secondary 2'-OH and 3'-OH. The introduction of heteroatoms (e.g., the two methoxy groups of DMTr) was necessary to tune the rate of cleavage by acids.

Except for the H-phosphonate method, all phosphorus needs protection during the oligomer assembly. At the end of the synthesis, the protecting groups (usually cyanoethyl) on phosphorus need to be removed before all the other groups, because a triester function is very sensitive to nucleophiles and to intramolecular attack by 5'-OH, 3'-OH and internal 2'-OH functions.

1.6.3 Phosphodiester chemistry

Khorana and co-workers (1961) opened the way to synthetic sequence-defined oligonucleotides in 1958, using phosphodiester chemistry. Their synthesis of dinucleotides TpTp is shown in Figure 1-10 using DCC as a coupling agent.

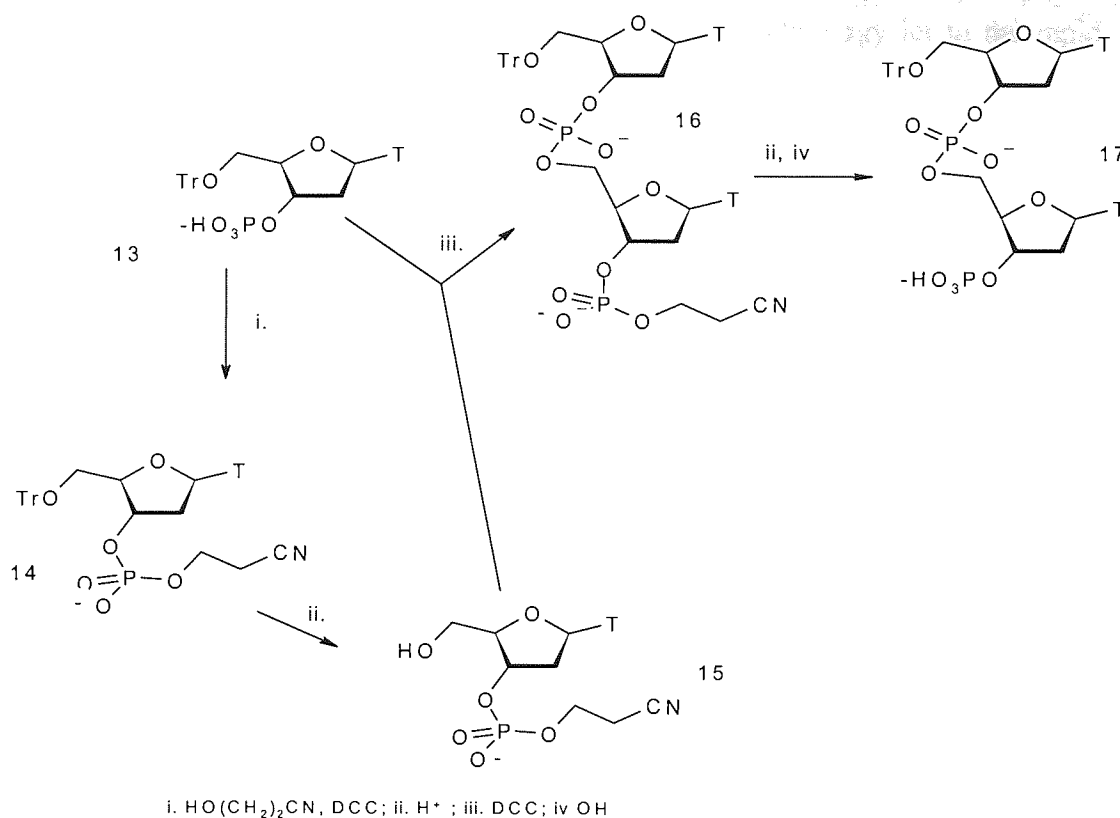


Figure 1-10. Synthesis of oligonucleotides by phosphodiester method

The nature of the chemistry involved in the phosphodiester coupling reaction with DCC and sulfonyl halides has never been wholly elucidated, but it was clear that pyrophosphate, trimetaphosphate, and higher polyphosphate esters were quickly formed and more slowly alcoholised (Sudhir 1993a). The fact that yields were good and also presumably the greater ease, at that time, gave an impetus to diester chemistry that lasted well into the 1970s.

1.6.4 Phosphotriester chemistry

The first synthesis of unsymmetrical dinucleotides phosphates was effected by triester chemistry in 1955 (Letsinger *et al.* 1965; Reese 1978) (Figure 1-11). The phosphotriester method of oligonucleotide synthesis realized the need to protect the internucleotidic phosphodiester groups during synthesis, revolted the preparation of

oligonucleotides. Application of solid-phase synthesis technology led to the rapid and efficient synthesis of oligonucleotides.

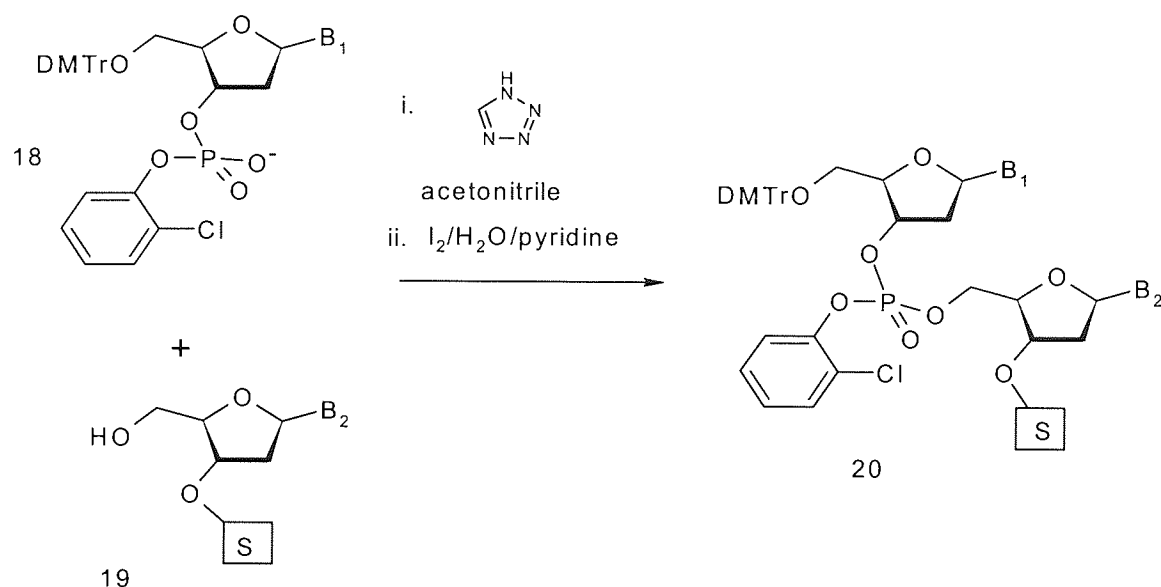


Figure 1-11. Phosphotriester approach

The phosphotriester method of synthesis may be regarded as the method of choice for the large-scale preparation of oligodeoxyribonucleotides. Although this method is perhaps not so readily adaptable to fully automated machine-aided synthesis, it is superbly amenable to scale-up processes. A major advantage of the phosphotriester method is that excesses of the phosphodiester monomer building blocks used in chain assembly can be recovered, which cannot be done in phosphoramidites or H-phosphonate method (discussed later). If kilogram amounts of short oligonucleotides were to be made, then the losses and cost of monomer building blocks and other reagents may be a primary factor in developing large-scale synthesis.

The most significant criterion for the successful synthesis of oligodeoxyribonucleotides by the solid-phase phosphotriester approach is the purity of solvents and reagents, and it is essential to ensure the highest purity for all reagents. The advantage of the phosphotriester method is that medium length oligodeoxyribonucleotides can be prepared reasonably quickly using simple and inexpensive equipment with a minimum number of steps per cycle (Sudhir 1993a).

1.6.5 H-Phosphonate chemistry

After the successful application of phosphotriester chemistry, a new development occurred: two groups of workers observed that a nucleoside 3'-H-phosphonate could be activated and then coupled rapidly to give a diester H-phosphonate in high yield (Froehler *et al.* 1986; Garegg *et al.* 1986). This versatile H-phosphonates can be oxidized to phosphate, thiated to the phosphorothioate (24), or aminated to give phosphoramidates (25) (Figure 1-12). Furthermore, H-phosphonate coupling of oligonucleotides was the shortest time used in all such chemical syntheses known (Froehler *et al.* 1986). Although, this chemistry offers some advantages over the phosphoramidite method, the H-phosphonate approach to oligonucleotide synthesis has not nearly been used as heavily as the phosphoramidite chemistry.

The synthesis of H-phosphonate monomers is quite easy by using phosphilytating agent (Marugg *et al.* 1986). The most reliable method for characterization of the product H-phosphonates is ^1H and ^{31}P NMR. Hydrogen-phosphonates have a characteristic coupling constant of the P-H ($J_{\text{P-H}}=600\text{-}605\text{ Hz}$) that can be seen in the ^1H NMR spectrum as well as in the ^1H -coupled ^{31}P NMR.

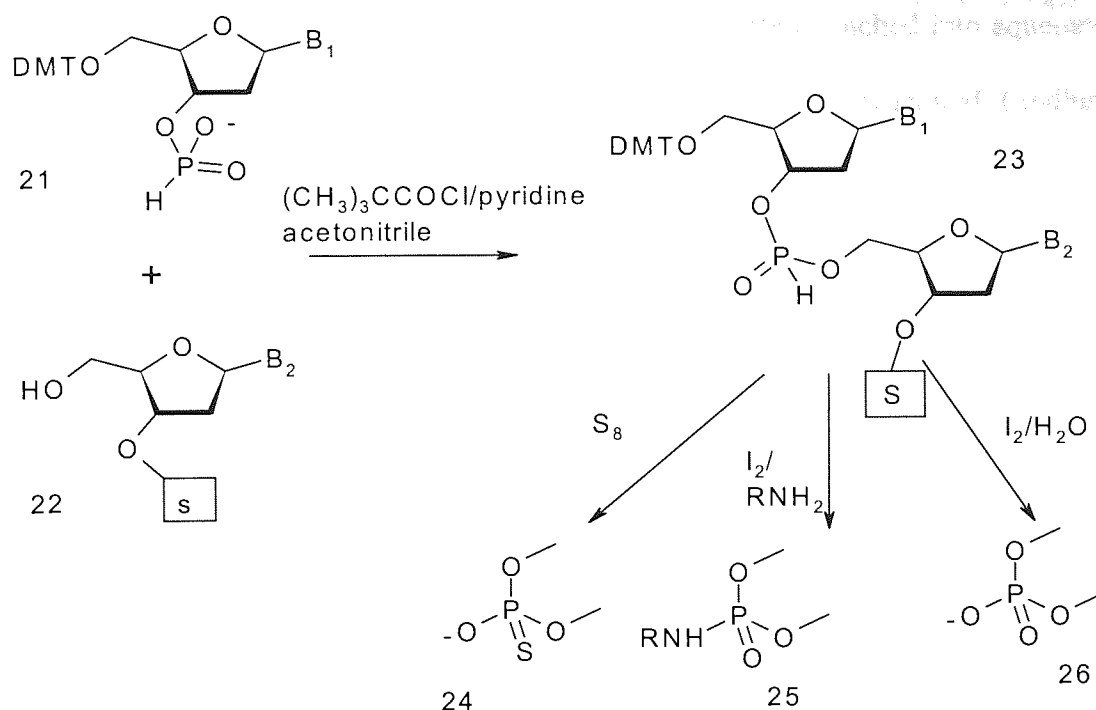


Figure 1-12. H-phosphonate approach

Furthermore nucleoside H-phosphonates are very stable hygroscopic solids that can be stored under Ar at 0 °C for more than 3 years (Froehler *et al.* 1987). It is also very stable in pyridine/ CH_3CN solution. Nucleoside H-phosphonates are resistant to oxidation and are only oxidized by strong oxidants and H-phosphonate monoesters are stable to hydrolysis under acidic and basic conditions (Brown *et al.* 1960). Thus they offer distinct advantages over phosphoramidite reagents for the synthesis of DNA.

Synthesis of oligodeoxynucleotides via H-phosphonates consists of a DMT-deprotection reaction, a coupling reaction and an optional capping step just like using the phosphoramidite method. However, only one single oxidation at the end of the synthesis is used to convert the H-phosphonate linkage to phosphodiester. The other advantage is that excess nucleoside H-phosphonate can be recovered.

This is because, when activated nucleoside H-phosphonate quenched into aqueous TEAB, the starting nucleoside H-phosphonate is therefore generated (Sudhir 1993a).

1.6.6 Phosphoramidite chemistry

It has long been known that the reactivity of P^{III} derivatives is much greater than that of the corresponding P^V . Naturally, one way to decrease coupling times in oligomer synthesis would be to operate at the P^{III} oxidation level. Following by oxidation, P^{III} is converted into P^V .

The first step of phosphoramidite approach involved formation of phosphoramidite monomer. Deoxyribunucleotides were protected with chloro-(*N,N*-dimethylamino) methoxyphosphine in the presence of *N,N*-diisopropylethylamine. The phosphoramidite approach entailed the conversion of the relatively stable deoxyribunucleoside phosphoramidite derivatives to reactive intermediate suitable for the oligonucleotides synthesis (Figure 1-13).

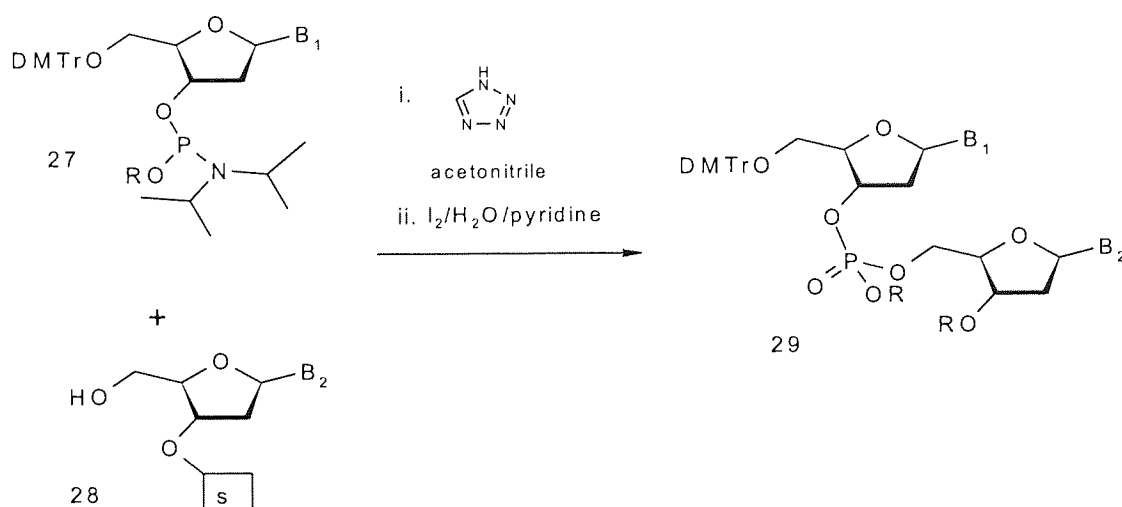


Figure 1-13. Phosphoramidite approach

Of the four methods discussed above, phosphoramidite synthesis is the most popular one. Today almost all synthetic oligonucleotides are prepared by this technique. The first nucleoside at the 3'-terminus is attached by means of a linker arm to a solid support, usually a bead of borosilicate glass (control-pore glasses). In the first step the support-bound nucleoside is deprotected to provide a free 5'-hydroxyl group for the attachment of the second nucleotide. An excess of the second nucleotide, protected at the 5'-hydroxyl position to prevent self-polymerization and activated at the 3'-phosphate position to facilitate condensation, is then added. This results in the formation of a support-bound dimer, which must be oxidized. A capping step renders any remaining mononucleotide inert to further monomer additions. Oligomer is assembled by repeating the whole procedure several times until the desired sequence has been constructed. Finally, the fully assembled oligonucleotide is cleaved from the solid support, deprotected, and purified by HPLC or other methods (Eckstein 1991). As far as the present needs of synthetic oligonucleotides are concerned, this strategy is still a viable method for scale-up process without being too cost prohibitive because of ease of solid-phase synthesis in manipulating all the steps and efficient coupling during chain elongation.

The preparation of deoxyribonucleoside phosphoramidite is facile and occurs in high yields. Moreover, the efficacy of the deoxyribonucleoside phosphoramidites can be adapted by solid-phase synthesis. Compared with other approaches, the phosphoramidite approach requires strictly anhydrous conditions.

The most commonly used activator in coupling step is 1*H*-tetrazole (Kumar *et al.* 1984). The mechanism of activation of phosphoramidite by 1*H*-tetrazole has attracted considerable attention (Berner *et al.* 1989; Froehler *et al.* 1983; Hostomsky *et al.* 1987). It has been argued that the protonation of the phosphoramidite function by 1*H*-tetrazole was rapid and followed by the reversible and slower formation of a phosphorotetrazolide intermediate. The intermediate is then converted to desired product. Among various activators available, 1*H*-tetrazole still remained the most commonly used reagent for the activation of phosphoramidites.

Despite of its easiness, this approach still has some shortcomings. One of them is the chain extension step did not occur in quantitative yields even under optimum conditions. Therefore the acetylation of unphosphorylated chains (capping step) is a necessity in a stepwise manner to terminate the elongation of these truncated oligomers (Farrance *et al.* 1989). Compared with H-phosphonate approach, oxidation after each step of coupling means (a) more time needed for the synthesis (b) large quantity of monomer was consumed because they can't be recovered afterwards.

During the discussions of four different approaches of oligonucleotide synthesis, one method is constantly mentioned—solid phase synthesis. It is the common methods used in phosphoramidite and H-phosphonate approaches. Solid-phase synthesis of oligonucleotides stemmed back more than two decades ago. It was developed from the idea of automated solid-phase synthesis of peptide during 1980s. The principle of solid phase synthesis was developed by Bruce Merrifield at

the Rockefeller Institute in the 1950s and 1960s (Saiki *et al.* 1985). The key to successful peptide synthesis is the anchoring of the first amino acid to an insoluble polymeric support. Other amino acids can then be attached to the previous amino acid, one by one, until the full length of polypeptide is reached. At the end of the sequence, the whole chain can be detached from the insoluble polymer and purified by various means. This invention has greatly accelerated the research on hormones, enzymes and many commercial peptide-based drugs. Indeed, the impact and importance of Merrifield's work was such that he was awarded the Nobel Prize for Chemistry in 1984. Soon after the solid phase method had been shown to be valuable for peptide synthesis, the technique was applied to the synthesis of oligonucleotides (Letsinger *et al.* 1975).

The Figure 1-14 shows all stages of solid phase synthesis of a dimer. Generally, a protected phosphoramidite monomer is attached to a solid-phase support, usually, CPG. The protected monomer is first undergoing a deprotecting procedure by trichloroacetic acid, which removes the trityl protecting group to make the free hydroxyl group available for the attachment of the second nucleotide. An excess of second monomer is then added. Under the catalyst action of tetrazole, phosphoramidite is coupled with the first nucleotide. In theory, not all the monomer reacted with the first nucleotides. Thus, there is 2-3% of the former nucleotides unreacted. Those un-reacted nucleosides must be protected, otherwise, they would keep on reacting with the following phosphoramidite, resulting in n-1, n-2... by-products. Those failure sequences are difficult to remove even by high resolution HPLC technology. However, after treatment with acetic anhydride, those unreacted monomer resulting in ester structure, which prevents further reactions. Now the

dimer is treated with iodine to transfer P^{III} into P^V . The reaction cycle is carried on until the desired sequence is obtained. The oligonucleotides are then treated with conc. ammonia to remove the protecting groups. Once this step has been done, the oligonucleotides must undergo further purification like desalting, HPLC to obtain pure oligonucleotides.

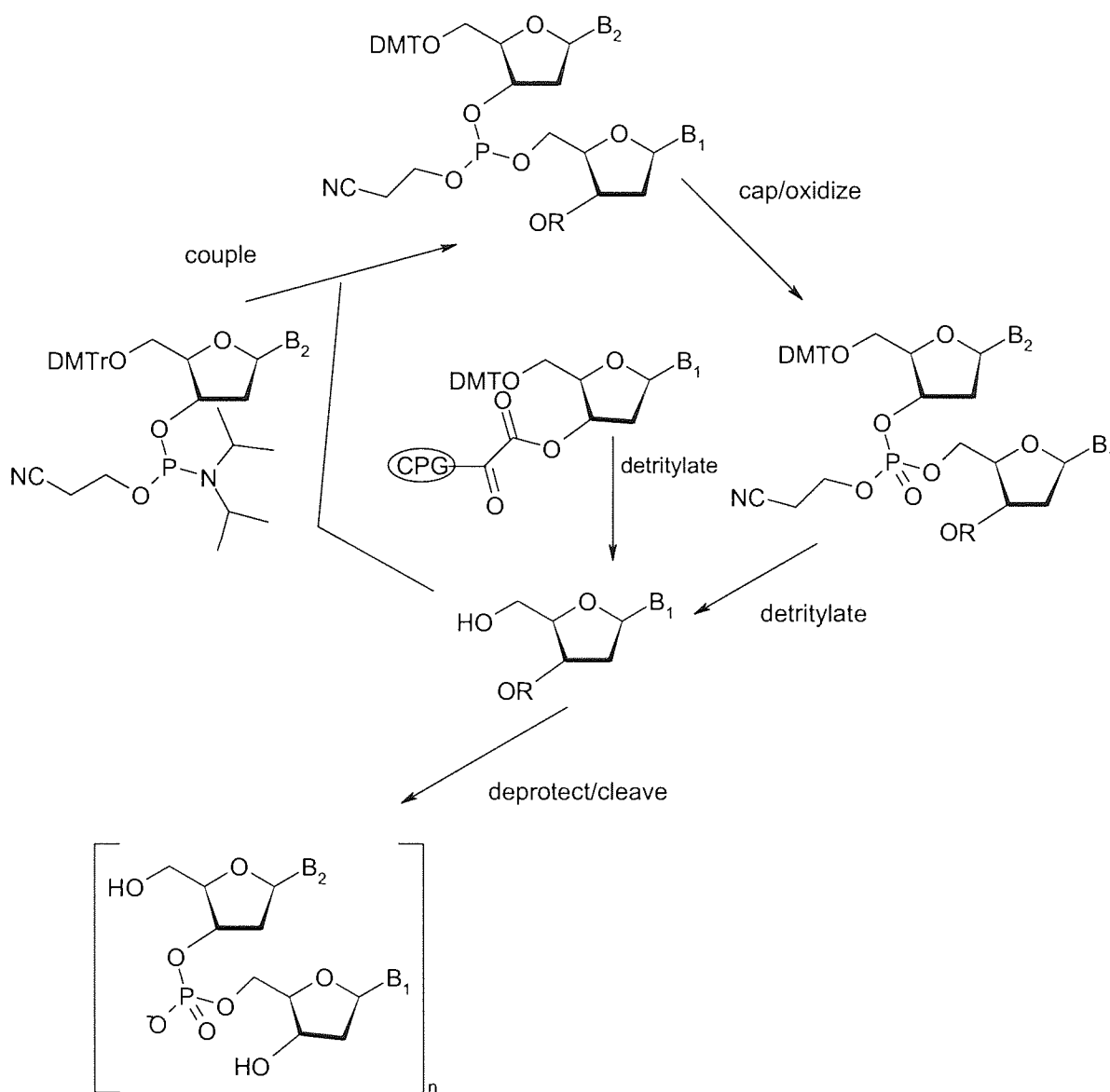


Figure 1-14. Solid phase phosphoramidite approach for oligonucleotides synthesis.

