CHEMICAL SYNTHESIS OF ISOTOPICALLY LABELLED (M+4) PURINE NUCLEOSIDES AND THEIR INCORPORATION INTO DNA OLIGOMERS

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A thesis submitted for the degree of Doctor of Philosophy

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Summary

Development of accurate and sensitive analytical methods to measure the level of biomarkers, such as 8-oxo-guanine or its corresponding nucleoside, 8-oxo-2'-deoxyguanosine, has become imperative in the study of DNA oxidative damage *in vivo*. Of the most promising techniques, HPLC-MS/MS, has many attractive advantages. Like any method that employs the MS technique, its accuracy depends on the use of multiply, isotopically-labelled internal standards. This project is aimed at making available such internal standards.

The first task was to synthesise the multiply, isotopically-labelled bases (M+4) guanine and (M+4) 8-oxo-guanine. Synthetic routes for both (M+4) guanine and (M+4) 8-oxo-guanine were designed and validated using the unlabelled compounds. The reaction conditions were also optimized during the "dry runs". The amination of the 4-hydroxy-2,6-dichloropyrimidine, appeared to be very sensitive to the purity of the commercial [15]N benzylamine reagent. Having failed, after several attempts, to obtain the pure reagent from commercial suppliers, [15]N benzylamine was successfully synthesised in our laboratory and used in the first synthesis of (M+4) guanine.

Although (M+4) bases can be, and indeed have been used as internal standards in the quantitative analysis of oxidative damage, they can not account for the errors that may occur during the early sample preparation stages. Therefore, internal standards in the form of nucleosides and DNA oligomers are more desirable.

After evaluating a number of methods, an enzymatic transglycolization technique was adopted for the transfer of the labelled bases to give their corresponding nucleosides. Both (M+4) 2-deoxyguanosine and (M+4) 8-oxo-2'-deoxyguanosine can be purified on micro scale by HPLC. The challenge came from the purification of larger scale (>50 mg) synthesis of nucleosides. A gel filtration method was successfully developed, which resulted in excellent separation of (M+4) 2'-deoxyguanosine from the incubation mixture. The (M+4) 2'-deoxyguanosine was then fully protected in three steps and successfully incorporated, by solid supported synthesis, into a DNA oligomer containing 18 residues.

Thus, synthesis of 8-oxo-deoxyguanosine on a bigger scale for its future incorporation into DNA oligomers is now a possibility resulting from this thesis work. We believe that these internal standards can be used to develop procedures that can make the measurement of oxidative DNA damage more accurate and sensitive.

Key words: *DNA oxidative damage; internal standards; 8-oxo-dG; isotopically labelled guanine; DNA oligonucleotide.*

To my parents and my husband

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ABBREVIATIONS

^{1}H NMR	proton nuclear magnetic resonance
2D/3D- NMR	2/3 dimensional nuclear magnetic resonance
5-OHC	5-hydroxycytosine
8-oxo-dA	8-oxo-2'-deoxyadenosine
8-oxo-dG	8-oxo-2'-deoxyguanosine
8-oxo-dGMP	8-oxo-2'-deoxyguanosine monophosphate
8-oxo-dGTP	8-oxo-2'-deoxyguanosine triphosphate
8-oxo-G	8-oxo-guanosine
8-oxo-Gua	8-oxo-guanine
A	adenine
AIDS	acquired immunodeficiency syndrome
AP site	apurinic/apyrimidinic site
ATP	adenosine triphosphate
BER	base excision repair
C	cytosine
CIL	Cambridge Isotope Laboratory
CPG	controlled pore glass
CSPD	calf spleen phosphodiesterase
DEAE	diethylaminoethyl
dG	2'-deoxyguanosine
DMEA	dimethylethylacetate

DMSO	dimethyl sulphoxide
DMT(r)	dimethoxytrityl-
DNA	deoxyribonucleic acid
dNMPs	deoxynucleotide monophosphates
dNTPs	deoxynucleotide triphosphates
dT	thymidine
EI	electron impact
ELISA	enzyme-linked immunosorbent assay
ESCODD	European Standards Committee on Oxidative DNA Damage
Fapy	formamidopyrimidine
Fapy-Ade	4,6-diamino-5-formamidopyrimidine
Fapy-Gua	2,6-diamino-4-hydroxy-5-formamidopyrimidine
Fpg	formamidopyrimidine DNA N-glycosylase
G	guanine
GC-MS	