The advantages of solid phase synthesis are listed below:

- Oligonucleotides can be assembled rapidly. When cleavage from the solid support and removal of protecting groups is taken into account, it is possible to prepare a 50-mer residues oligonucleotides within one day.
- As oligonucleotides chain is attached to a solid support, all excess reagents are simply washed away into waster bottle, thus avoiding laborious purification after each monomer addition.
- All chemical reactions can be driven to completion by the use of large excesses of solution reagents relative the polymer-bound oligonucleotides.

1.6.7 Silyl-phosphoramidite method for RNA synthesis

It is necessary to point out that, compared with a 2'-deoxyribonucleoside, the presence of a single extra hydroxyl group at the 2'-position of a ribonucleoside has caused lots of troubles for chemists attempting to assemble oligoribonucleotides chains. For example, whereas machine-aided assembly of oligodeoxyribonucleotides has been established for some years, it was only possible to assemble oligoribonucleotides satisfactorily using mechanized solid phase procedures a decade later. The problem was raised because of the 2'-protecting groups, while it must remain intact throughout all steps of oligoribonucleotide assembly and must be removed specifically at the end of synthesis without leading to chain migration or internucleotidic cleavage. Further problems stem from RNA itself. Since RNA sequences are very sensitive to chemical and enzymatic degradation, all procedures involved in the assembly of RNA chains must respect the delicacy of the assembled chain. The assembly of RNA sequences is also complicated by the presence of a 2'-hydroxyl group in the

monomeric ribonucleosides that form the starting materials for the chemical assembly of RNA chain.

The research of RNA synthesis was started as early as 1968 (Sudhir 1993a). However, completely satisfactory solutions to all those problems mentioned above are not yet available. Nevertheless, it has now become possible to synthesize oligoribonucleotides of moderate length in reasonable yield and purity by machine-aided methods and by use of commercially available reagents.

The most commonly used method for RNA synthesis is the phosphoramidite approach with *tert*-butyldimethylsilyl group protecting the 2'-OH groups. The same procedure as DNA assembly is followed except the coupling time being extended to 600 s instead of 30 s. After the assembly, the oligomers are cleaved from the solid phase support with addition of one step to remove the TBDMS group either with TBAF or triethylamine trihydrofluorides. The later one was found to be more mild than the former one and has been used to deprotect the oligoribonucleotides with delicate modified bases (Adams *et al.* 1994a; Adams *et al.* 1994b; Adams *et al.* 1995a; Adams *et al.* 1995b). Nowadays, synthesis of RNA with normal sequence can be performed in most laboratories.

1.7 Aims of the project

As automated DNA/RNA synthesiser are widely used, one would image taking advantage of a synthesiser to incorporate modified nucleosides into oligonucleotides. To achieve this aim, there are two different strategies. The first is to design a protected modified nucleoside and then use the synthesiser to

incorporate it into an oligomer (method a in Figure 1-15). If a suitable protected nucleoside derivative is available, incorporation of a non-standard residue into an oligomer can be easily achieved by solid-phase synthesis. However this strategy has been proved to be a difficult task even for a skilful chemist because of the diversities and high restriction in synthesis process. Each new modified base requires the synthesis of a modified monomer because these are normally not commercially available and even a minor change in the structure of a modified nucleoside, for example from O^6 -methylguanine to O^6 -ethylguanine, needs multi-step synthesis of a new monomer, followed by synthesis and purification of the new modified DNA/RNA. Preparation of the protected monomer, is not easy and sometimes they can't withstand the conditions of solid-phase synthesis and even a monomer which can survive the assembly procedure may be damaged by post-synthetic procedures, such as deprotection by ammonolysis.

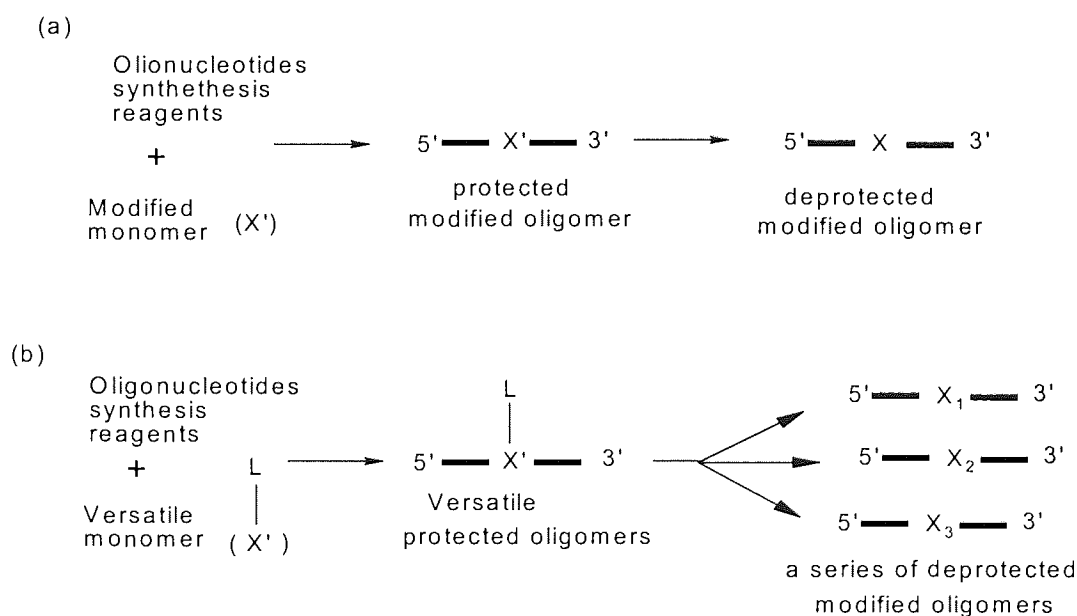


Figure 1-15. Strategies for modified oligonucleotides synthesis.

To overcome these difficulties, a second method emerged, i.e. post-synthetic modification (method b in Figure 1-15). This strategy is to incorporate a versatile monomer into DNA/RNA which combines properties of stability to the normal procedure of oligonucleotide synthesis with sufficient chemical reactivity to allow one to convert it into a number of desirable products after the synthesis of the oligomer. The strategy has at least the following potential advantages: (a) a single synthesis of an oligomer containing the versatile nucleoside could provide a source of oligomers each containing a different modified nucleoside; (b) it offers the possibility of making DNA/RNA containing a labile or chemically reactive nucleoside. To develop this strategy for the synthesis of oligonucleotides containing modified nucleosides using 6-thioguanine as an attachment site is one of the aims of this project.

Sulphur modified oligonucleotides have been very useful tools for studies of carcinogenesis, protein or nucleic acid structures and functions, protein-nucleic acid interactions, and for antisense modulation of gene expression [for latest reviews see (Chambert & Decout 2002; Micklefield 2001; Xu 2000; Luyten & Herdewijn 1998)]. Sulphur containing oligonucleotides are also useful intermediates for the synthesis of other modified oligonucleotides (Zheng *et al.* 2003; Wang & Zheng 2000a; Wang & Zheng 2000b; Xu 1998; Xu *et al.* 1997; Coleman *et al.* 1997; Meyer & Hanna 1996; Coleman & Kesicki 1994). Over the last decade numerous methods have been developed for the synthesis of oligonucleotides bearing a thiol function on the base, and the use of these oligomers for various studies has also been widely reported (Chambert & Decout 2002).

Photochemical cross-linking is a proven method for probing RNA-protein or RNA-RNA interactions at the atomic level as cross-linking can only take place between the molecules that are at the interface of these bio-molecules (Hanna *et al.* 1996; Zheng *et al.* 1997). 4-Thiopyrimidine and 6-thiopurine nucleosides possess several desirable properties for such approach. The sulphur atom is only slightly larger than oxygen, but it otherwise chemically resembles oxygen, hence the introduction of these thionucleosides into oligonucleotides should not appreciably disturb the interaction between these molecules. Furthermore, these nucleosides are photoactive at long wavelength UV light (330-350 nm), which is well away from the usual absorption maxima of proteins (280 nm) and nucleic acids (260 nm), thus cross-linking can be carried out at a wavelength which there is no appreciable detrimental effect on proteins and nucleic acids.

In our efforts to develop the methods for chemical synthesis of oligonucleotides containing thio-bases, we have reported the synthetic incorporation of 4-thiothymine (Xu *et al.* 1992c), 6-mercapopurine (Xu *et al.* 1992b) and 6-thioguanine (Xu *et al.* 1992a) into DNA. Like many other modifications of oligonucleotides, the chemical incorporation of thio-nucleosides has largely been carried out on DNA, mainly because RNA synthesis is more difficult than DNA. Although several methods have been reported for chemical synthesis of oligoribonucleotides containing thio-substituted pyrimidine nucleotides (Kumar & Davis 1997; Adams *et al.* 1994a, 1994b; Murray *et al.* 1994; McGregor *et al.* 1996; Becker *et al.* 1998; Kumar & Davis 1997), methods for thio-substituted purine nucleosides are limited. For example, to our knowledge there has been one report detailing the chemical incorporation of 6-thioguanosine into oligoribonucleotides,

using a cyanoethyl group for the protection of the thiocarbonyl function (Adams *et al.* 1995a). The synthesis of the phosphoramidite involved eight synthetic procedures starting with 2'-deoxyguanosine. Although the authors stated that the composition of the modified oligomers was confirmed by base composition analysis, HPLC chromatogram confirming the identity of the oligomers was not provided. In that method, the cyanoethyl group was used to protect sulphur function. The problem with the cyanoethyl protecting group is that it requires tedious washing with acetonitrile/methanol to remove the deprotection reagent DBU. Even tiny amounts of DBU left in the solution could cause the RNA degradation in the later stage. Second problem is that using DBU as the deprotecting reagent, one cannot step-wisely remove cyanoethyl group without affecting the other parts of the oligomers. Because the phosphate is also protected with cyanoethyl group. Thus it restricts the application of the method. More recently, enzymatic ligation was applied to incorporate 6-thioguanosine into oligoribonucleotides using its 3',5'-bisphosphate derivative (Kadokura *et al.* 2000). To develop an alternative chemical synthetic method for oligoribonucleotides containing 6-thioguanosine, and time permitting, to preliminarily evaluate the thionucleoside as a "peg site" for introduction of modifications into the oligomers is the second aim of this project.

Chapter 2 Post-synthetic modification of oligonucleotides containing 6-thioguanine

2.1 Introduction

As mentioned in the introduction, the availability of base-modified oligodeoxynucleotides has greatly stimulated research in many areas including carcinogenesis, DNA repair and DNA-protein interactions (English *et al.* 1991; Beaucage & Iyer 1993). It is widely accepted that post-synthetic modification is an easy way to synthesize base-modified oligodeoxynucleotides. This method has been investigated by many groups (Xu *et al.* 1992a; Xu *et al.* 1992b; Xu *et al.* 1992c; MacMillan *et al.* 1991; Ferentz *et al.* 1991; Ferentz *et al.* 1992).

Xu *et al.* (1992a) reported that variety of modified oligomers, including those containing 6-thioguanine, could be easily obtained after a single synthesis of a versatile oligonucleotide. 6-Thio-2'-deoxyguanosine was synthesised in three steps from 2'-deoxyguanosine. The protected 6-thioguanine nucleoside was then converted to phosphoramidite and incorporated into DNA. Due to the attachment of 2,4-dinitrophenyl group, thiocarbonyl group became a good leaving group. Thus in the post-synthetic modification, it can be fully replaced by various groups (amino, methylamino, methoxyl, oxo).

Other thio-substituted nucleosides have also been investigated for the post-synthetic modification. Coleman *et al.* (1997) examined various modified nucleic acids as platforms upon which tethered chemically reactive functionalities. Among those 6-thio-2'-deoxyinosine has been proved to be successful. 6-Thio-2'-

deoxyinosine was first synthesised from 2'-deoxyinosine. The conversion of an amide carbonyl group to the corresponding thiocarbonyl has historically used P_2O_5 (Carrington 1944; Elion *et al.* 1955). While in that project, a different method was adopted by converting 2'-deoxyinosine to its 6-pyridyl derivative and then into corresponding 6-thio-2'-deoxyinosine. The thiocarbonyl group was protected with cyanoethyl group and the nucleoside was converted into phosphoramidite monomer and incorporated into an 8-mer. The oligodeoxynucleotides containing 6-thio-2'-deoxyinosine was then subjected to two electrophilic reagents (iodomethane, *N*-phenyl-bromoacetamide). These reactions produced oligomers containing S^6 -alkylated-6-thio-2'-deoxyinosine. Duplex stability studies showed no significant decrease in T_m values of the oligodeoxynucleotides containing the modified bases. The results were coherent with the former findings that single mismatches do not seriously destabilize duplex structure (Ke & Wartell 1996).

The first part of my project was to utilize 6-thio-2'-deoxyguanosine as a post-synthetic peg site to introduce various groups into oligonucleotides. Thioguanine is a drug used for the treatment of acute leukemia and as an immunosuppressive agent (Elion 1989). It produces a delayed cytotoxicity which is believed to be associated with thioguanine incorporated into DNA followed by chemical methylation by *S*-adenoxylmethionine to form S^6 -methylthioguanine (Swann *et al.* 1996). Furthermore, thioguanine incorporated in DNA also causes chromosome damage (Tidd & Paterson 1974). Although its mechanisms are not understood a route via sister chromatid exchange could be possible as thioguanine is an exceptionally good target for alkylating agents of S_N2 type. It has been demonstrated that the highest incidence of sister chromated exchange is achieved with bifunctional

alkylating agents (Latt 1981). Therefore studies on reaction of thioguanine in oligonucleotides with electrophilic reagents would be of interest. Furthermore the introduced group, particularly the bifunctional groups, could be used for chemical and enzymatic modification to give new tools for biological studies, such as DNA repair, sister chromatid exchange induction, and for diagnostic applications.

In this study we have developed an efficient and straightforward method for preparation of DNA containing various groups by one-step introduction of the individual groups onto the thio-function of thioguanine within synthesized and fully purified oligodeoxynucleotides. While this project was ongoing, we noticed a report using thioguanine as an attachment site for the introduction of biologically useful functional groups (Xu 1998).

2.2 Results and discussion

2.2.1 Automatic assembly of DNA

The dG^{SH} phosphoramidite, purchased from *Glen Research* and protected with a cyanoethyl group at the 6-thio function, was incorporated into a 12-mer oligodeoxynucleotide by *Beckmann Oligo 1000* DNA synthesizer using standard procedures recommended by the manufacturer. The sequences of parent, complementary and oligomers containing dG^{SH} are shown in Figure 2-1.

Parent oligomer (P-1)

5'-C-A-T, C-A-G, T-G-A, G-C-T-3'

Oligomer containing G^{SH} (G-1)

5'-C-A-T, C-A-^{thio}G, T-G-A, G-C-T-3'

Oligomer with complementary sequence (C-1)

5'-A-G-C, T-C-A, C-T-G, A-T-G-3'

Figure 2-1. The sequence of the synthesised oligonucleotides.

All phosphoramidite monomers were dissolved by anhydrous acetonitrile just before the synthesis and used up within one week. During the synthesis, the trityl group was removed from 5'-position and the process was monitored by UV measurement of the released DMT group at 495 nm to make sure the initiation and continuation of the coupling. One coupling cycle usually takes 6-9 min. Synthesis of a 12-mer oligomer therefore took about 2 h. The column was dried under vacuum afterwards and the solid support with oligomers attached was carefully transferred to Eppendorf tubes and ready for deprotection.

Ideally, the coupling time for the modified sequence should be longer than those for the normal sequences. Because of the introduction of thiocarbonyl and its protecting group, the coupling efficiency would also be lower than normal sequences. However, changing program with Beckmann 1000 synthesizer requires help from specialists. No change was made to the synthesis program. Modified nucleoside phosphoramidite was subjected to coupling for 60 s. The deprotection and measurement carried out afterwards showed: on 1 μ mol scale, normal sequence yielded about 35-40 O.D. DNA, while for modified sequence, the yield was about 25-30 O.D. These results proved that the coupling time could affect yield

of DNA containing *S*-cyanoethyl-6-thio-2'-deoxyguanosine phosphoramidite but it was not significant and could be ignored.

2.2.2 Deprotection & purification

The support-linked oligonucleotide containing 6-thioguanine was first treated with 1.0 M 1,8- diazabicyclo-[5,4,0]-undec-7-ene (DBU) in acetonitrile to remove the *S*- and *O*-cyanoethyl groups prior to its cleavage from the solid support. The mechanism was thought of attack by DBU to hydrogen at α carbon on the cyanoethyl group (Figure 2-2). Because DBU is a base, it attracted a proton from the α -carbon of the cyanoethyl group leaving a lone pair electrons. The shift of these electrons resulted in the broken of *S-O* bond which led to the formation of thiocarbonyl group. This step of deprotection took 4 hours at room temperature. No change of the amount of the oligomer was observed after leaving the oligomer in DBU/acetonitrile a bit longer. The results suggested that 4 h was enough for deprotection of cyanoethyl group.

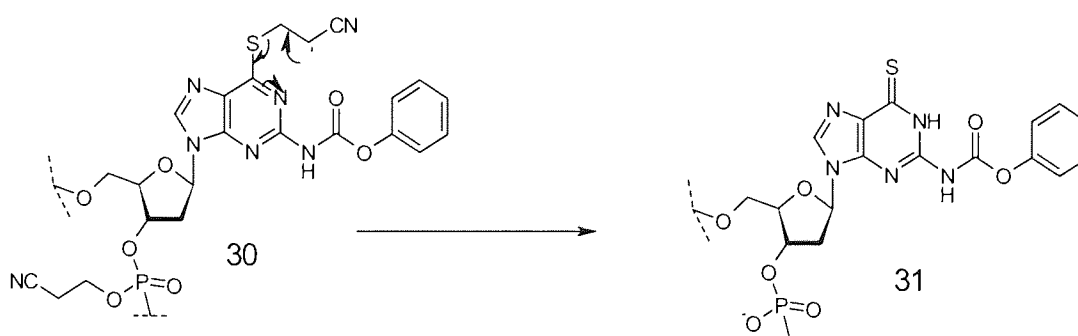


Figure 2-2. Formation of thiocarbonyl group

The treatment with DBU removed the cyanoethyl group on the thiol ester, while the oligomer were still attached to the solid support. This gave us an advantage to wash away DBU physically without involving laborious purification work. DBU

needs to be carefully removed, as any DBU remaining in the column could cause the degradation of thiocarbonyl group when treated with ammonia (Adams *et al.* 1995a).

This was followed by NaSH/ammonium to remove other protecting groups and release the oligomer from CPG support. NaSH was included in order to minimize the hydrolysis of thiol group. This strategy has been previously employed for 6-thioguanosine concomitant with S-cyanoethyl deprotection by Christopherson & Broom (1991). For the unmodified oligomers, a single-step deprotection was needed: treatment of oligomers with conc. ammonia at 55 °C for 4 hours.

Up to this stage, the DMT group was still attached to the 5'-end of the oligomer, as the purification with NENSORBTM column requires the oligomer with the dimethyltrityl group on. NENSORBTM column is a reverse phase column. DNA oligomers with DMT group have a high lipophilic affinity, while other failure sequences and most of by-products do not have such character. Most of failure sequence and by-products were washed away by acetonitrile/0.1 M TEAA [1:9 (v:v), 10 ml]. TFA (0.5%) was then added to the column to cleave the DMT group from 5'- end of oligomers. The column was then washed by 0.1M TEAA to remove the acid (TFA). Oligomers was finally retrieved by 35% MeOH. During the whole experiment, 0.1 M TEAA was used to maintain the ion strength which was crucial for the polar strength that keep the oligomer on the cartridge. The methanol eluate was collected into eppendorf tubes (1ml/tube) and each fraction was diluted by 20 times and subjected to UV measurement at 260 nm. A typical result is showing

below (Figure 2-3). DNA was mainly in the fractions 4 and 5. These fractions were pooled together and dried with Speed-Vac and ready for the HPLC test.

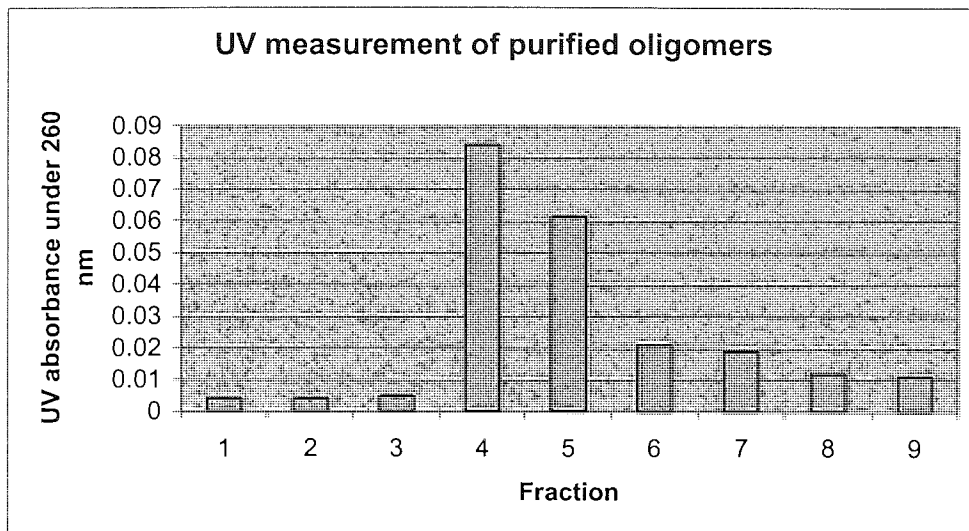


Figure 2-3. UV absorbance of fractions of NEN-SORB column purified oligomers

An UV spectrometry was also carried out to identify the existence of DNA containing dG^{SH}. The result is shown in Figure 2-4. Comparing the UV spectrum of the unmodified oligomer (Figure 2-4, dotted line) with the dG^{SH} containing oligomer, one can see a small peak at the wavelength of 345 nm. This is due to the presence of the -SH group.

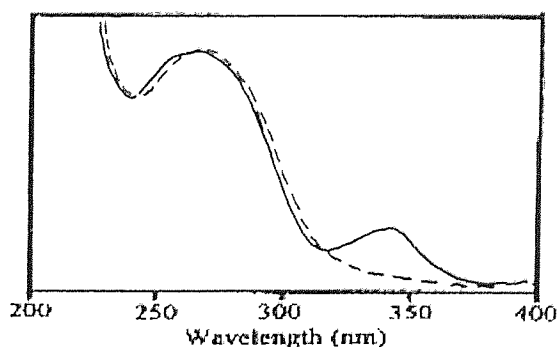


Figure 2-4. UV spectrum of the oligomer containing dG^{SH} (G-1) (solid line) and that of the parent oligomer (P-1) (dotted line).

2.2.3 HPLC Purification

HPLC technique can provide adequate separation for biopolymers of both proteins and nucleic acids. Usually gradient elution can provide high-resolution separation. Gradient elution can be set as % of buffer B at a particular time. In the present program, it was found that the gradient elution from 0%-30% buffer B over 20 min gave satisfactory resolution in separation. Depending on the properties of oligomers, the gradient might be set a bit lower to separate close shoulder peaks.

The detection system used in the present project was UV detection. One advantage of ultraviolet detector is its simplicity of the design. All required is to connect the column output to the flow cell of the detector and the output from the detector to a fraction collector or to waste. In the present experiments, the oligonucleotides have a strong absorbance peak at around 260 nm. The other advantage is that the wavelength of UV detector can be easily changed to fit for the further requirement. For example, in our experiment, 340 nm was also chosen to specifically detect the existence of thiocarbonyl group, while 324 nm was chosen for substituted thiol ether group.

In deciding which column to use, there are several factors that should be considered. These are (a) the composition of the solid support, (b) size and shape of the particles, (c) pore size, (d) bonded phase, and (e) column length. Another factor is the column diameter. The vast majority of RP-HPLC supports are fully porous micro-particulate silica. The silica-based particles provide excellent rigidity under pressure. Lower pH buffers generally provide the best results. Due to the instability alkaline conditions the use of buffers above pH 8 results in great decrease of the column life. The pH value chosen in the current project was pH 7.0. Most HPLC

columns available are packed with 3- to 10- μ m diameter particles. The next variable to consider is the hydrocarbon coated on the silica particles. The alkyl straight chain columns (C_8 or C_{18}) are commonly used.

Although NENSORB column gives a quick way to purify oligomers, HPLC technique was used to further purify the oligomer to obtain high purity DNA. An HPLC chromatogram of an HPLC purified oligomer is shown in Figure 2-5

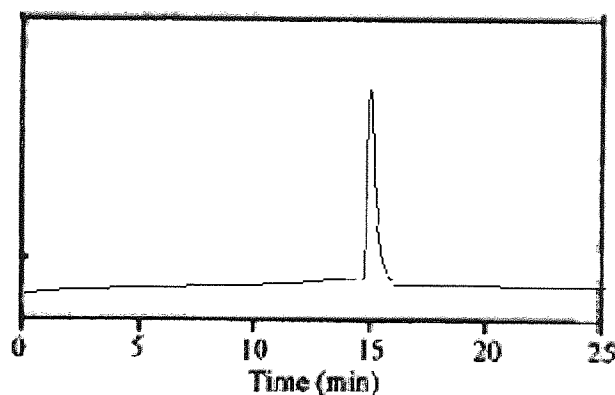


Figure 2-5. HPLC profile of DNA after HPLC purification

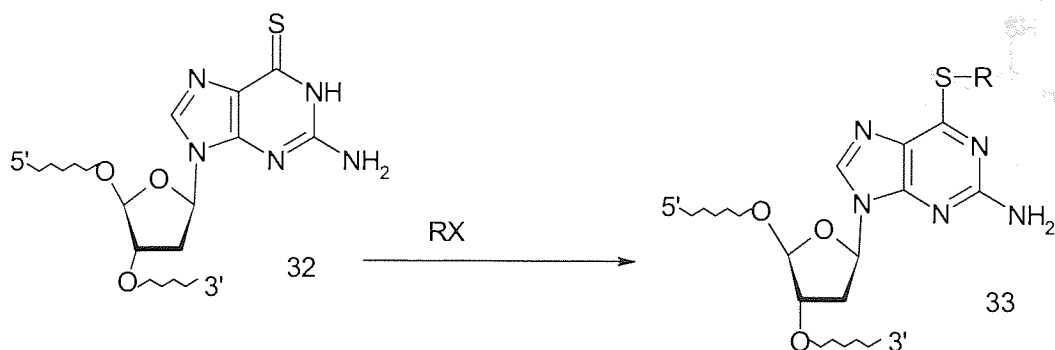
The HPLC program was specially designed for the current oligodeoxynucleotides. The two buffers used in the present experiments were buffer A and buffer B as seen the experiment section. Thus adjustment of the composition of buffer A and buffer B can change the lipophilicity of the mobile phase, resulting the compounds being eluted according to their polarities. From 0 min to 20 min, the composition of buffer B was increased so that the elution power was increased. This is so called gradient elution. The order of elution is dependent on the order of polarity or lipophilicity of the compounds being analysed. This character is extremely useful when electrophilic substitution was carried out later. Changing the mobile-phase

ionic strength has little effect on the retention behaviour of nucleosides and bases. This was confirmed by the results obtained during the project.

2.2.4 Post-synthetic substitution of DNA containing G^{SH}

One of the aims of this project was to modify the dG^{SH} oligonucleotides post-synthetically at the thiocarbonyl group of the 6-thioguanine in a chemoselective fashion. The thiocarbonyl group, which was introduced into oligomers, is an electron rich group so that it can attract electrophilic reagents such as alkyl halides. Various halides were tested (Figure 2-6).

Different reagents needed different reaction times. Generally, benzyl halides reacted easily with the thiocarbonyl group. Most of such reactions were completed in 5 min. Other alkyl halides were less reactive. For example, iodomethane took 60 min to fulfil the reaction while iodoethane took approximately 24 h. When a reagent turned from 1° carbon to 2° carbon, extended reaction time was needed. For example, only two third of starting oligomers had been converted to substituted one after 36 h when 2-iodopropane was used.



X= Br OR I

R= $-\text{CH}_2\text{CH}_3$, $-\text{CH}(\text{CH}_3)_2$, $-\text{CH}_2\text{CN}$, $-\text{CH}_2\text{COOH}$
 (a) (b) (c) (d)

$-\text{CH}_2\text{COOCH}_3$, $-\text{CH}_2\text{CONH}_2$
 (e) (f)

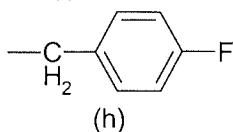
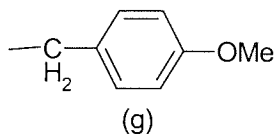


Figure 2-6. Alkylation of the oligomer (G-1) with various alkylating agents

These reactions were usually carried out in the phosphate buffer (pH 7.5 - 9.0). The optimal range is pH 8.0 - 8.5. Basic reaction condition was adopted because thiocarbonyl group can undergo a keto-enol isomerisation. In a basic condition, the enol tautomer is more favoured (Figure 2-7 compound 35). The enol tautomer can be converted to thiol anion form (Figure 2-7 compound 36), which is a strong nucleophilic reagent so that $\text{S}_{\text{N}}2$ nucleophilic substitution can occur at the carbon of an alkyl halide.

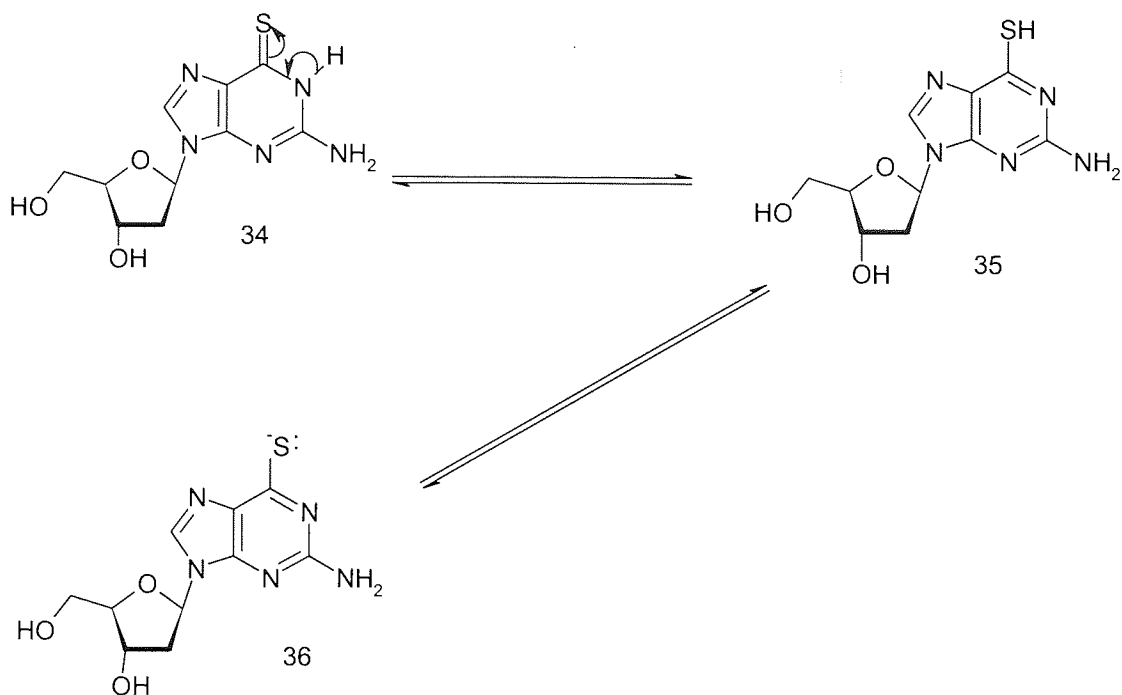


Figure 2-7. Keto-enol isomerisation of thioguanosine

The reaction was followed by HPLC as shown in Figure 2-8, 2-9, 2-10 for the reaction with iodoethane. Two major peaks were seen in the Figure 2-8: the first one (Rt: 15.19 min) was the unalkylated oligomer with a free thiol group, the second major peak (Rt: 22.34 min) was the alkylated oligomer. Its longer retention time (comparing with the unalkylated one) in RP-HPLC was due to the presence of ethyl group. During the course of the reaction, more and more DNA was converted into substituted DNA. This was reflected in the Figure 2-9: peak 1 decreased while peak 2 increased. The reaction was finished in 2 hrs, where one can see the peak corresponding to the oligomer containing dG^{SH} (G-1) disappeared (Figure 2-10).

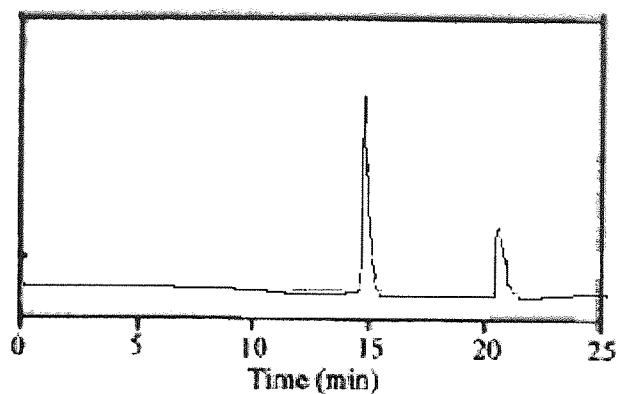


Figure 2-8. HPLC trace of conversion of the oligomer containing dG^{SH} (G-1) to the one containing S⁶-ethylguanine after 5 min. The first peak represents original DNA, the second peak represents substituted DNA which is more hydrophobic and has a longer retention time.

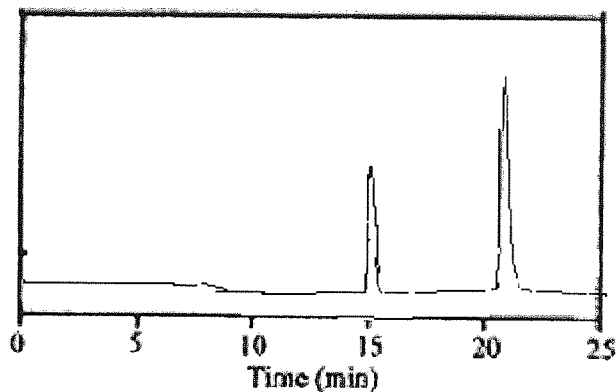


Figure 2-9. HPLC trace of the same reaction as Figure 2-8 after 50 min

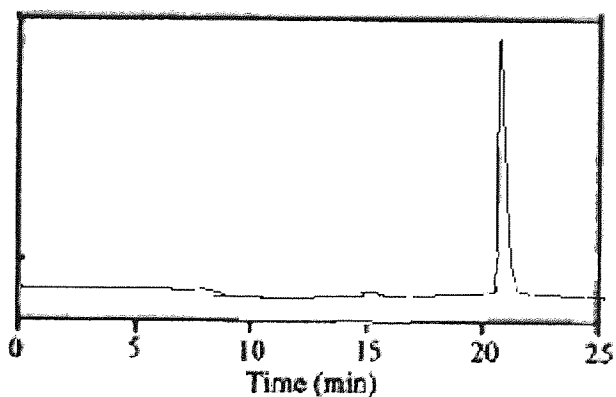


Figure 2-10 HPLC trace of the same reaction as Figure 2-8 after 2 hrs

The reaction was also confirmed by UV spectrometry. Thiocarbonyl group gave a peak around 340 nm, while sulfide only showed a shoulder peak around 320 nm. Thus two peaks corresponding unsubstituted and substituted DNA were collected

and subjected to UV spectrometry. The results are shown in Figure 2-11. Solid line represents unsubstituted DNA with clear peak around 340 nm. Dotted line represents substituted DNA, where peak around 340 nm was replaced by a shoulder peak around 320nm.

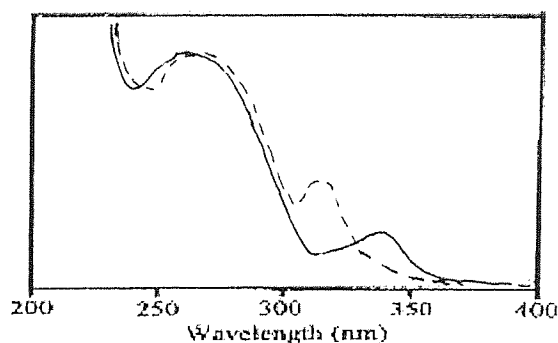


Figure 2-11. UV spectra of unsubstituted DNA (solid line) and substituted DNA

Reactions with iodoethane, iodobutane, 2-iodopropane usually took 24-48 hrs. Iodoethane reacted with DNA at room temperature and was completed within 48 hrs. The same results were observed on iodobutane and 2-iodopropane.

Another type of halide used in this work was benzyl halides (Figure 2-6). Those benzyl halides were more reactive than their aliphatic counter-parts. Most reactions completed within 5 min at room temperature. Several bi-functional reagents (Figure 2-6) were also tested on the oligomers. The produced oligomers can be very useful because once a bi-functional reagent has been attached to the oligomers further reaction could be carried out with these functional groups. All the results were confirmed by HPLC and UV spectrometry and the results were similar to those in Figure 2-8, 2-9, 2-10, 2-11.

2.2.5 Nucleoside analysis of electrophilic substitution

The identity of the modified oligomers was further confirmed by base analysis, where DNA was subjected to enzymatic condition to decompose to nucleosides. The digest was at two wavelengths 260 nm and 320 nm. The results of nucleoside analysis of DNA containing 6-ethylthio-2'-deoxyguanosine is shown in Figure 2-12, which prove that ethyl group has been attached to thiocarbonyl group.

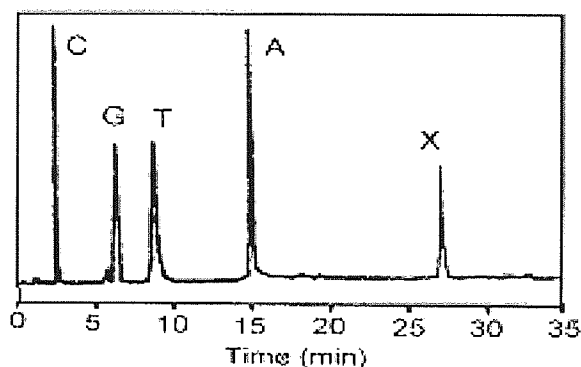


Figure 2-12 Nucleoside analysis, substituted DNA (X standing for 6-ethylthio-2'-deoxyguanosine)

2.3 Conclusion

6-Thio-2'-deoxyguanosine was incorporated into a 12-mer oligomer via conventional methods. The deprotection of DNA was carried out in mild conditions at room temperature and NENSORB column was proved to be efficient in purification of crude DNA. HPLC analysis showed that DNA after NENSORB column purification could be applied directly to application with no need of further refinement.

Thiocarbonyl group on the position 6 of guanine is an active reacting site. Various electrophiles i.e. alkyl halides can be attached to that position. In the present project, both aliphatic and aromatic halides were tested on the thiocarbonyl group and the reaction course was monitored by HPLC. DNA was converted into alkylated DNA

with change of the retention time in the HPLC trace because of the extra attachment of hydrophobic groups. Generally speaking, aromatic halides are much more active than their aliphatic counter-parts. The HPLC trace showed that the attachment of aromatic groups i.e. benzyl bromide could be completed within 5 min, while reaction of modified DNA with ethyl iodide was longer than 48 h. The eluate was collected and subject to UV spectrophotometry. The UV spectrum showed that the peak around 340 nm was replaced by a new peak around 320 nm. Peak around 340 nm is the identity peak for thiocarbonyl group while peak around 320 nm is due to the existence of the sulphide functional group. Thus the UV results further proved that thiocarbonyl group was attached by alkyl groups. This post-synthetic modification on DNA opens a new way to introduce various functional groups into DNA.

Chapter 3 Synthesis of oligoribonucleotides containing 6-thioguanine and preliminary investigation of their post-synthetic modification

3.1 Introduction

Sulphur modified oligodeoxynucleotides have been very useful tools for studies of carcinogenesis, protein or nucleic acid structures and functions, protein-nucleic acid interactions, and for antisense modulation of gene expression [(for latest reviews see (Chambert & Decout 2002; Micklefield 2001; Xu 2000; Luyten & Herdewijn 1998)]. Sulphur containing oligonucleotides are also useful intermediates for the synthesis of other modified oligonucleotides (Zheng *et al.* 2003; Wang & Zheng 2000a; Xu 1998; Xu *et al.* 1997; Coleman *et al.* 1997; Meyer & Hanna 1996; Coleman & Kesicki 1994; Fidanza & McLaughlin 1992). Over the last decade numerous methods have been developed for the synthesis of oligonucleotides bearing a thiol function on the base and the use of these oligomers for various studies has also been widely reported (Chambert & Decout 2002).

One successful example has been the synthesis and study of oligodeoxynucleotides containing 6-thioguanine or 6-thiomethylguanine. Both 6-thioguanine and mercaptopurine are presently among the most widely used anti-leukemia agents (Elion 1989), and azothioprine, a pro-drug which is converted *in vivo* to mercaptopurine, is a useful immunosuppressant for transplant surgery. It is thought that these thiopurines exert their cytotoxic, therapeutic effects by being incorporated into DNA as deoxy-6-thioguanosine. Methods have been developed for the synthesis of DNA containing 6-thioguanine (Clivio *et al.* 1993; Rao *et al.*

1995; Christopherson & Broom 1991; Rappaport 1988; Rao *et al.* 1992; Nikiforov & Connolly 1992; Waters *et al.* 1992; Xu *et al.* 1992a; Kadokura *et al.* 2000) or 6-methylthioguanine (Xu *et al.* 1995; Xu 1998; Wang & Zheng 2000b; Zheng *et al.* 2003). The study of 6-methylthioguanine containing oligonucleotides revealed that the cytotoxicity of these agents depends upon *S*-methylation of the thiobase by *S*-adenosylmethionine after incorporation into DNA (Swann *et al.* 1996; Waters & Swann 1997). Similarly a recent study of the solution structure of thioguanine-modified DNA duplex provided new mechanistic insight into the effects of thioguanine incorporation into DNA at the level of DNA structure and dynamics, and provided explanations for the effects of thioguanine incorporation on the activity of DNA-processing enzymes (Somerville *et al.* 2003).

Photochemical cross-linking can be used to probe RNA-protein or RNA-RNA interactions. This technique can be applied at the atomic level as cross-linking only take place between the molecules that are close to each other (Hanna 1996; Zheng *et al.* 1997). During research to find the proper compounds, 4-thiopyrimidine and 6-thiopurine nucleosides came into light. Because the sulphur atom, which was introduced into the molecular contains some special characters. It is only slightly larger than oxygen, but its other chemical properties resembles oxygen very much. Incorporation of these thionucleosides into oligonucleotides would not significantly alter the interaction between these molecules. On the contrary, these nucleosides are photoactive at long wavelength UV light (330-350 nm), which is far from the usual absorption maxima of proteins (280 nm) and nucleic acids (260 nm). Thus cross-linking carried out at such wavelength would have almost no detrimental effect on proteins and nucleic acids. Finally, the contact of cross-linking only occurs between the photoactivable thiocarbonyl functions so that the photo-cross-

linking approach using oligoribonucleotides bearing these thiobases has made it possible to probe the structure and mechanism of hammerhead ribozymes (Wang & Ruffner 1997) and to build the plausible three-dimensional models of biomolecules such as U snRNA (Yu & Stertz 1997), rRNA (Baravov *et al.* 1998) and hammerhead ribozymes (Laugaa *et al.* 1995). In addition, it has also been reported that these photo-cross-linkable oligonucleotides are very useful probes for *in situ* hybridization assays (Huan *et al.* 2000) and for investigation of RNA-protein interactions (McGregor *et al.* 1996). The use of these oligomers for studying the mechanism of hammerhead ribozyme cleavage has also been documented (Adams *et al.* 1995a).

In the efforts to develop the methods for chemical synthesis of oligonucleotides containing thiobases, some progress has been reported like, 4-thiothymine (Xu *et al.* 1992c), 6-mercapopurine (Xu *et al.* 1992b) and 6-thioguanine (Xu *et al.* 1992a) into DNA, and in subsequent postsynthetic modifications of the non-natural bases, reactive functionality was pegged in a site-specific, chemoselective manner via the thiol function by *S*-alkylation without effecting other bases within the DNA strand (Xu *et al.* 1995; Xu *et al.* 1997; Zheng *et al.* 2003). This strategy makes it possible to incorporate reactive functionality that would otherwise be incompatible with conditions of solid-phase synthesis.

As other modification of oligonucleotides, the chemical synthesis of oligonucleotides containing thio group has been largely carried out in DNA mainly because RNA synthesis is more difficult than DNA. However methods have been reported for chemical synthesis of oligoribonucleotides containing 2-thiouridine (Kumar & Davis 1997), 4-thiouridine (Adams *et al.* 1994b; Murray *et al.* 1994;

McGregor *et al.* 1996; Becker *et al.* 1998; Kumar & Davis 1997), 6-thioinosine (Adams *et al.* 1995b), and 6-thioguanosine (Adams *et al.* 1995a). More recently, 3',5'-bisphosphates of 6-thioguanosine, 6-thioinosine, and 4-thiouridine were synthesised by improved methods and were enzymatically incorporated into RNA (Kadokura *et al.* 2000). In this chapter the synthesis of thioguanosine phosphoramidite (42), using 2,4-dinitrophenyl as the masking group for the thiol function, and its incorporation into oligoribonucleotides of mixed-base composition are described, together with the initial results of chemoselective postsynthetic modification of the thiocarbonyl group of 6-thioguanosine residue. The methods and results detailed in this report clearly demonstrate the ease of the synthesis of 6-thioguanosine-modified oligoribonucleotides, and the effectiveness of the thiocarbonyl group as a peg site for site-specific post-synthetic modifications.

Although the success of incorporation of 6-thioguanosine into RNA has been reported by Adams *et al.* (1995a). This strategy was not totally satisfactory for two reasons. First problem was that cyanoethyl group was used to protect sulphur group. The problem with cyanoethyl group is that it requires tedious washing with acetonitrile/methanol to remove DBU which was the first deprotection reagent. Even a trace of DBU left in the solution could cause the RNA degradation in the final step. Second problem was that with cyanoethyl protection one cannot remove step-wise the thiol-protecting group without affecting the other parts of the oligomers, because phosphoramidite was also protected by cyanoethyl group. Thus, after DBU deprotection, oligomers could not be put back into synthesiser to extend its length in order to introduce multiple modified nucleosides. That restricts the application of the method. Unlike cyanoethyl group, DNP (2,4-dinitrophenyl)

group can be removed specifically by 2-mercaptoethanol. The deprotecting condition is much milder and specific to the DNP group. After removed of the DNP from the thiol group, other parts of oligonucleotides remained intact. This provides the possibility to introduce of structural diversity into oligonucleotides at multiple-site via on-column conjugation (Zheng *et al.* 2003). A paper reporting the work described in this chapter has now been accepted by Bioorganic & Medical Chemistry Letters.

3.2 Results & discussion

3.2.1 Synthesis of 6-thioguanosine phosphoramidite (42)

6-Thioguanosine (37) is commercially available from Aldrich (UK). It was converted into a protected phosphoramidite monomer (42) as shown in Figure 3-2. 6-Thioguanosine has 5 reactive groups which need to be protected before automatic assembly. These are the thiol group at position 6, the amine group at position 2, the three hydroxyl groups at positions 2', 3', 5' of the sugar ring (Figure 3-1).

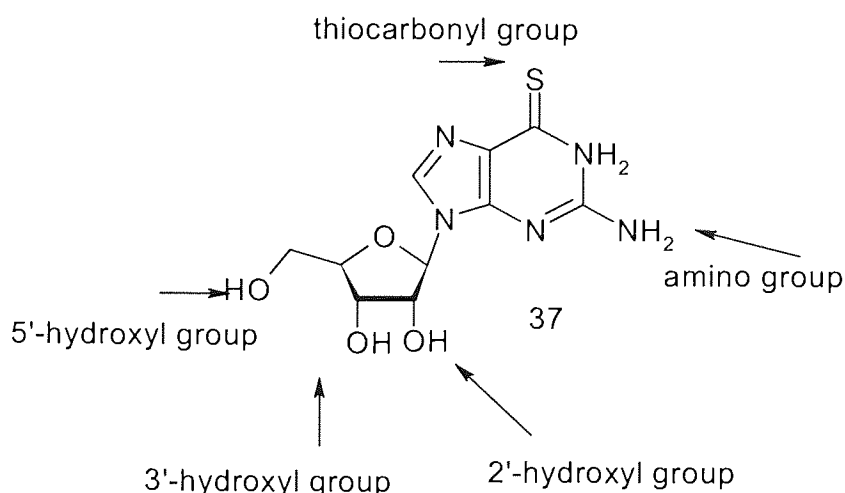


Figure 3-1. Protection of thioguanosine

The whole synthetic procedure is shown below (Figure 3-2). 6-Thioguanosine was first reacted with 2,4-dinitrofluorobenzene. The resulting compound was then reacted with trimethylsilyl chloride to transiently protect the hydroxyl groups. The amino group on position 2 of the base was not reactive under that reaction condition. Phenoxyacetyl chloride was then used to protect the amino group, followed by ammonia deprotection of trimethylsilyl group on the hydroxyl groups. The three hydroxyl groups were then protected, following the order 5', 2', 3'. MMT or DMT group was used to protect 5'-hydroxyl group. The resulting compound (40) was then reacted with TBDMS-Cl (*tert*-butyldimethylsilyl chloride). The mixture was then separated by silica gel and the isolated 2'-TBDMS-protected-isomer was then phosphitylated leading to the desired phosphoramidite monomer (42).

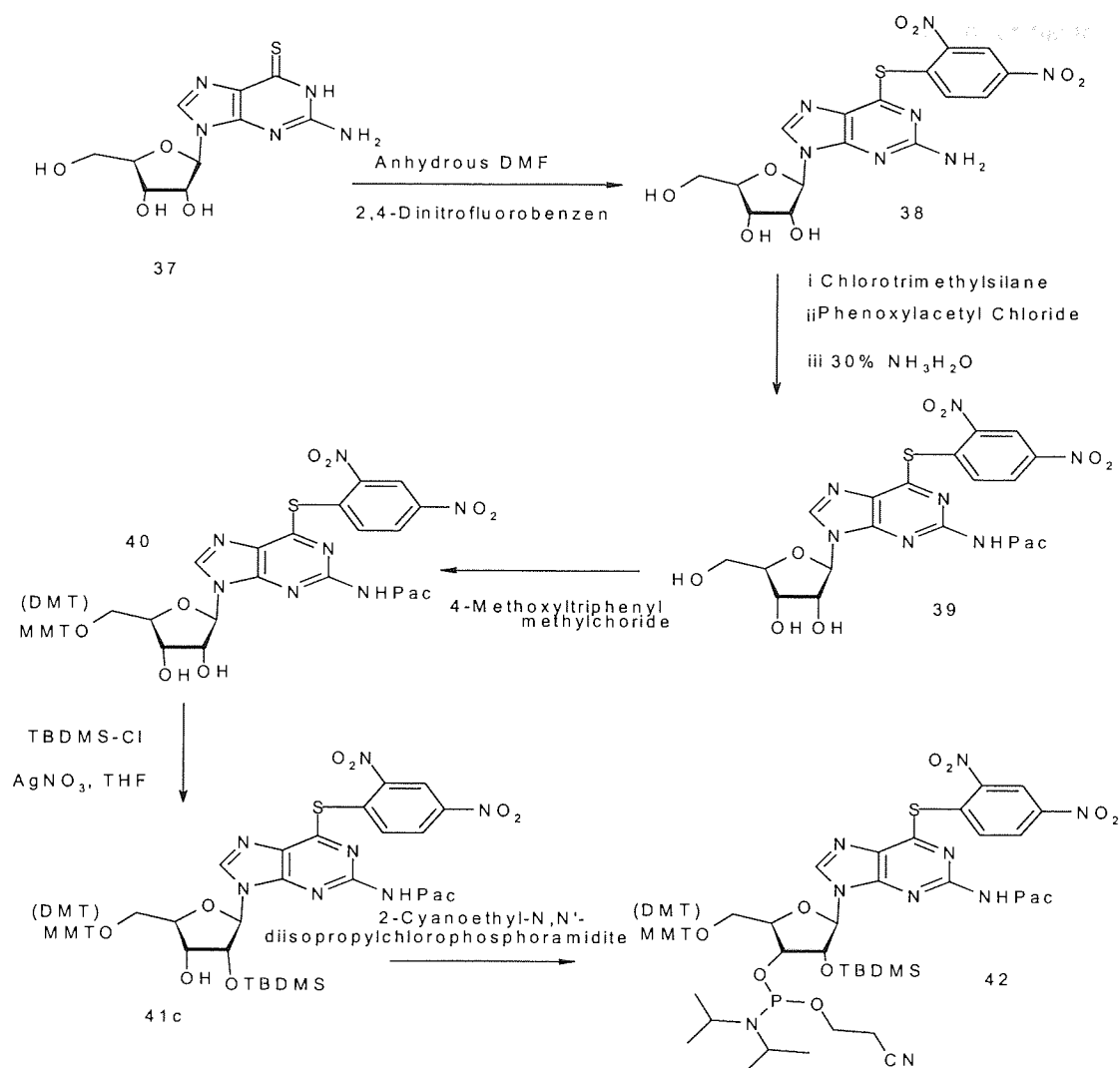


Figure 3-2. Synthetic route for the synthesis of the protected thioguanosine phosphoramidite

3.2.1.1 Synthesis of 6-(2,4-dinitrophenyl)thioguanosine (38)

6-Thioguanosine (37) (2-amino-6-mercaptopurine riboside) was treated with 2,4-dinitrofluorobenzene to form compound (38). 2,4-Dinitrophenyl group (DNP) has been successfully used in protecting the thiocarbonyl group in DNA (Xu *et al.* 1992a; Xu *et al.* 1992b).

In the present project, the thiocarbonyl group is the most active group in the whole thioguanosine molecular so that it reacted chemoselectively with 2,4-

dinitrofluorobenzene (Figure 3-4). The reaction was usually carried out under basic conditions, because it can undergo a keto-enol isomerisation (Figure 3-3):

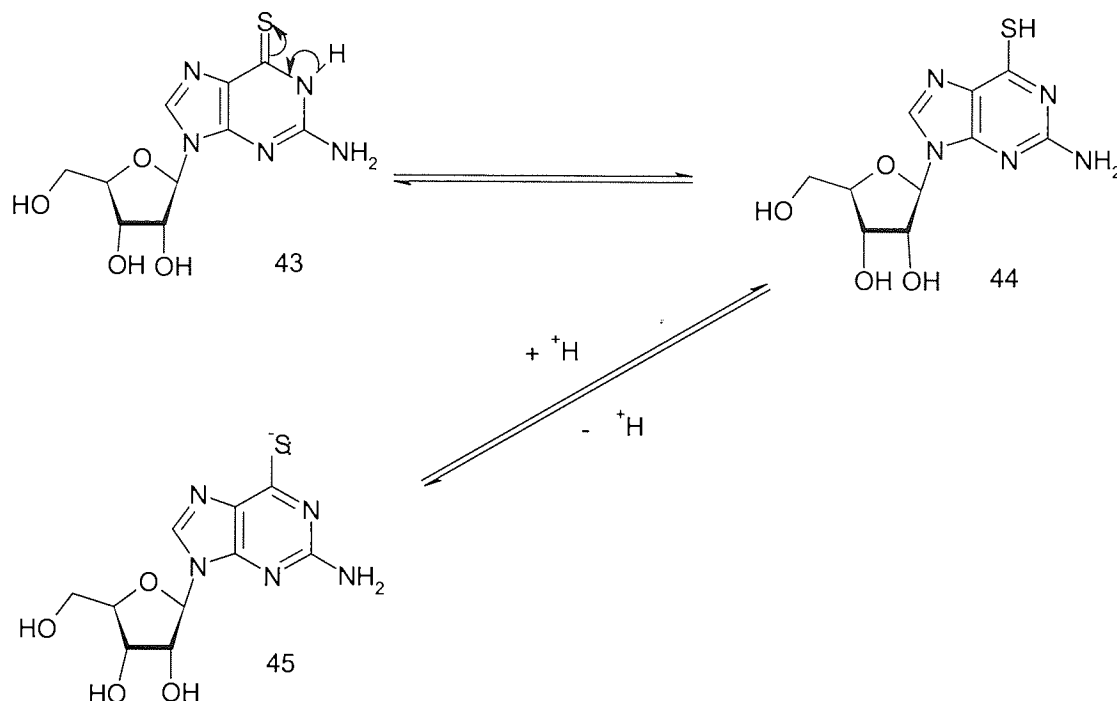


Figure 3-3. Keto-enol isomerisation of thioguanosine

Thiocarbonyl group adopts enol form (compound 44) or thiol anion (compound 45), under basic conditions. Thiol anion is a good nucleophilic reagent. Triethylamine was included in the reaction. There are two purposes to use triethylamine (a) to provide basic conditions, (b) to scavenge the hydrogen fluoride formed in the reaction.

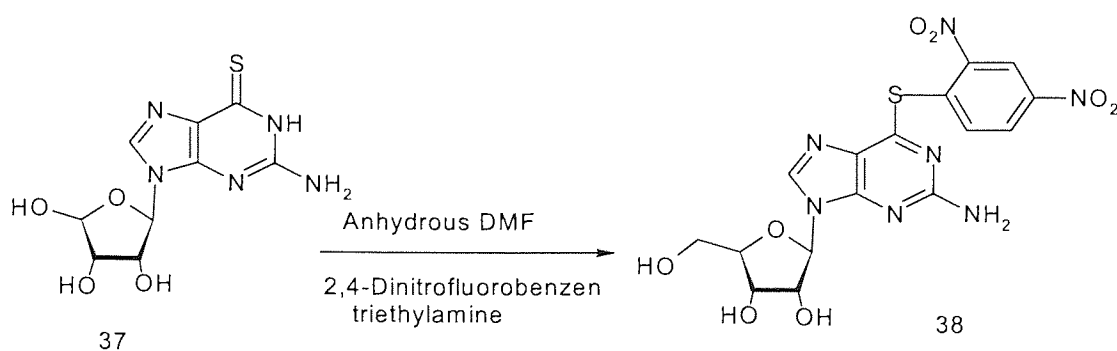


Figure 3-4. Protection of the thiocarbonyl group

The reaction was carried out in DMF solution. Two nitro groups at *ortho*- and *para* positions and the fluoro group at the position 1 of 2,4-dinitrofluorobenzene make the position 1 electron deficiency (Figure 3-5). Thus thioguanosine (37) can attack this position to fulfil S_N2 nucleophilic substitution reaction

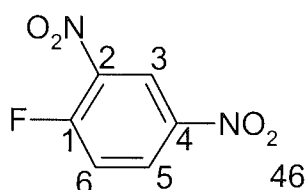


Figure 3-5. Structure of 2,4-dinitrofluorobenzene

One practical difficulty encountered in the chemical synthesis of compound 38 was the poor solubility of the reactant -- 6-thioguanosine. To find out a suitable solvent for the reaction a solubility test of 6-thioguanosine was carried out as below: 10 mg of thioguanosine was put into each of several Eppendorf tubes. Small amount of various solvents (1 ml) was added into each tube. Tubes were shaken for 30 min at room temperature. The results were observed by naked eyes. A number of commonly used solvents were tested but only *N,N*-dimethylformamide was found to dissolve 6-thioguanosine completely. The reaction was carried out smoothly using DMF as a solvent and confirmed by TLC which showed the disappearance of original spot of nucleosides and appearance of a new spot with a high R_f value. The compound became more hydrophobic because thiol group had been replaced by an alkyl thiol group so that it has a higher R_f value.

3.2.1.2 Synthesis of *N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (39)

The 2-amino group on thioguanine base was the next on the line to be protected according to its reactivity. Because of the introduction of 2,4-dinitrophenyl group, the solubility of the thioguanosine was improved dramatically. 6-(2,4-Dinitrophenyl)thioguanosine (38) could be dissolved in pyridine. This provided advantage for the whole reaction because pyridine can serve as a scavenger for hydrogen chloride formed during the reaction.

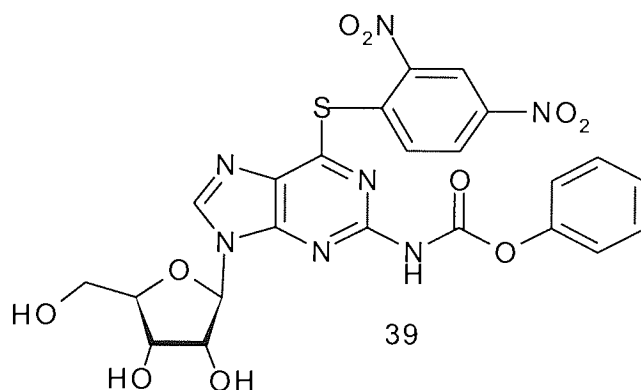


Figure 3-6. Structure of *N*²-phenoxycarbonyl-6-(2,4-dinitrophenyl)thioguanosine

Since hydroxyl groups can also react with phenoxycarbonyl chloride, they need to be temporary protected in order to make sure that they wouldn't interfere with the whole reaction. Trimethylsilyl chloride was chosen to temporarily silylate the hydroxyl groups. Phenoxycarbonyl chloride was then added into the mixture after the silylation had been completed without isolation of the intermediate. TLC showed only one resultant spot with a higher *R_f* value.

The amino groups of nucleosides are usually protected in an amide intermediate form because amide is much less reactive than other carboxylic acid derivatives. It is stable enough to withstand the synthetic conditions of oligonucleotides. Either benzoyl group (47) or Pac group (48) can be used to protect the amino groups.

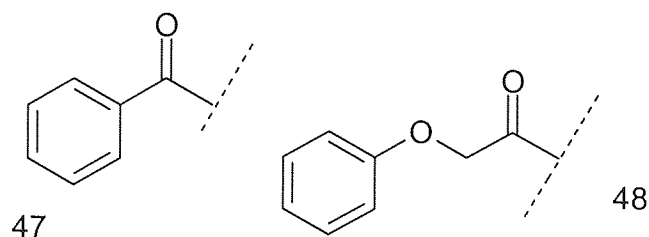


Figure 3-7. Benzoyl group (left) & phenoxyacetyl group (right)

Benzoyl group was well documented as an amino protecting group. However, benzoyl group needs severe conditions to be cleaved off after the RNA assembly. Those severe conditions might destroy sensitive groups, like thiol in the present project, on the modified nucleosides. Thus protecting group with milder deprotecting conditions is used. This phenoxyacetyl group (Figure 3-7, 48) can be removed under much milder conditions and was chosen to protect the amino group of thioguanine. After the phenoxyacetyl group reacted with the amino group, the three trimethylsilyl groups were removed by 30% ammonium. Cautions needed to be taken when treating the protected thioguanosine with ammonia solution. Heating must be avoided during the ammonia treatment because of the sensitivity of the sulphur group to ammonia. Even a mild condition like 30 °C could cause the degradation of the nucleoside.

The trimethylsilyl group can be cleaved by reaction with aqueous acids, fluoride ion, or weak bases to regenerate the alcohol. With shielding from three methyl groups, it is usually difficult for ammonia to attack the atom in the centre. However, silane is a third row atom so that it has larger atom diameter than its second row counterpart. Thus ammonia can attack silane in trimethyl ester, resulting a free hydroxy and an ammonised substituted silane (Figure 3-8).

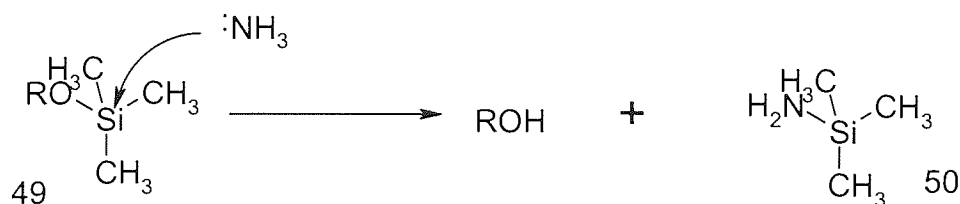


Figure 3-8. Deprotection of the trimethylsilyl groups

The whole procedure is summarised in Figure 3-9. 6-(2,4-Dinitrophenyl)-thioguanosine (38) was first converted into a protected intermediate (51). The phenoxyacetyl (Pac) group was then brought to the amino group resulting in compound (52). TMS groups were then cleaved by 30% ammonia yielding the desired compound (39).

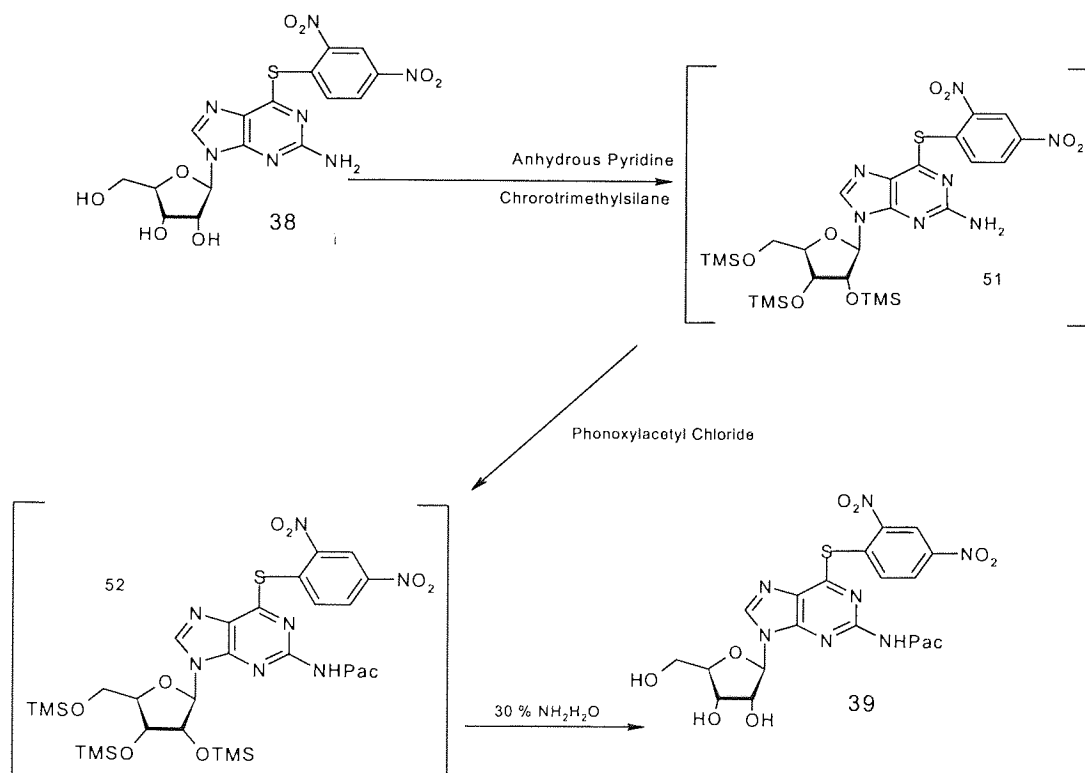


Figure 3-9. Addition of the Pac group to the amino group of compound (38). TMS: trimethylsilyl; Pac: phenoxyacetyl.

3.2.1.3 Synthesis of 5'-methoxytrityl-*N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (40)

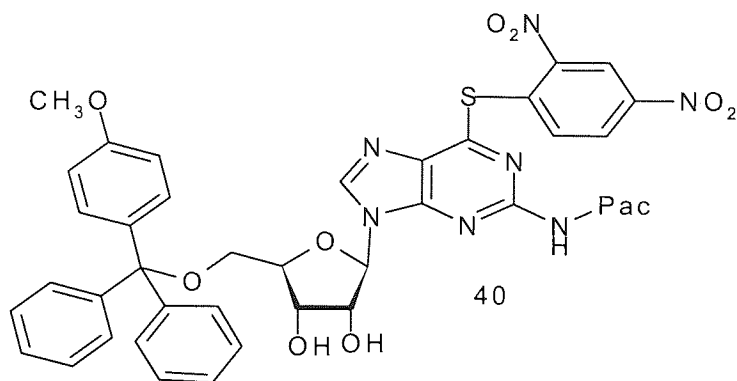


Figure 3-10. Structure of 5'-MMTr-6-(2,4-dinitrophenyl)-2-phenoxyacetyl thioguanosine

The N^2 -phenoxyacetylated compound (39) was tritylated with monomethoxytrityl chloride (MMT-Cl) to form 5'-methoxytrityl- N^2 -phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (compound 40, Figure 3-10). The reaction took place smoothly with little difficulty. Although there are three free hydroxyl groups in compound (38), due to its bulky structure, the MMT group dominantly reacted with 5'-OH group (1° OH group) in regioselective fashion. The mechanism lies behind is S_N1 substitution. The reaction is shown in Figure 3-11.

It is widely accepted that 4,4'-dimethoxytrityl (DMT) group is better than 4-monomethoxytrityl (MMT) group for protecting the 5'-hydroxyl group because DMT group needs less time to be removed than MMT group. This can make the whole procedure of automatic assembly quicker. DMT group was also tried in the present project but it failed to produce the phosphoramidite in the last phosphitylation step (details described later, section 3.2.2).

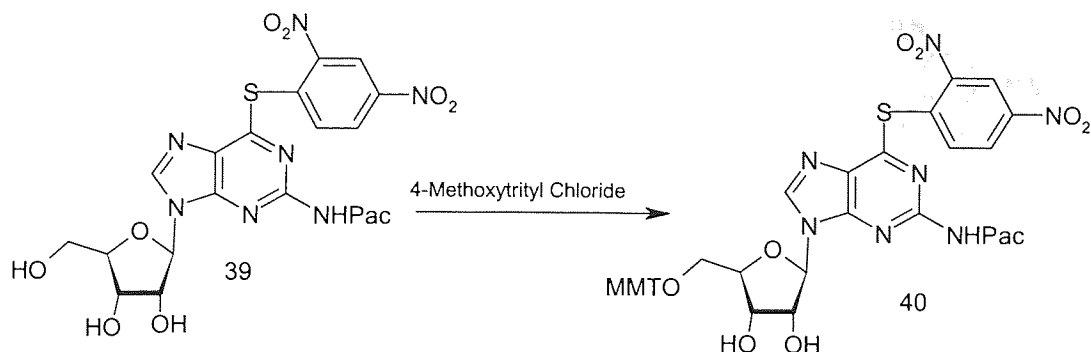


Figure 3-11. Addition of methoxyltrityl group

3.2.1.4 2'-*tert*-Butyldimethylsilyl-5'-methoxytrityl-*N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (41c)

Two groups have been commonly used for the protection of 2'-hydroxyl group: the *tert*-butyldimethylsilyl group (TBDMS) and the triisopropylsilyl group (TIPS). Usually, the TBDMS group is used when MMT is chosen for the 5'-OH and Bz (benzoyl), PhOAc (phenoxyacetyl) or iBu (isobutyl) are used for the amino group of the nucleosides. The TIPS group is used for the protection of *N*-Bz (or PhOAc)-5'-MMT-G in preference to the TBDMS group because of the ease of separation of the resulting 2'-TIPS from 3'-isomer.

In the current project, attempts to use TIPS for the protection of 2'-OH group was not successful. Once the protected thioguanosine was dissolved into DMF/imidazole, TLC showed that compound decomposed. This method was discarded and TBDMS method was adopted.

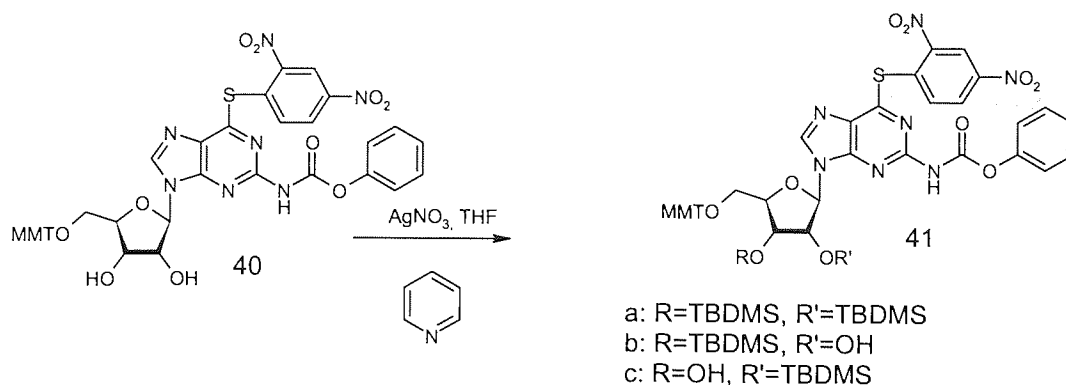


Figure 3-12. Reaction of forming 2'-*tert*-butyldimethylsilyl-5'-methoxytrityl-*N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (41).

Tetrahydrofuran (THF) was used as solvent (Figure 3-12). The lone pair of electrons on the nitrogen of pyridine can attract protons formed during the reaction so that it scavenges the acid formed. The purpose of silver nitrate was to catalyse the TBDMS groups. Silver cation is attractive to chloride anion, thus it made silicon atom more electrophilic.

The silylation reaction was extremely moisture sensitive. Thus, all solvents and reagents must be anhydrous, all solid chemicals were dried overnight before their use over P₂O₅ under vacuum. Reaction vessels were dried and TBDMS-Cl reagent (stored at -20°C) was warmed to room temperature in desiccator before use.

2', 3'-Hydroxyl groups can both react with the TBDMS group. However, only TBDMS connected with 2'-hydroxyl group is the desired product (Figure 3-12, compound 41). During the reaction, the 2'-hydroxyl group was more favourable one than the 3' hydroxyl group, which was shown by the TLC with a larger spot. This is probably due to the introduction of the MMT group on the 5' position. The steric hindrance of the MMT group slows the reaction on 3'-hydroxyl group.

Despite the fact that 2'-silylated compound (41c) was the main product, this step of reaction was still the most problematic one among all steps in synthesising the phosphoramidite monomer. This was not unexpected, because 2'- and 3'- hydroxyl groups have similar reactivity. The reaction yielded three silylated products, 2'-TBDMS (41c), 3'-TBDMS (41b), 2',3'-TBDMS-isomer (41a) (Figure 3-13). It was not easy to separate the mixture by just one-step silica gel chromatography because of the narrow gap of polarities between those three products and the other reactants. Thus, we adopted two steps separation. First step was to separate 41c and 41b from 41a. Then using less polar solution system, 41c and 41b were separated completely. After two steps column chromatography, the desired product was obtained with a satisfactory yield (56%).

3.2.1.5 Synthesis of 2'-tert-butyl dimethylsilyl-5'-methoxytrityl-*N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanine-3'-*O*-[2-cyanoethyl-(*N,N*-diisopropylamino)] phosphoramidite (42)

The last step is to convert compound 41c into its phosphoramidite form (42) by phosphitylation (Figure 3-13).

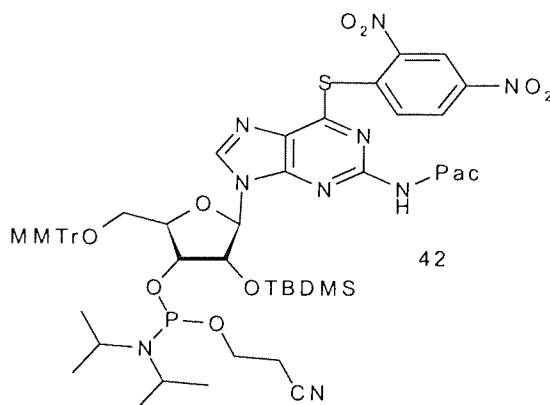


Figure 3-13. Structure of phosphoramidite monomer

As the silylating reaction, the phosphitylation of compound (41c) was also extremely moisture sensitive. Therefore, all solvents, reagents and apparatus must be anhydrous. To the reaction mixture, *N,N*-diisopropyl-(2-cyanoethyl)phosphoramidic chloride and diisopropylethylamine in dry THF were added to produce the phosphoramidite in 59.6% yield. ^{31}P NMR of the phosphoramidite showed two signals characteristic of a pair of diastereomers.

The reaction was illustrated in Figure 3-14. Apart from diisopropylethylamine, other weak bases, such as 4-dimethylaminopyridine was also tried but without any success.

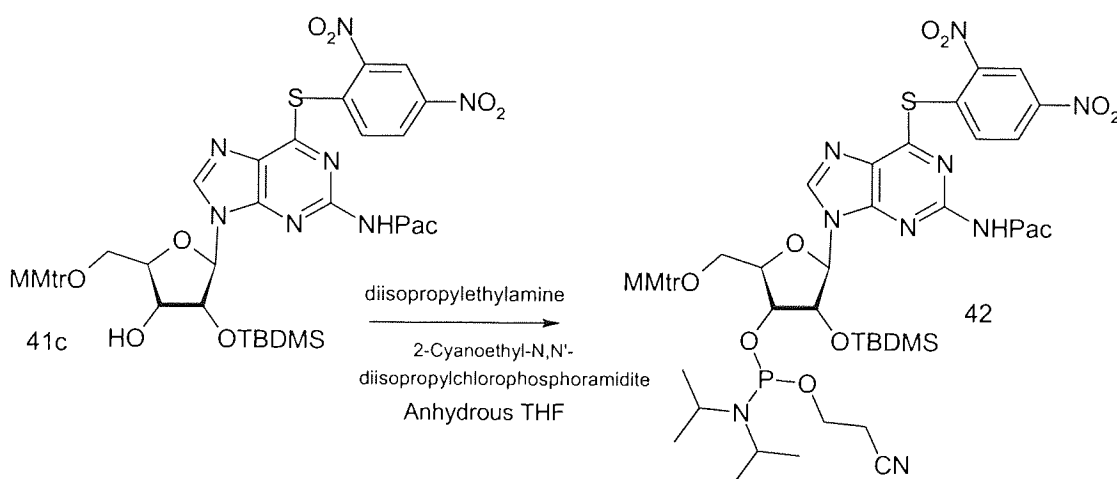


Figure 3-14. Phosphitylation reaction

3.2.2 Attempt on using DMT group for the protection of 5'-OH group.

DMT group is easier to be removed than MMT group so that it is conventionally used in protecting 5'-OH group in DNA/RNA automatic synthesis. Efforts were also made in this project trying to utilize DMT group in the synthesis of monomer but without success.

The first two synthesis procedures were identical as those of section 3.2.1.1 & 3.2.1.2. As described above, the sulphur group was first protected with 2,4-dinitrophenyl fluoride, followed by the protection of amino group with Pac group. DMT group was then introduced into 5'-hydroxyl group. Afterwards, the monomer was subject to normal procedures of tritylation and phosphitylation. Discussion in this section will start from *N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine. The route for the synthesis of phosphoramidite monomer with DMT group was composed of three steps from this point: Step 1, introducing dimethoxytrityl group into 5'-hydroxyl position; Step 2, introducing *tert*-butyldimethylsilyl group into 2'-hydroxyl position; Step 3, introducing *N,N*-diisopropyl(2-cyanoethyl)phosphoramidite into 3'-hydroxyl position.

3.2.2.1 Synthesis of 5'-dimethoxytrityl-*N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (53)

*N*²-Phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (39) was treated with 4,4'-dimethoxytrityl chloride at room temperature with anhydrous pyridine as solvent. Pyridine was also used as scavenger for the HCl formed during the reaction. The reaction finished within 12 hours as indicated by TLC.

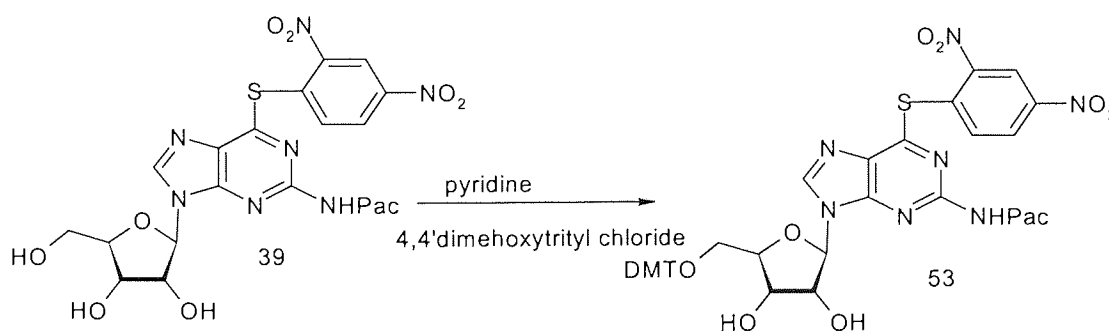


Figure 3-15. Tritylation reaction

The reaction condition for the conversion (Figure 3-15) was similar to that when MMT group was used in the place of DMT group as the introduction of one extra methoxyl group does not significantly affect the characteristic of the trityl group.

The reaction required strict anhydrous condition since any existence of water can dramatically slow down the reaction. All the reactants were co-evaporated with pyridine twice to remove any moisture in the compound and then dissolved in anhydrous pyridine. 4,4'-Dimethoxytrityl chloride was added at once. The mixture solution was turned into dark red due to DMT-Cl. As the reaction went on, the colour became darker. The reason of this phenomenon was not clear. Nevertheless, this indicated the process of the reaction and was a good indicator to monitor the reaction.

The reaction course was monitored by TLC. It showed that the reaction was running slowly at the initial stage. During the first 4 hours, only tiny amount of the starting material was converted into a new compound with a higher R_f value. The colour of the mixture remained the same in light orange. During the following 6 hrs, the colour of the mixture became darker and darker, TLC also showed that 90% of the start material had been converted into a new compound. After 12 hours, TLC showed that the conversion had been completed. All the starting material had been converted into a new compound with a higher R_f value. The structure of the product 5'-dimethoxytrityl- N^2 -phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (53) was confirmed by ^1H NMR.

3.2.2.2 Synthesis of 2'-*tert*-butyldimethylsilyl-5'-dimethoxytrityl-*N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (54b)

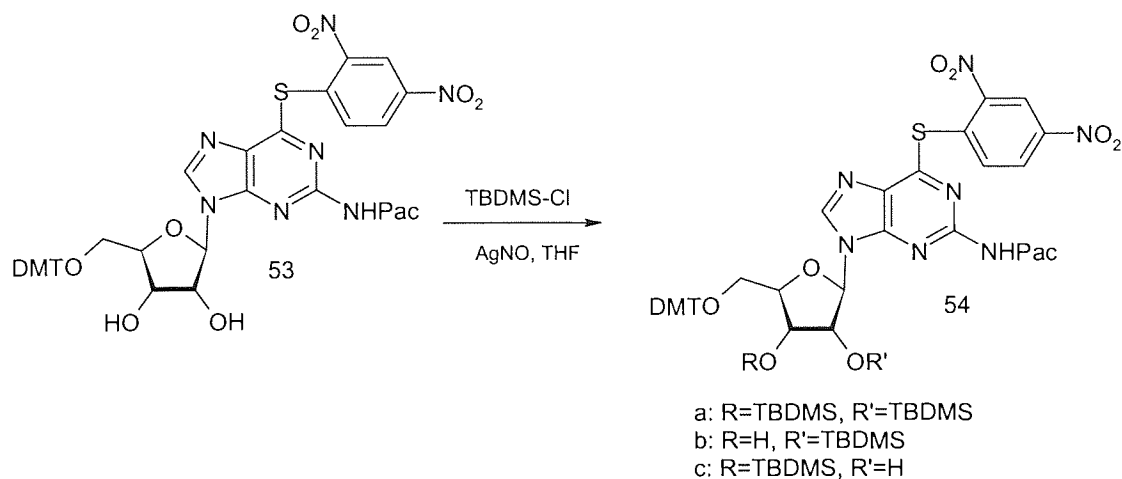


Figure 3-16. Silylation of 2'-hydroxyl group of 5'-dimethoxytrityl-*N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine

5'-Dimethoxytrityl-*N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine was treated with *tert*-butyldimethylsilyl chloride (Figure 3-16). The procedure followed the successful procedure for synthesising compound (41c).

The reaction took place smoothly and no trouble was encountered by introduction of DMT group on the 5' position. As with compound (41), both of the 2'-, 3'-hydroxyl groups reacted with the TBDMS group, thus resulting in three compounds with similar *R_f* value i.e. 2',3'-di TBDMS nucleoside (54a), 2'-TBDMS nucleoside (54b), 3'-TBDMS nucleoside (54c). TLC showed that compound 54b was the main spot. Compound 54a was only tiny trace in the front of the compound 54b on TLC. The ratio of 54a/54b was less than its counterpart with MMT (41a/41c). The reason could be that an extra methoxyl group on the trityl group of the 5'-hydroxyl position creates more steric hindrance in the compound, thus

caused the reaction less favourable towards the formation of the 2'3'-TBDMS di-substituted nucleoside.

3.2.2.3 Attempt to synthesize 2'-*tert*-butyldimethylsilyl-5'-dimethoxytrityl-*N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanine-3'-*O*-[2-cyanoethyl-(*N,N*-diisopropylamino)] phosphoramidite (55)

The structure of the final compound 2'-*tert*-butyldimethylsilyl-5'-dimethoxytrityl-*N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanine-3'-*O*-[2-cyanoethyl-(*N,N*-diisopropylamino)] phosphoramidite (55) is shown in Figure 3-17. Several mild bases (diethylamine, diisopropylethylamine, *N*-methyl pyridine) were tried for the synthesis of compound 55. But they all failed. The possible reason is given below.

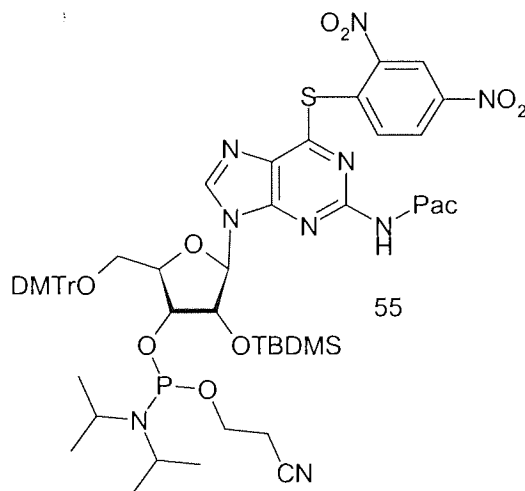


Figure 3-17. Structure of targeted phosphoramidite monomer

As in the synthesis of MMT protected phosphoramidite monomer, the first chosen reaction condition was anhydrous THF with diethylamine and the phosphorylation reagent 2-cyanoethyl-*N,N*-diisopropyl chlorophosphoramidite. Diethylamine was added to scavenge HCl formed during the reaction. This reaction is illustrated in Figure 3-18.

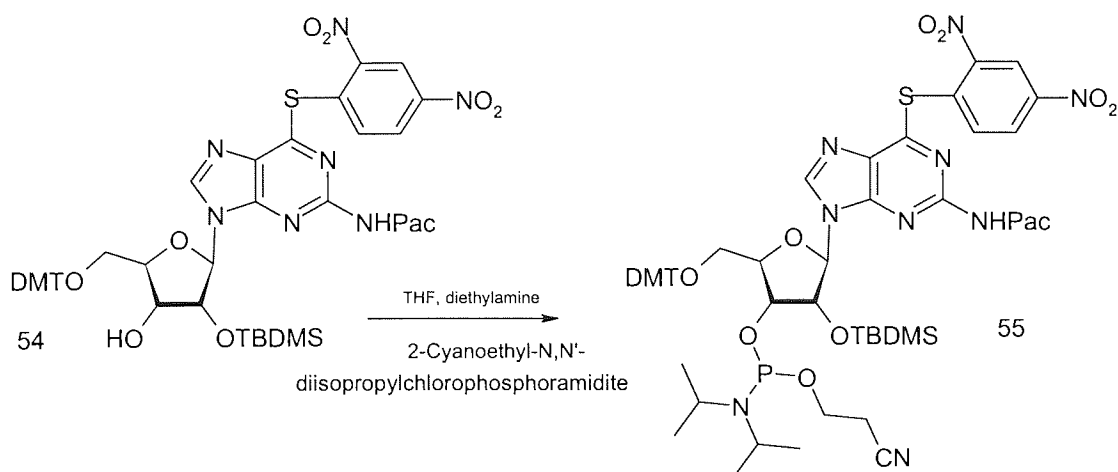


Figure 3-18. Phosphitylation reaction

However, TLC showed that compound (54b) was unstable under those conditions and underwent degradation resulting in many spots on TLC with lower R_f values. Many efforts were made (changing the reagent supplier, catalyst, refining the solvent *etc*) to overcome this problem. However, the reactant still degraded each time. The compound was even unstable in DMF. Once the compound was dissolved in DMF, TLC showed that several new compounds appeared at low R_f values. Attempt was also made to control the temperature or the speed of addition of the reagents. None of these efforts produced any positive result.

The possible reason for the failure of the reaction might be due to the acidic-sensitivity of the DMT group. Since the DMT group has one more methoxyl group, it is much more sensitive to acidic conditions than the MMT group. When cyanoethyl-*N,N*-diisopropylchlorophosphoramidite was used, the resulting HCl could cause the cleavage of the DMT and resulted in the degradation of the

monomer. The possible way to overcome it is to use another phosphorylation reagent which has no chloro groups so that no acid would be generated.

In the RNA synthesis, the program can be amended to suit the existence of MMT group. The yield was satisfactory. Thus no further efforts were made to try to use the DMT group.

3.2.3 Oligoribonucleotide synthesis and post-synthetic modification

3.2.3.1 Incorporation strategy

6-Thioguanosine phosphoramidite (39) was incorporated into RNA (a 5-mer and a 12-mer) with *Expedite 8909* automatic synthesizer. Standard procedures were followed except for the coupling of the modified phosphoramidite monomer. 6-Thioguanosine phosphoramidite was dissolved with anhydrous acetonitrile at the same concentration as G-phosphoramidite monomer (100 mg/ml) and put in bottle 7. Changes were made in the program to direct the machine to deliver the monomer from bottle 7 when incorporating the modified monomer into RNA. According to a previous report detailing the incorporation of 6-thio-2'-deoxyguanosine into DNA (Xu *et al.* 1992a), modified monomers usually need longer coupling time. This was reflected in the program. Coupling time for 6-thioguanosine phosphoramidite was extended from 600 s to 1800 s. All the conventional phosphoramidite monomers used the DMT group for protecting the 5'-OH. The default setting in the program for cleavage 5'-end group is 60 s. In the current project, the MMT group was used to protect the 5'-OH of the modified monomer. Because the MMT

is more stable than the DMT group, its cleavage time was extended to 180s to accommodate the difference.

The coupling efficiency was monitored by machine via "trityl monitoring" procedure. Results are shown in Figure 3-19 for the synthesis of the 12 mer [(3'-UCG AGU 7AC CAU-5'); 7 represents the modified nucleoside, 6-thioguanosine]. DMT of the last nucleotide was attached to the RNA when all the synthesis was finished. Thus only 11 data was obtained. The first bar represents the DMT group that was cleaved from the first nucleotide that attached to the support Its absorbance is usually smaller than the others. Position 7 represents G^{SH}. As shown in the figure, there was no significant difference on the coupling either before or after G^{SH}. This proved a good coupling yield of the modified nucleoside. Nevertheless the bars corresponding to the second and last nucleotide "C" showed unusually high UV absorbance (Figure 3-19).

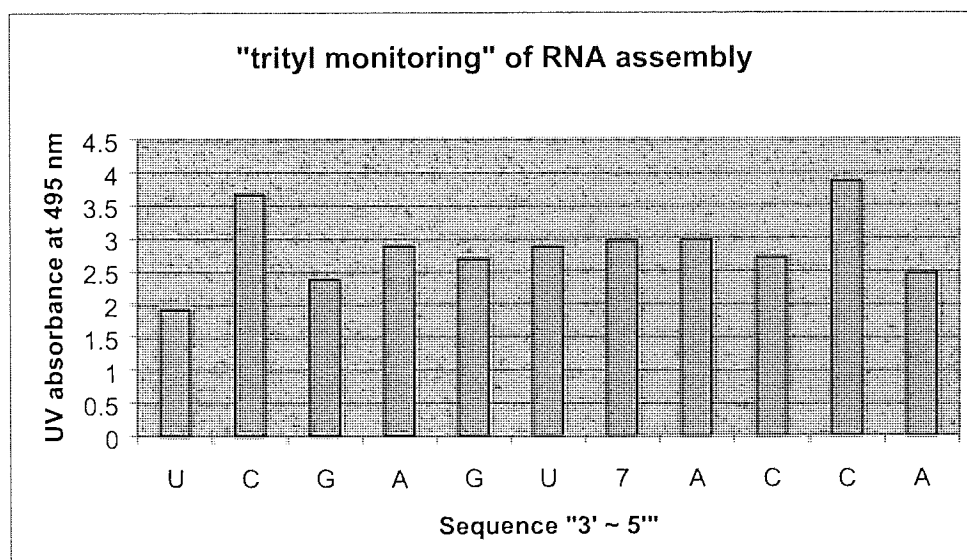


Figure 3-19. "Trityl monitoring" of RNA assembly. Sequence 3'-UCG AGU 7AC CAU-5', 7 = G^{SH}

Therefore, concern was raised considering the absorbance of MMT cleaved from the modified base. To make sure that practical error in the Expedite machine won't affect the results of the modified nucleotide, a "negative" experiment was carried out. The modified phosphoramidite was replaced by a blank sample (acetonitrile). A short sequence was designed (3'-UAb CG-5', b standing for blank sample). Results are shown in Figure 3-20. The result clearly indicated that the absorbance of blank sample was almost zero compared with the first two nucleotides. From Figure 3-19 and Figure 3-20, it can be concluded that 6-thioguanosine phosphoramidite was incorporated into RNA with a satisfactory coupling yield.

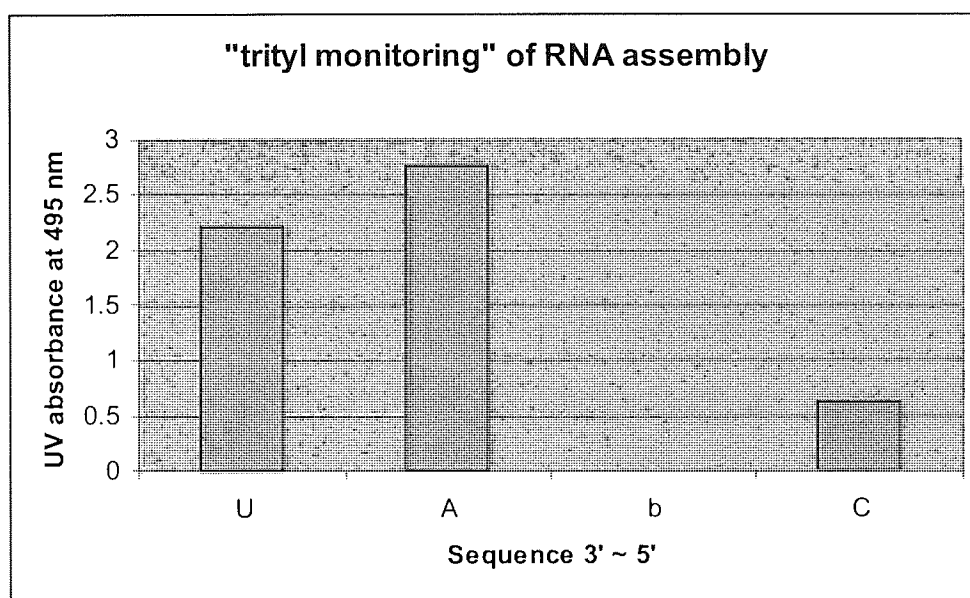


Figure 3-20. "Trityl monitoring" experiments of RNA assembly. Sequence 3'UAbCG5', b = acetonitrile.

3.2.3.2 Deprotection of RNA containing 6-thioguanine

Usually, RNA deprotection requires same conditions as for DNA except an extra step to remove the TBDMS group on the 2'-OH of ribosides with tetrabutylammonium fluoride (TBAF). However, it was reported by Admas *et al.* (1995a) that TBAF was detrimental towards RNA containing thiocarbonyl group.

In that report, triethylamine/trihydrofluoride with equal volume of DMSO was used and thiocarbonyl group was proved to be intact at the end of the deprotection. To confirm their results, DNA oligomer containing 6-thioguanine was subjected to the same deprotecting procedures and the results were satisfactory. The deprotection with RNA was then carried out.

The deprotecting procedures for RNA involved three steps:

- 1) Treating RNA with 10% 2-mercaptoethanol/acetonitrile with drops of triethylamine for 4 hrs to remove the 2,4-dinitrophenyl group.
- 2) Treating RNA with methanolic ammonia at 30 °C for 14 hrs to remove the exocyclic protecting groups and to cleave the oligomers from CPG support.
- 3) Treating RNA with triethylamine, trihydrofluoride/DMSO (50:50) at room temperature for 24 h to remove the TBDMS group on the 2'-OH position.

RNA was cleaved from CPG by the second-step deprotection and could be subject to UV spectroscopy afterwards. Thus, UV spectrophotometric detection of RNA after each step of treatment was carried out from step 2 deprotection to confirm the intactness of the RNA oligomers. It was also noted that the existence of RNase was ubiquitous in the circumstances. Therefore sterile condition was adopted when dealing with RNA to prevent the degradation of the RNA oligomers.

3.2.3.2i Treatment with 2-mercaptoethanol

The 2,4-dinitrophenyl group can be removed by treatment with 2-mercaptoethanol. In the literature, two methods have been used to deprotect the 6-thiol group. They are:

- 1) Treating oligomers with 10% 2-mercaptoethanol/acetonitrile with drops of triethylamine for 4 hrs.
- 2) Combining the deprotection of the 2,4-dinitrophenyl with that of the exocyclic protecting groups, i.e. treating oligomers with 10% 2-mercaptoethanol/ammonia (conc.)

In the current project, both methods were tested and worked well. Nevertheless, for the purpose of step-wise deprotection of the thiocarbonyl group, method 1 was adopted.

The efficiency of the deprotection of the 2,4-dinitrophenyl group was tested at the nucleoside level. Compound (38) was subjected to the deprotection condition and the results were monitored by UV spectrometry. As shown in Figure 3-21, the nucleoside (38) carrying the DNP group showed a shoulder peak around 320 nm (solid line). After the treatment with 2-mercaptoethanol, it was converted into a compound showing a peak around 340 nm (dotted line) for the thiocarbonyl group. The results demonstrated that 2-mercaptoethanol could remove the DNP group within 4 hrs.

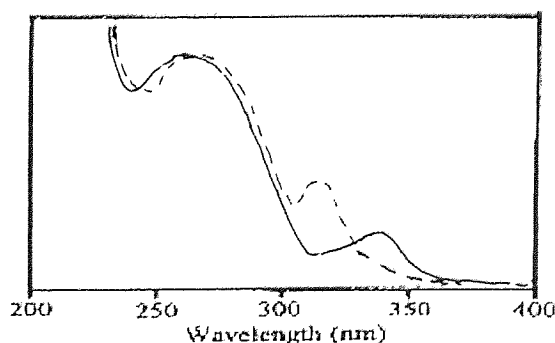


Figure 3-21. UV spectrum of compound (38) (solid line) and that of the solution of compound (38) after being treated with 2-mercaptoethanol (dotted line)

The possible mechanism for the deprotection is shown in Figure 3-22. Nucleophilic sulfur atom of 2-mercatopethanol attacked the position 1 of the 2,4-dinitrophenyl group resulting in the formation of the thiocarbonyl group.

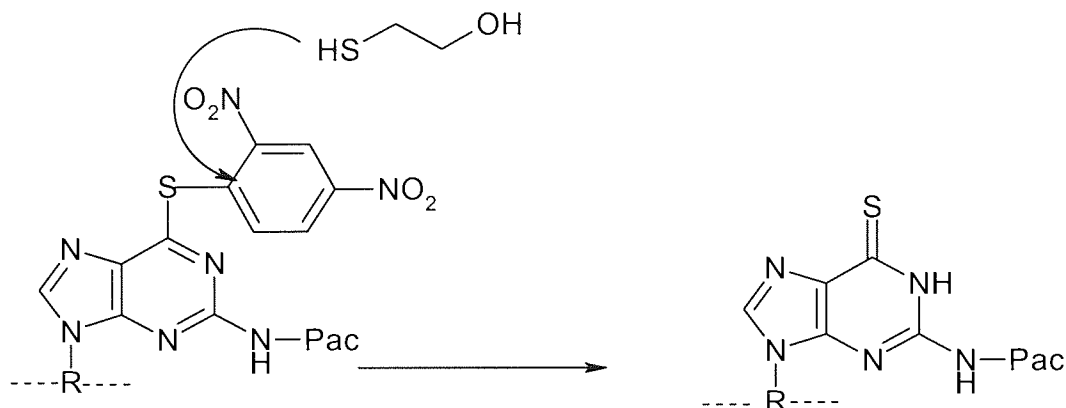


Figure 3-22. Deprotection of the DNP group

Since the oligomers were still attached to the solid support after the first-step deprotection, mercaptoethanol was removed simply by washing the support with anhydrous acetonitrile followed by washing with methanol (10×2 ml). The solid residue was then dried with speed-vac for 4 hours under room temperature to remove methanol completely.

3.2.3.2ii Deprotection of exocyclic protecting groups and cleavage of RNA

Concentrated aqueous ammonia used to remove *N*-acyl protecting groups from synthetic oligoribonucleotides can cause problems with alkylsilyl protecting groups on the 2'-hydroxyl and lead to chain cleavage (Wu *et al.* 1989). Although, this problem never existed in DNA, this can cause big problem in RNA synthesis. Thus, a labile *N*-acyl protecting group is needed in order to avoid any damage to the 2'-alkylsilyl group. In the present project, Pac group was used to protect the amino position. It was proved that Pac group could be removed even with mild methanolic ammonia. Under those conditions, no damage was caused to the 2'-alkylsilyl group.

As for the procedures of deprotecting the 2,4-dinitrophenyl group, there are several methods available for the deprotection of exocyclic protecting groups:

- 1) Treating oligomers with 50mM NaSH/Ammonia (conc.) for 24 hrs
- 2) Treating oligomers with saturated methanolic ammonia (prepared by bubbling ammonia into anhydrous methanol for 30 min at 0°C) for 14 hrs at 30 °C
- 3) Treating oligomers with 10% 2-mercaptoethanol/ammonia (conc.) for 48 hrs.

Preliminary experiments were carried out with each of the above three deprotection methods. It was found that method 2 worked perfectly well with RNA so that this method was adopted in this project. The possible mechanism of the deprotection is shown in Figure 3-23. Because the treatment of ammonia also broke the linkage between oligomers and solid support CPG, the oligomers were now existing freely in the supernatant, which made it possible to use UV spectrophotometry to monitor the identity of thioguanine. UV spectrum of the supernatant (Figure 3-24) showed clear evidence which supported the existence of oligomers with the thiocarbonyl group (peak 260 nm for oligomers, peak 340 nm for thiocarbonyl function).

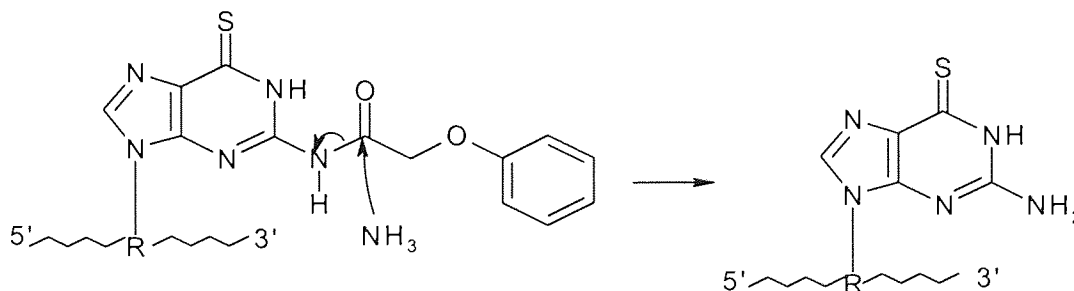


Figure 3-23. Mechanism of deprotection with methanolic ammonia.

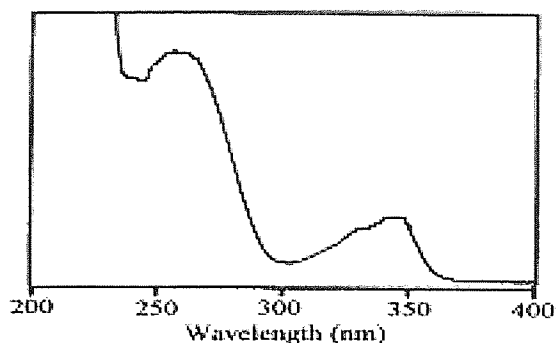


Figure 3-24. UV spectrum of the supernatant after the step 2 deprotection.

The supernatant was then separated from the solid-support by filtration and dried by Speed-Vac. The residue was now ready for the step-three deprotection.

3.2.3.2iii Deprotection of the 2'-TBDMS group

There are two commonly used methods for removal of the TBDMS group: TBAF (tetrabutylammonium fluoride)/THF, or $\text{NEt}_3 \cdot 3\text{HF}$ (triethylamine/trihydrofluoride). Degradation of RNA has been reported when using TBAF (Sproat *et al.* 1989; Adams *et al.* 1995a; Adams *et al.* 1995b). Thus, in the current project, no risk was taken of using TBAF. Instead $\text{NEt}_3 \cdot 3\text{HF}$ was used in the place of TBAF.

$\text{NEt}_3 \cdot 3\text{HF}$ /DMSO was used in this project. The purpose of inclusion of DMSO was to increase the solubility of long chain RNA oligomers. It was also found that the stability of RNA was also improved by the inclusion of DMSO (Adams *et al.* 1995a; Adams *et al.* 1995b). Oligomers were treated with $\text{NEt}_3 \cdot 3\text{HF}$ /DMSO (50:50) for 24 hrs at room temperature. As the previous deprotection steps, the integrity of the thiocarbonyl group was confirmed by UV spectrophotometry (Figure 3-25). The strong UV absorbance around 200-220 nm was due to the existence of DMSO.

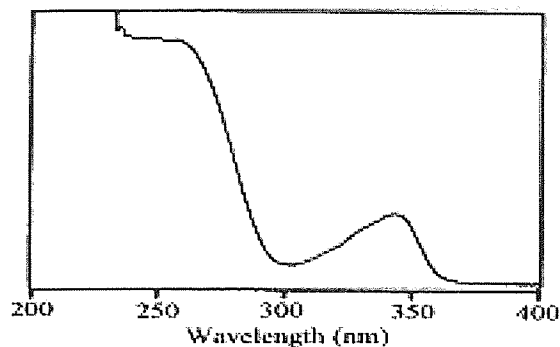


Figure 3-25. UV spectrum of supernatant after treatment with $\text{NEt}_3 \cdot 3\text{HF}/\text{DMSO}$.

The only problem caused by addition of DMSO was its high boiling point, which made it difficult to remove it even with freeze-drier (vacuum 10^{-6} pa, temperature, -40°C). This did cause some problem for purification. Nevertheless, the problem was overcome by NENSORB column purification. When the oligomers were loaded onto the NENSORB column, only oligomers with MMT group could be retained by the column. DMSO was washed away together with failure sequences and any possible by-products.

3.2.3.3 Purification and desalting

RNase exists ubiquitously so that it is critical that sterile equipment, reagents, and handling techniques are used in handling deprotected oligoribonucleotides. All water, silanized glassware, and plasticware must be autoclaved in the presence of diethylpyrocarbonate (DEP) as follows:

- 1) Deionized sterile water: Sterilize double-distilled water by treatment with DEP (1% solution) followed by autoclaving at 120°C for 20 min. When cooled sodium azide (0.001%) was added to inhibit microbial growth. Water sterilized in this way can be used safely for 2 weeks. Whenever in doubt, discard water and sterilize a new batch.

- 2) Glassware and plastic-ware: Glassware used in the deprotection of nucleotides was silanized prior to sterilization using Sigmacote (Sigma, St. Louis, MO). Small glassware and plasticware were sterilized by autoclaving at 120°C for 20 min. Large pieces of glassware were sterilized by washing with double-distilled water (1% DEP), and drying in an oven (110°C).
- 3) Buffers: Filter buffer through a 0.45-micron filter, and degas before use.

There are several methods available for purification; NENSORB column, OPC cartridge (oligonucleitdes purification cartridge, Applied Biosystme), HPLC, PAGE-SDS gel electrophoresis. Three purification methods were tested in this project: NENSORB column, OPT column and HPLC. HPLC was used as both an analytic method to evaluate the results of the other two purification methods and a method for further purification of the oligomers.

NENSORB column purification worked well as the first step purification, while OPC method failed to provide any positive results. An additional advantage of using NENSORB column is its capacity. It was found that one NENSORB column could hold as many as 50 O.D. of oligonucleotides, while OPT column can only hold small quantity of the oligomers (< 5 O.D.).

The purification procedure was carried out by following the instructions of the manufacturers (same as those in Chapter 2, DNA purification). The final eluate (35% methanol) was collected into Eppendorf tube (1ml/tube). The fractions containing the desired oligomers were identified by UV spectrophotometry at 260 nm. The results are shown in Figure 3-26. Oligomers were mainly contained in

fractions 5, 6 and 7. These fractions were pooled together and dried using freezer drier.

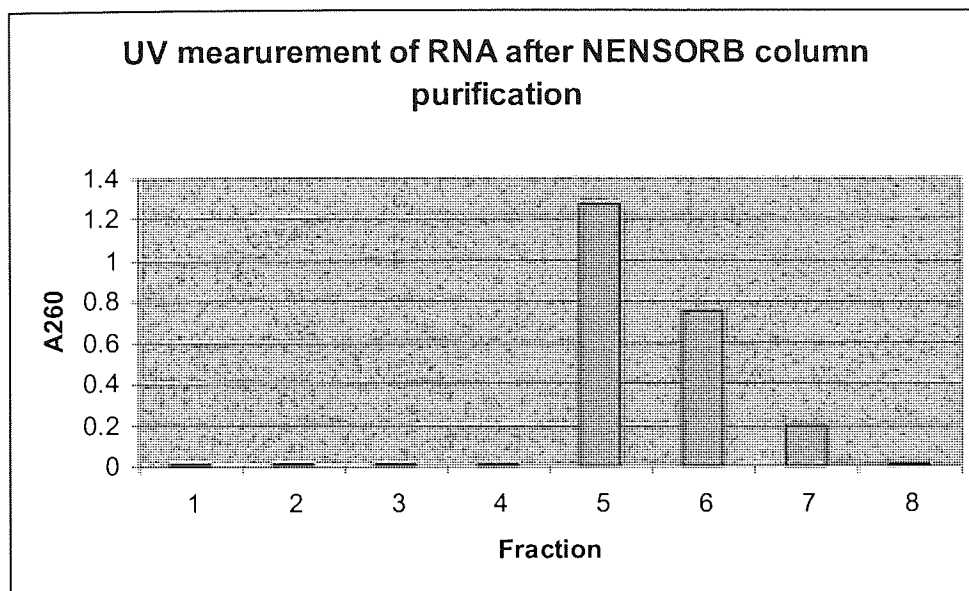


Figure 3-26. UV absorbance of fractions after NENSORB purification (260 nm).

Further purification of the oligomers was carried out with HPLC. The HPLC trace of the NENSORB column purified oligomer is shown in Figure 3-27. The main peak around 15.9 min was carefully collected and its purity was checked again with HPLC (Figure 3-28).

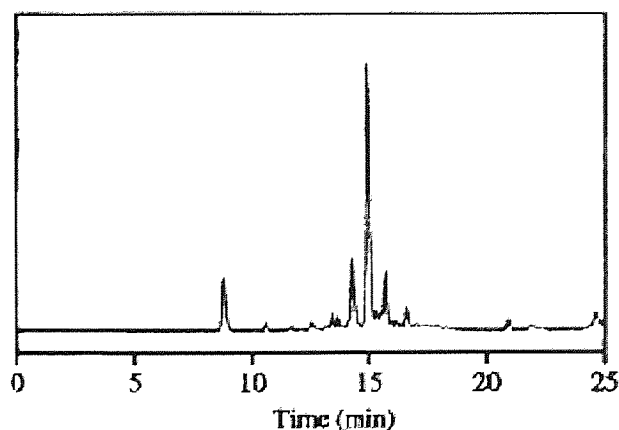


Figure 3-27. HPLC trace of oligoribonucleotides after NENSORB column purification

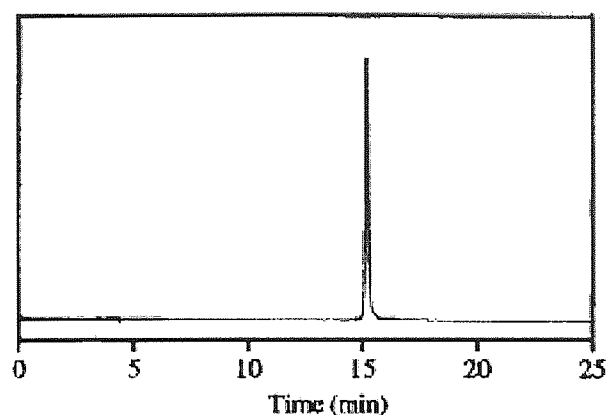


Figure 3-28. HPLC trace of oligoribonucleotides after further purification by HPLC

3.2.3.4 Nucleoside composition analysis

Nucleoside composition analysis is a most useful method to confirm the integrity of oligomers. Two enzymes, phosphodiesterase and phosphatase, were used. Snake venom phosphodiesterase (SVPDE) was used to cut the phosphodiester linkage, while alkaline phosphatase (AP) was used to cleave the phosphate from the nucleotides, leaving only nucleosides. Due to the long incubation time for the nucleoside composition analysis, adenosine was converted into inosine by adenosine deaminase, which was a contaminant in the enzyme preparations.

Standard nucleosides (G, C, U, I, G^{SH}) were injected into HPLC separately in order to determine their retention time. It was found that the order of their retention time (from short to long) was cytosine, uridine, inosine, guanosine, thioguanosine and adenosine. While all showed a peak at 260 nm, only G^{SH} showed a peak at 340 nm. The oligomers were subjected to enzymatic digestion and the digest was injected into HPLC and the results are shown in Figure 3-29 for the 5 mer oligoribonucleotide. Peaks on the HPLC trace were identified by retention time comparison with the authentic samples. No adenosine was observed due to the

deamination of adenosine by adenosine deaminase. This has been previously observed by others (Damha & Ogilvie 1993). The adenosine deaminase is a common contaminant in the enzyme preparations. When monitored at 340 nm only one peak was observed with the retention time corresponding to an authentic sample of 6-thioguanosine (Figure 3-30).

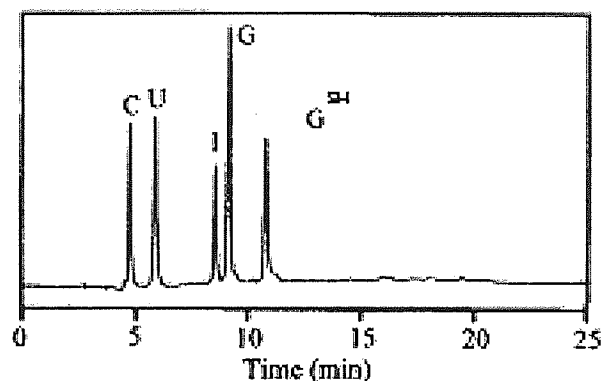


Figure 3-29. Nucleoside composition analysis of 5'-GCG^{SH} AU-3'. The nucleosides generated from digestion with snake venom phosphodiesterase and alkaline phosphatase were separated by HPLC with monitoring at 260 nm. The identity of the peaks was confirmed by retention time comparison with the authentic samples. Inosine was resulted from the deamination of adenosine by adenosine deaminase, which is a contaminant in the enzyme preparations.

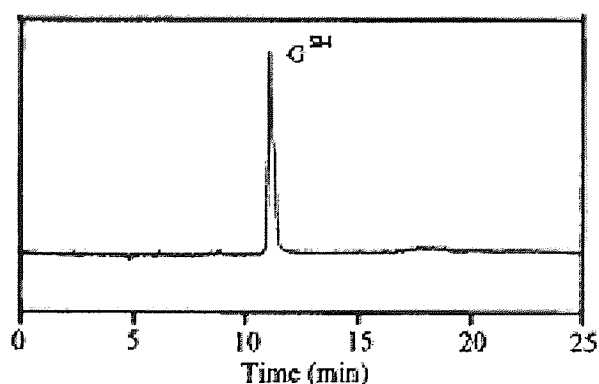


Figure 3-30. Nucleoside composition analysis of 5'-GCG^{SH} AU-3'. The nucleosides generated from digestion with snake venom phosphodiesterase and alkaline phosphatase were separated by HPLC with monitoring at 340 nm.

3.2.3.5 Post-synthetic modification, preliminary results

As discussed in the introduction section, one of the purposes of the current project is to investigate the post-synthetic modification of the thiocarbonyl group in the RNA oligomers. Thus the purified RNA was tested against several electrophilic reagents (Figure 3-31).

The post-synthetic modification reaction is an S_N2 reaction involving thiocarbonyl group reacting with a electrophilic reagent, such as alkyl chloride or alkyl bromide. During the investigation of post-synthetic modification on DNA, the optimal condition was found as follows: pH 8.5 (phosphate buffer), reaction times varied from 5 min for benzyl bromide to 72 h for 2-iodopropane. It was also found that bi-functional groups could be attached to the DNA.

The post-synthetic modification of RNA containing 6-thioguanine was carried out similarly for the DNA counterpart. The HPLC trace (Figure 3-32) showed one of the successful examples of introduction of a methyl group (CH_3 -). A new peak appeared with a longer retention time than the original RNA peak at 260 nm (Figure 3-32). This peak was collected and analysed by UV spectrometry (Figure 3-35 dotted line). The spectrum clearly showed that the peak for thiocarbonyl was disappeared, while a new peak around 324 nm appeared. These results proved that thiocarbonyl group (340 nm) had been converted into a sulfide groups (324nm). As the reaction went on, the original peak for unsubstituted RNA decreased (Figure 3-33) and eventually disappeared (Figure 3-34).

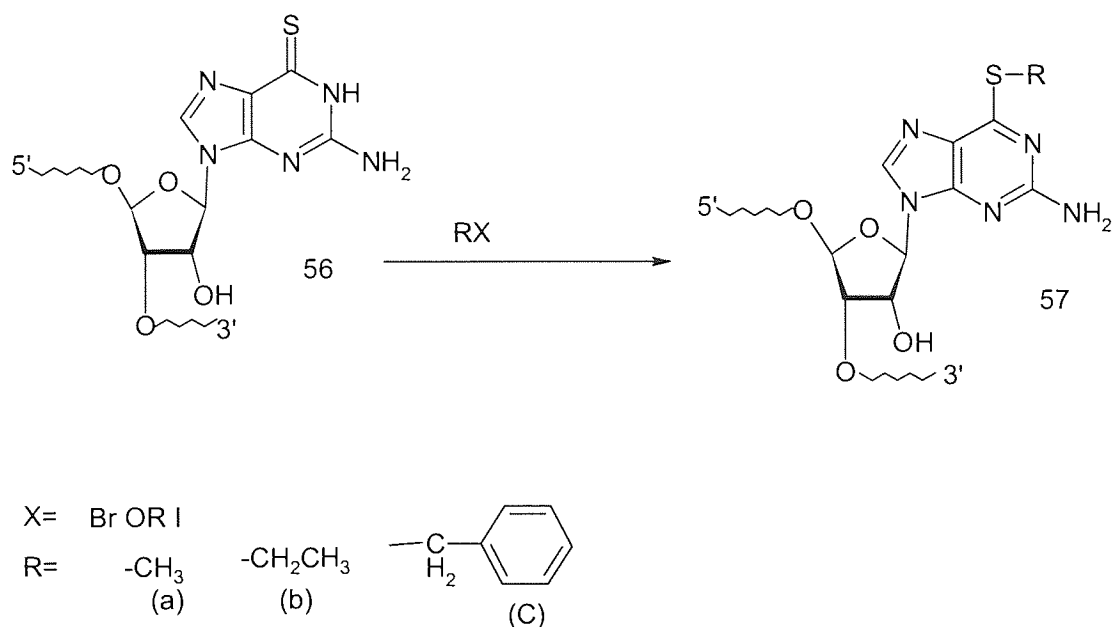


Figure 3-31. Post-synthetic modification of oligoribonucleotides containing 6-thioguanosine

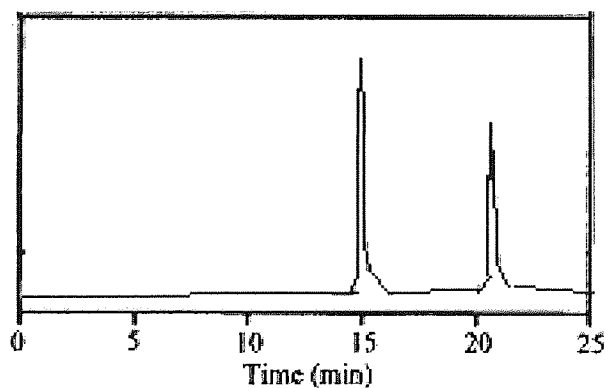


Figure 3-32. HPLC profile of the reaction between the 5-mer oligomer with iodomethane after 5 min

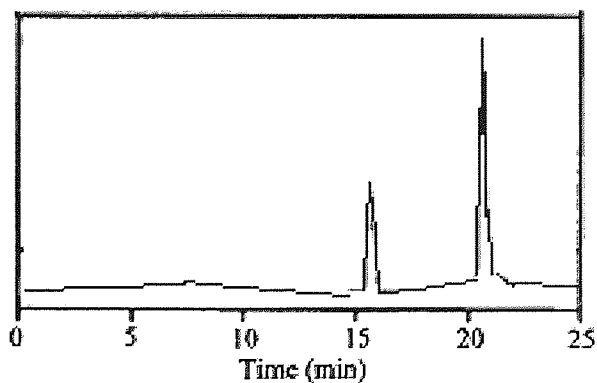


Figure 3-33. HPLC profile of the reaction between the 5-mer oligomer with iodomethane after 50 min

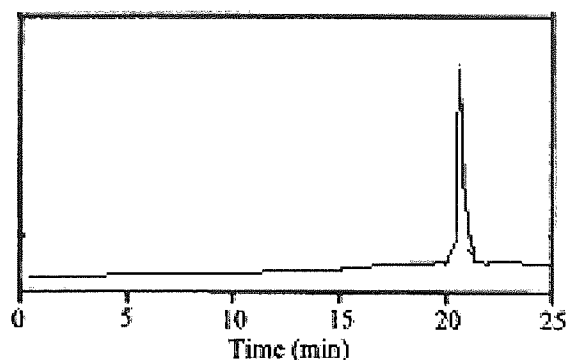


Figure 3-34. HPLC profile of the reaction between the 5-mer oligomer with iodomethane after 4 hrs

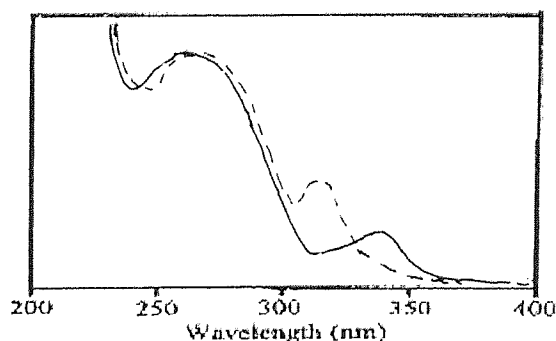


Figure 3-35. UV spectra of the unalkylated RNA (solid line) and the alkylated RNA (dotted line).

*S*⁶-Alkylated thioguanine nucleosides were synthesised in order to provide standards for nucleoside composition analysis. It was surprising that a commonly used reaction condition (DMF as solvent with Et₃N as base) did not work for the alkylation of thioguanosine. Later an alternative reaction condition (with phosphate buffer pH 10.25) was used. After the reaction, the mixture was co-evaporated with ethanol to remove water. Small amount of ethanol was added to the residue, and undissolved materials were removed by filtration. The remaining ethanol was evaporated to dryness. The obtained white powder was checked with TLC, showing only one spot. NMR spectrum further confirmed the identity of the nucleoside

derivatives. Nevertheless, due to the time limitation, base analysis of the substituted RNA has not been carried out.

3.3 Conclusion

The present work illustrates two accomplishments: 1) synthesis of the 6-thioguanosine phosphoramidite with 2,4-dinitrophenyl as a protecting group for the thio-function and the effective incorporation of this modified nucleoside into synthetic oligoribonucleotides; 2) demonstration of the feasibility of a simple, chemoselective and versatile method for preparation of oligoribonucleotides containing S^6 -functionalized thioguanine. Although the postsynthetic modification was carried out on a 5-mer and a 12-mer oligoribonucleotides, it can be reasonably predicted that these groups, including other biological useful functions, such as fluorescence groups, could be introduced on the thiocarbonyl function in longer oligomers, as the 6-thioguanosine can be enzymatically incorporated into long chain oligoribonucleotides via its 3',5'-bisphosphate by two-step ligation (Kadokura *et al.* 2000). It is worth pointing out that removal of the thiol-protecting group (2,4-dinitrophenyl) in 6-thioguanosine does not affect other parts of the oligomers, the post-synthetic modification could be performed "on-column", a strategy with several potential advantages (Zheng *et al.* 2003). The described protocol for the post-synthetic modification, after some modifications, could also be applied to oligoribonucleotides bearing other thio-ribonucleosides.

Chapter 4 Experiments

4.1 General methods

NMR spectra were recorded on a *Bruker AC250* at ^1H (250.1 MHz), ^{31}P (101.1 MHz). Positive chemical shifts are downfield of tetramethylsilane for ^1H , downfield of H_3PO_4 for ^{31}P . Mass spectrometry was carried out by Mrs. Karen Farrow in Aston (*HP 5989B MS engineering, HP 59987A Electrospray accessories*) and high-resolution mass spectrometry was carried out by Mr. Peter Ashton in Birmingham University. Ultraviolet spectra were recorded with *Unicam PU8730* Spectrophotometer. Flash column chromatography was performed using *Sorbsil C60* silica gel. TLC was performed using alumini-backed G60 silica gel plates containing a fluorescent indicator and visualised under UV (260nm). *Ethanolc anisaldehyde-H₂SO₄* was used where appropriate to visualise TLC plates to indicate the position of nucleosides.

4.2 Synthesis, purification and post-synthetic modification of DNA containing 6-thioguanine

4.2.1 General methods

Automated solid phase oligonucleotides synthesis was carried out on a 1 μmol scale using a *Beckmann Oligo 1000 DNA* Synthesiser following the standard protocol recommended by the manufacturer. 2'-Doxyribonucleotide cyanoethyl-phosphoramidites of T, A^{Bz}, C^{Bz (or Ac)} and G^{Bz} and conventional LCAA-CPG supports were purchased from *Beckmann* or *Glen Research*. Molecular sieves (3Å) were purchased from *Beckmann* and were used to dry all phosphoramidites reagents.

NENSORB columns were purchased from *Du-Pont*. OPC (oligonucleotides purification cartridge) was purchased from *Applied Biosystems*. All sequences were synthesised in "DMT-ON" mode, unless otherwise stated.

4.2.2 Synthesis and deprotection of DNA

Synthesis of oligonucleotides was typically carried out on 1 μ mol scale using phosphoramidite and succinyl CPG support. DNA sequences are shown in Figure 4-1. Phosphoramidite monomer containing dG^{SH} was dissolved in anhydrous acetonitrile (1 mg/ml). The solution was put in position X of the Beckmann synthesizer. Symbol X was entered the sequence in the control panel to represent dG^{SH}. The normal synthesis procedure was followed.

Parent sequences (P-1)
5'-CAT CAG TGA GCT-3'
Oligomer containing G^{SH} (G-1)
5'-CAT CAG^{SH} TGA GCT-3'
Complementary sequences (C-1)
5'-AGC TCA CTG ATG-3'

Figure 4-1. Sequences of DNA

After solid-phase synthesis, DNA needs to undergo a deprotection procedure to release the free oligomers. There is an extra deprotection step for modified oligomers. It was first deprotected with 1.0 M DBU in anhydrous CH₃CN for 5 h. The DBU solution was removed and the support was washed with anhydrous CH₃CN several times. The oligomers-attached support was put into the Speed-Vac to remove any remaining acetonitrile. The dried oligomers were then treated with 1 ml NaSH

(50mM) in concentrated ammonia at room temperature for 2-3 days. The ammonia was removed by Speed-Vac at room temperature. The residue was then ready for purification. Unmodified oligomers were treated with conc. ammonia at 55 °C for 5 hrs. Samples were cooled down and ammonia was removed by Speed-Vac.

4.2.3 Purification by NENSORB™ PREP column

Triethylammonium acetate 0.1 M (TEAA) was used in NENSORB column. Usually, 1.0 M TEAA stock solution was made and diluted to the 0.1 M solution before the experiments. 1.0 M TEAA stock solution was made as follows: 64 ml of triethylamine is diluted into 300 ml water. Glacial acetic acid was added to the solution drop-wise until it reached pH 7.0 and then deionised water was added to bring the volume up to 400 ml. 0.1 M TEAA was made by diluting of 1 portion 1.0 M TEAA with 9 portions of water.

Each NENSORB column can purify up to 50 O.D. The purification procedure was carried out following the manufacturer's protocol. All solutions were made freshly before usage and kept in refrigerator 4°C and consumed in one week.

NENSORB column was first activated by 10 ml methanol. The flow rate of methanol should not exceed 10 ml/min in order to get the best results. Liquid should be stopped when the meniscus reaches the top of the resin bed. The column was then washed through with 5 ml TEAA (0.1 M). The oligomers, which had been dissolved in 0.1 M TEAA, were then added onto the column. Liquid was carefully sucked out of the column to let the oligomers bound to the support. Acetonitrile:TEAA (0.1 M) [1:9 (v:v), 10 ml] was then applied to remove the failure sequences, salts, and any other

possible by-products, while the trityl-on oligonucleotides remained in the cartridge. After this step, 25 ml of 0.5% TFA was used to wash through the column. The trityl group was removed in this step whereas the trityl-off oligonucleotides remained in the resin. The cartridge was then washed with 10 ml 0.1 M TEAA, pH 7.0, to remove the acid.

The oligomers was finally eluted out with 35% MeOH/water and fractions were collected with Eppendorf tubes. Each fraction was then diluted by a factor of 20 in methanol solution (35%) for UV measurements (260 nm). Fractions containing desired oligomer were pooled and dried and stored at -20°C.

4.2.4 HPLC purification

HPLC analysis was carried out using a *Hewlett Packard Series 1100* instrument with a UV detector at a wavelength of 260 nm or 340 nm. Gradient elution was performed on a C18 reversed phase column (*HPLC technology* 250 × 4.6 mm) at a flow rate of 1 ml/min [buffer A: 0.1 M TEAA (98%)/CH₃CN pH 7.0; buffer B: TEAA (20%)/CH₃CN at pH 7.0]. Gradient was formed from 0% of buffer B to 30% of buffer B within 20 min. Semi-preparative purification was carried out as following: the oligonucleotides obtained after NENSORB column was dissolved in H₂O (1 ml) and aliquots (typically 0.2-0.5 ml) was injected into HPLC using a 1 ml injection loop. The peaks were collected manually in Eppendorf tubes. The fractions containing desired products were re-injected into the HPLC to assess the purity of oligomers. During the UV measurements, sample was dissolved in proper solvent (either water or 35% methanol).

4.2.5 Post-synthetic substitution of dG^{SH} oligomers & nucleoside composition analysis

Typically, 0.5 O.D. of oligomer was mixed with an electrophilic reagent in phosphate buffer (pH 8.5) and shaken for a varied period of time (5 min to 2 days) at room temperature. The reaction course was followed by HPLC and desired peaks were collected and quantified by UV spectrometry.

4.2.6 General method for enzymatic digestion of modified oligodeoxyribonucleotides

The oligodeoxyribonucleotides substrate was purified with reverse-phase HPLC. The oligomer (\approx 0.5 O.D. unit) was then evaporated to dryness in Eppendorf tube. The residue was redissolved in 160 μ l H₂O and 20 μ l 600 mM Tris-HCl, 60 mM MgCl₂, pH 8.5. Snake venom phosphodiesterase I (10 μ l, 10 μ g protein) was added and the mixture incubated (37 °C, 30 min), then alkaline phosphatase (10 μ l, 5 μ g protein) was added and incubation continued for 60 min. the nucleosides were separated by HPLC using 97% buffer A (0.05 M aqueous KH₂PO₄, pH 4.5) and 3% buffer B (0.05 M aqueous KH₂PO₄/35% CH₃CN, pH 4.5) for the first 8 min, then with a linear gradient from 3 to 35% of buffer B over the following 17 min followed by linear gradient from 35 to 70% of buffer B over the remaining 10 min. The eluate was monitored at 260 nm for the first 20 min for detection of dC, dG, dT and dA and the remainder at 310 nm for detection of the modified nucleosides. Peaks on the HPLC traces were identified by retention time comparison with authentic samples.

4.3 Synthesis of 6-thioguanosine phosphoramidite (42)

4.3.1 6-(2,4-Dinitrophenyl)thioguanosine (37)

The test on solubility of 2-amino-6-mercaptapurine riboside [6-thioguanosine, compound (37)] was carried out first in Eppendorf tube to find out a proper solvent for the reaction. About 10 mg of 6-thioguanosine was put into several tubes and 1 ml of various solvents (Acetonitrile, pyridine, tetrahydrofuran, *N,N*-dimethylformamide) were added to each tube. The tubes were shaken for 10 min at room temperature. Results were observed by naked eyes. It was found that 6-thioguanosine could only be dissolved in *N,N*-dimethylformamide (DMF).

2-Amino-6-mercaptapurine riboside (37) (5g, 9 mmol) was co-evaporated with anhydrous pyridine (2 × 50 ml). The compound was then dissolved in 20 ml anhydrous *N,N*-dimethyl-formamide (DMF). 2,4-Dinitrofluorobenzene (4 ml, 10.8 mmol, 1.2 eq.) and 5 ml triethylamine were added to the solution. After 4 hrs, TLC showed that starting material had been converted into a new spot with a higher R_f value. *N,N*-dimethylformamide was removed under vacuum. The remaining solid mixture was recrystallized with methanol and yellow solid crystal was obtained (5.66 g, yield 75%). R_f 0.63 (20% MeOH/CH₂Cl₂). mp 214.6°C. NMR spectrum (d₆-DMSO), 3.47-3.64 (m, 2H, 5'-H, 5''-H); 3.89 (m, 1H, 4'-H); 5.07 (t, 1H, 5'-OH); 5.20 (d, 1H, 3'-OH); 5.50 (t, 1H 2'-OH); 4.47 (m, 1H, 2'-H); 4.10 (m, 1H, 3'-H); 5.80 (s, 1H, 1'-H); 7.96 (d, 1H, 6-H of 2,4-dinitrophenyl); 8.32 (s, 1H, 8-H); 8.40 (d, 1H, 5-H of 2,4-dinitropheny l); 8.88 (s, 1H, 3-H of 2,4-dinitropheny).

HRMS calculated for C₁₆H₁₅N₇O₈S [M+Na]⁺ 488.0601, found 488.0592.

4.3.2 *N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (39)

6-(2,4,-Dinitrophenyl)thioguanosine (38) (4g, 2.12 mmol) was co-evaporated with anhydrous pyridine twice (100 ml). The compound was re-dissolved in 50 ml anhydrous pyridine. Chlorotrimethylsilane (5 ml, 10.6 mmol) was added. After 5 hours, TLC (17% MeOH/CHCl₃) showed that new compound with a higher R_f value was formed. Phenoxyacetyl chloride (3.8 ml, 2.12 mmol) was then added. The mixture was left overnight under stirring. TLC (10% MeOH/CHCl₃) showed that a new compound was formed with a higher R_f value.

The reaction mixture was cooled in an ice-bath and 9 ml of water was added followed by 9 ml of 35% ammonia (NH₃•H₂O). The mixture was allowed to stir under this condition for 5 min and then poured into 200 ml dichloromethane (CH₂Cl₂). TLC (17%MeOH/CHCl₃) showed that a new compound was formed with a lower R_f value. The solution was washed by brine (2 × 400 ml), dried over anhydrous Na₂SO₄ and purified by silica gel G60 (CH₂Cl₂; 0-4%MeOH/CH₂Cl₂). The fractions containing desired product were combined and evaporated to a light yellow solid (1.94 g, 35%). R_f: 0.53(17%MeOH/CHCl₃). mp 176.3°C. NMR spectrum (d₆-DMSO): 3.34-3.58 (m, 2H, 5'-H, 5''-H); 3.94 (m, 1H, 4'-H); 4.18 (m, 1H, 3'-H); 4.59 (m, 1H, 2'-H); 4.79 (s, 2H, PhOCH₂CO); 5.94 (s, 1H, 1'-H); 6.80-6.92 (m, 3H, Ar); 7.25 (m, 2H, Ar); 8.21 (d, 1H, 6-H of 2,4-dinitrophenyl); 8.37 (d, 1H, 5-H of 2,4-dinitrophenyl); 8.72 (s, 1H, 8-H); 8.87 (s, 1H, 3-H of 2,4-dinitrophenyl); 10.87 (s, 1H, NH).

HRMS calculated for C₂₄H₂₁N₇O₁₀S [M+Na]⁺ 622.0968, found 622.0975.

4.3.3 5'-Methoxytrityl-*N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (40)

*N*²-Phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (39) (0.5 g, 1 mmol) was co-evaporated with anhydrous pyridine (2 × 50 ml) and then dissolved in 30 ml

anhydrous pyridine. 4-Methoxytrityl chloride (0.37 g, 1.2 mmol) was added to the solution. The mixture was stirred for 18 hours. TLC (40% EtOAc/CH₂Cl₂) showed that a new compound with a higher R_f value was formed. The reaction mixture was concentrated to 20 ml under vacuum and poured into 150 ml CH₂Cl₂ and washed with brine (2 × 100 ml) and dried over anhydrous Na₂SO₄. The solution was concentrated under vacuum to yield a solid product which was further purified by silica gel G60 (15% EtOAc/CHCl₃). The fractions containing the desired product were combined and evaporated to a pale yellow solid (0.35g, 49%). R_f: 0.46(10%MeOH/CH₂Cl₂). mp 117.3°C. NMR spectrum (d₆-DMSO): 3.13-3.16 (m, 2H, 5'-H, 5''-H); 3.35 (m, 1H, 4'-H); 3.69 (s, 3H, CH₃O-); 4.05 (m, 1H, 3'-H); 4.29 (m, 1H, 2'-H); 4.73 (s, 2H, PhOCH₂CO); 5.24 (d, 1H, 3'-OH); 5.68 (d, 1H 2'-OH); 6.01 (s, 1H, 1'-H); 6.75-6.96 (m, 4H, Ar); 7.15-7.29 (m, 15H, Ar); 8.15 (d, 1H, 6-H of 2,4-dinitrophenyl); 8.46 (d, 1H, 5-H of 2,4-dinitrophenyl); 8.61 (s, 1H, 8-H); 8.89 (s, 1H, 3-H of 2,4-dinitrophenyl); 10.84 (s, 1H, NH).

HRMS calculated for C₄₄H₃₇N₇O₁₁S [M+Na]⁺ 894.2169, found 894.2150.

4.3.4 2'-*tert*-Butyldimethylsilyl-5'-methoxytrityl-*N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (41c)

5'-Methoxytrityl-*N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (40) (0.5 g, 0.5 mmol) was co-evaporated with anhydrous pyridine and dried overnight over P₂O₅ *in vacuo*. The compound was dissolved in 20 ml dry tetrahydrofuran (THF). Anhydrous pyridine (2 mmol) and AgNO₃ (0.11 g, 0.65 mmol), which had been dried over P₂O₅ *in vacuo*, were then added and the mixture was stirred for 5 min until almost all AgNO₃ dissolved. *tert*-Butyldimethylsilyl chloride (0.097 g, 0.65 mmol.) was added at once and the resulting cloudy-milky solution was stirred at room

temperature for 10 hours. TLC showed spots corresponding to 2',3'-diTBDMS, 2'-TBDMS, 3'-TBDMS isomers with higher R_f values than the starting compound (40). The solution was filtered off into saturated brine and extracted with CH_2Cl_2 (2×50 ml). The combined organic solution was dried over anhydrous Na_2SO_4 and evaporated to yield pale yellow foam.

Purification was carried out by two-steps column chromatography with silica gel. First, the column was eluted by slow elution of ether: CHCl_3 (gradient 10%-15%). Trace amounts of 2', 3'-TBDMS isomer (41a) eluted first from the column, followed by pure 2'-TBDMS isomer (41c) and mixture of 2'-TBDMS isomer and 3'-TBDMS isomer (41b). The pooled fractions containing the mixture of 2' and 3' isomers were evaporated and re-purified by silica gel (5-10% Ether/ CHCl_3). The fractions containing 2'-TBDMS isomer were pooled together and evaporated under vacuum to a pale yellow solid (0.159g 56%). R_f : 0.63 (50%EtOAc/Hexane) NMR spectrum (CDCl_3): 3.70 (m, 2H, 5'-H, 5''-H); 3.70 (m, 3H, CH_3O -); 3.85 (m, 1H, 4'-H) 4.21 (m, 1H, 3'-H); 4.33 (m, 1H, 2'-H); 4.50 (s, 2H, PhOCH_2CO); 4.98 (s, 1H, 3'-OH); 6.04 (d, 1H, 1'-H); 6.77-6.87 (m, 4H, Ar); 7.18-7.38 (m, 15H, Ar); 8.21 (s, 1H, NH); 8.29 (m, 2H, 5-H and 6-H of 2,4-dinitrophenyl); 8.80 (s, 1H, 8-H); 8.89 (s, 1H, 3-H of 2,4-dinitrophenyl).

HRMS calculated for $\text{C}_{50}\text{H}_{51}\text{N}_7\text{O}_{11}\text{S}$ $[\text{M}+\text{Na}]^+$ 1008.3034, found 1008.3063.

4.3.5 **2'-tert-Butyldimethylsilyl-5'-methoxytrityl- N^2 -phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanine-3'-O-[2-cyanoethyl-(N,N -diisopropylamino)]phosphoramidite (42)**

2'-*tert*-Butyldimethylsilyl-5'-methoxytrityl-*N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (42) (0.1 g, 0.084 mmol) was co-evaporated with anhydrous pyridine and dried overnight with P₂O₅ *in vacuo*. Compound 47 was then added to dry tetrahydrofuran (THF) together with 3.0 equivalents of phosphitylating reagent [*N,N*-diisopropyl(2-cyanoethyl)phosphonamidic chloride] (0.059 0.252 mmol) and 10 equivalents of diisopropylethylamine (0.108 0.84 mmol). The mixture was stirred for 12 hours at room temperature. TLC (10% EtOAc/Hexane) showed a new compound with slightly high R_f value. The reaction mixture was then poured into 20 ml CH₂Cl₂ and washed with brine (2 × 50 ml) and dried over anhydrous Na₂SO₄. The solution was evaporated under vacuum to a solid which was further purified with silica gel G60 (15-19% EtOAc/Hexane). Fractions containing the desired product were pooled and evaporated to a yellow solid (0.07g, 59.6%). R_f: 0.23(50%EtOAc/Hexane). ³¹P NMR spectrum (CDCl₃): 153.79, 152.82.

HRMS calculated for C₅₉H₆₉N₇O₁₂SSiP [M+Na]⁺ 1186.1707 found 1186.1723.

4.3.6 5'-Dimethoxytrityl-*N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (53)

*N*²-Phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (39) (6.89 g, 1.0 mmol) was co-evaporated with anhydrous pyridine (2 × 50 ml) and then dissolved in 30 ml anhydrous pyridine. 4',4-Dimethoxytrityl Chloride (0.387 g, 1.2 mmol) was added to the solution. The mixture was stirred over night. TLC (40% EtOAc/Hexane) showed a new compound with a higher R_f = 0.44. The reaction mixture was poured into 50 ml CH₂Cl₂ and washed by brine (2 × 75 ml) and dried over anhydrous Na₂SO₄. The solution was concentrated under vacuum to yield a solid product, which was further purified by silica gel G60 (35-65% EtOAc/Hexane). The fractions containing desired

product were combined and evaporated to a pale yellow solid (9.19g, 90%). R_f : 0.44(30%CH₂Cl₂/EtOAc). mp 117.3°C. NMR spectrum (d₆-DMSO): 3.13-3.16 (m, 2H, 5'-H, 5''-H); 3.35 (m, 1H, 4'-H); 3.69 (s, 3H, CH₃O-); 4.05 (m, 1H, 'H); 4.29 (m, 1H, 2'-H); 4.73 (s, 2H, PhOCH₂CO); 5.24 (d, 1H, 3'-OH); 5.68 (d, 1H 2'-OH); ; 6.01 (s, 1H, 1'-H); 6.75-6.96 (m, 4H, Ar); 7.15-7.29 (m, 15H, Ar); 8.15 (d, 1H, 6-H of 2,4-dinitrophenyl); 8.46 (d, 1H, 5-H of 2,4-dinitrophenyl); 8.61 (s, 1H, 8-H); 8.89 (s, 1H, 3-H of 2,4-dinitrophenyl); 10.84 (s, 1H, NH)

4.3.7 2'-*tert*-Butyldimethylsilyl-5'-dimethoxytrityl-*N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (54b)

5'-Dimethoxytrityl-*N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (53) (0.5 g, 0.554 mmol) was co-evaporated with anhydrous pyridine and dried overnight over P₂O₅ *in vacuo*. The compound was dissolved in 20 ml dry tetrahydrofuran. Dry pyridine (2.216 mmol) and AgNO₃ (0.077 g, 0.72 mmol), which had been dried over P₂O₅ *in vacuo*, were added and the mixture was stirred for 5 min until almost all AgNO₃ dissolved. *tert*-Butyldimethylsilyl chloride (0.1 g, 0.72 mmol) was added at once and the resulting cloudy-milky solution was stirred at room temperature for 10 hours. TLC (diethyl ether) showed spots corresponding to 2',3'-diTBDMS, 2'-TBDMS, 3'-TBDMS isomers. The solution was filtered off into saturated brine and extracted with CH₂Cl₂ (2×50 ml). The combined organic solution was dried over anhydrous Na₂SO₄, filtered, and evaporated to yield a pale yellow foam.

Purification was carried out by two-steps column chromatography on silica gel. First, the column was eluted by slow elution of ether: CHCl₃ (gradient 10%-15%). Trace amounts of 2',3'-diTBDMS isomer eluted first from the column, followed by pure 2'-

TBDMS isomer and the mixture of 2'-TBDMS isomer and 3'-TBDMS isomer. The pooled fractions containing the mixture of 2' and 3' isomers were evaporated and re-purified by silica gel (5-10% Ether/CHCl₃). The pooled fractions containing 2'-TBDMS isomer were evaporated under vacuum to a pale yellow solid (0.159g 56%). R_f: 0.44 (40%EtOAc/Hexane) NMR spectrum (CDCl₃): 3.70 (m, 2H, 5'-H, 5''-H); 3.70 (m, 3H, CH₃O-); 3.85 (m, 1H, 4'-H); 4.21 (m, 1H, 3'-H); 4.33 (m, 1H, 2'-H); 4.50 (s, 2H, PhOCH₂CO); 4.98 (s, 1H, 3'-OH); 6.04 (d, 1H, 1'-H); 6.77-6.87 (m, 4H, Ar); 7.18-7.38 (m, 15H, Ar); 8.21 (s, 1H, NH); 8.29 (m, 2H, 5-H and 6-H of 2,4-dinitrophenyl); 8.80 (s, 1H, 8-H); 8.89 (s, 1H, 3-H of 2,4-dinitrophenyl).

4.3.8 Attempting to synthesis 2'-*tert*-butyldimethylsilyl-5'-dimethoxytrityl-*N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanine-3'-*O*-[2-cyanoethyl-(*N,N*-diisopropylamino)] phosphoramidite (55)

2'-*tert*-Butyldimethylsilyl-5'-dimethoxytrityl-*N*²-phenoxyacetyl-6-(2,4-dinitrophenyl)thioguanosine (54b) (0.1 g, 0.098 mmol) was co-evaporated with anhydrous pyridine and dried overnight with P₂O₅ *in vacuo*. Compound was then added to dry tetrahydrofuran (THF) together with 3.0 equivalents of phosphitylating reagent [*N,N*-diisopropyl(2-cyanoethyl)phosphonamidic chloride] (0.069 g, 0.294 mmol) and 10 equivalents of diisopropylethylamine (0.126 g, 0.98 mmol). The mixture was stirred for 12 hours. TLC plate showed a decomposed spot with lower R_f value.

4.3.9 Synthesis of *S*-methyl thioguanosine (58)

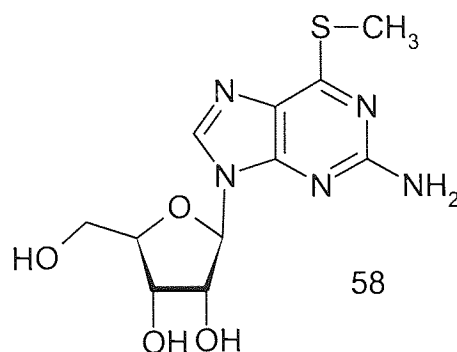


Figure 4-2. Structure of *S*-Methyl thioguanosine

6-Thioguanosine (37) (0.2 g, 0.668 mmol) was dissolved in 20 ml phosphate buffer (pH 10.25). Iodomethane (0.099 g, 0.7 mmol) was added. The mixture was stirred for 1 hr under RT. TLC showed the formation of a new compound. The mixture was co-evaporated with ethanol. The precipitate was filtered off. And the remaining solvent was dried to a white compound. $R_f = 0.23$ (MeOH/CH₂Cl₂) mp 190 °C. NMR ¹H (d₆-DMSO): 2.65 (3H, s, SCH₃); 3.32-3.58 (m 2H, 5'-H, 5''-H); 3.94 (m, 1H, 4'-H); 4.18 (m, 1H, 3'-H); 4.59 (m, 1H, 2'-H); 5.94 (s, 1H, 1'-H); 7.25 (m, 2H, Ar); 8.22 (s, 1H, 8-H).

4.3.10 Synthesis of *S*-(2-acetamido)-thioguanosine (59)

Thioguanosine (37) (0.2 g, 0.667 mmol) was dissolved in 20 ml phosphate buffer (pH 10.25). Iodoacetamide (0.12 g, 0.7 mmol) was added. The mixture was stirred for 2 hrs under room temperature. TLC showed the formation of new compound with lower R_f value. The mixture was co-evaporated with ethanol. The precipitate was filtered through and the remaining solvent was dried to white compound. $R_f = 0.23$ (MeOH/CH₂Cl₂) mp 190 °C. NMR ¹H (d₆-DMSO) 3.32-3.58 (m 2H, 5'-H, 5''-H); 3.89 (1H, s, 4'-H); 3.90 (2H, s, CH₂CO); 4.10 (m, 1H, 3'-H); 4.46 (m, 1H, 2'-H); 5.78 (d, 1H, 1'-H); 6.56 (2H, s, NH₂); 7.15~7.47 (m, 2H, CONH₂); 8.22 (s, 1H, 8-H).

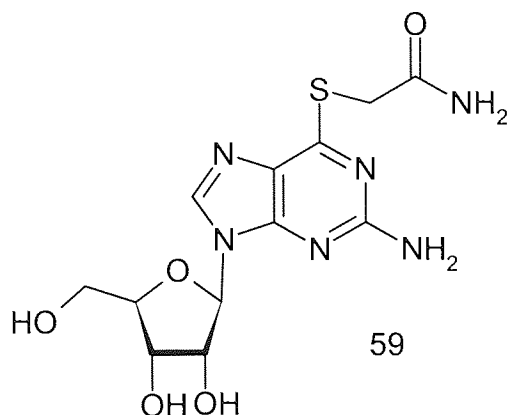


Figure 4-3. Structure of *S*-(2-acetamido)-thioguanosine

4.4 Synthesis and purification of RNA

4.4.1 General methods of synthesis

It is critical that sterile equipment, reagents, and handling techniques be used in handling free oligoribonucleotides all the time. Automated solid phase RNA assembly was carried out on a 1.0 μmol scale using a EXPETIDETM DNA/RNA synthesiser following the standard protocol recommended by manufacturer the deprotection time for removing thioguanosine phosphoramidite was extended to 180 s from 60 s and the coupling time was extended to 30 min from 10 min. G^{SH} phosphoramidite monomer was dissolved in anhydrous acetonitrile (1 mg/ml) and put into bottle 7. Details of RNA sequences are listed below (Figure 4-4).

12 mer

Parent Sequence 5'-UAC CAG UGA GCU-3'

Complementary Sequence 5'-AGC UCA CUG GUA-3'

Modified Sequence 5'-UAC CAG^{SH} UGA GCU-3'

5 mer

Parent Sequence 5'-GCG AU-3'

Complementary Sequence 5'-AUC GC-3'

Modified Sequence 5'-GCG^{SH} AU-3'

Figure 4-4. RNA sequences.

4.4.2 Deprotection of modified RNA

The deprotection of modified RNA involved three steps:

1) 10% mercaptoethanol/acetonitrile with drops of ethylamine was added to the oligomer which was still bound to the solid-support (CPG) to remove the 2,4-dinitrophenyl group. The suspension was shaken and left at room temperature for 4 hours. The supernatant was then removed and the solid support was washed with acetonitrile dried with Speed-Vac.

2) The oligomers were then treated with methanolic ammonia for 12 hours at 30°C. Methanolic ammonia was removed by Speed-Vac. Fresh methanolic ammonium was made by bubbling ammonia into anhydrous methanol for 30 min under 0°C.

3) Dried oligomers were then treated with triethylamine trihydrofluoride/DMSO (50:50, v:v) for 24 hours to remove the silyl group on 2'-hydroxyl position. The deprotection reagents were then removed by Freeze-Drier.

The deprotection of normal RNA sequence is easier than modified RNA monomer. Normal RNA sequence was first treated with anhydrous methanolic ammonia at 30°C for 14 hrs. The ammonia solution was then removed by Speed-Vac. The solid support

together with RNA oligomer was then treated with triethylamine trihydroufluoride/DMSO for at least 24 h under room temperature.

4.4.3 Stability of thioguanosine towards the deprotection solutions

The stability of 6-thioguanosine towards the deprotection conditions was tested by the following procedures. The nucleoside was dissolved in the above deprotection solutions. After certain period, the sample was checked by UV spectrometry to find out the integraty of 6-thioguanosine.

4.4.4 Purification of RNA

Method A

Method A used NENSORB column to purify RNA. The procedures were similar to those for DNA purifications, except that RNA in DMSO solution was diluted with 10-20 ml of 0.1 M TEAA.

Method B

OPC column was used to purify small quantity of RNA (< 5.0 O.D.) following the instructions of the manufacturer. The oligomers were loaded onto the column by pulling solvent through the column. Failure sequences and by-products were removed simultaneously because of their lack of hydrophobic groups "DMT". The column was then washed by 2.5% TFA to remove the DMT group the on 5'-hydroxyl position of the oligomers. Distilled water was then used to wash away the acid. Oligomer was eluted by 35% methanol/water. Nevertheless, the UV measurement of the eluate collected from each step of the purification showed that most oligomers came out at

the second step (still mixed with failure-sequences and by products), while there was few oligomers in the eluate (35% CH₃/H₂O) of the last step.

4.4.5 HPLC

Reverse phase HPLC was used both to detect and purify oligomers after NENSORB column purification. HPLC C-18 column was used in the experiments. The column was conditioned by 100% buffer A for 10 min. The oligomer samples were then injected into HPLC. The gradients elution was formed as follows: 0 min, 0% buffer B; 30 min, 30% buffer B; 35 min, 100% buffer B; 45 min, 100% buffer B; 50 min, 0% buffer B (Buffer A: 98% 0.1 M TEAA/CH₃CN pH 7.0; buffer B: 20% 0.1 M TEAA/CH₃CN pH 7.0). The flow rate was 1 ml/min and sample was detected at either 260 nm or 340 nm. The desired peak was collected and measured by UV spectrometry.

4.4.6 Post-synthetic modification of RNA

Post-synthetic modification was carried out using the HPLC purified oligomers in 50 mM phosphate buffer (pH 8.5). Electrophilic reagents were added to the solution. The mixture was shaken at room temperature for varied time according to the individual electrophilic reagent used. Usually, reaction with iodomethane was left for 2 hours at room temperature, while with benzyl bromide for 40 min.

The mixture was then analysed with HPLC with the same program as in section 4.3.5, and monitored at 260 nm. All peaks were collected in eppendorf tubes and UV measurement was carried out immediately to confirm the existence of oligomers.

4.4.7 Nucleosides composition analysis

Base composition analysis of the oligoribonucleotides made in this chapter was followed the method described by Allerson *et al.* (1997). 0.7 O.D. (A_{260} units) of oligomers were dissolved in 120 μ l of a solution containing 0.2 mM $ZnCl_2$, 16 mM $MgCl_2$, 250 mM Tris-HCl (pH 6.0). Snake venom phosphodiesterase I (0.2 unit) was added and the mixture incubated (37°C) for 30 min, then alkaline phosphatase (4 units) was added. The mixture was further incubated for 8 hrs. The nucleosides were separated by HPLC with a gradient elution from 0% buffer B to 20% buffer B over 30 min. The digest was divided into two equal parts and injected into HPLC in turn. The first injection was monitored at 260 nm, and the second part of HPLC at 340 nm. Retention times were: C, 4.5 min; U, 5.6 min; I (from enzymatic hydrolysis of A), 8.2 min; G 8.8 min; G^{SH} 10.5min (HPLC gradient; A: 98% 0.1 M TEAA/ CH_3CN pH 7.0; B: 20% 0.1 M TEAA/80% CH_3CN pH 7.0; 20 min 30% B).

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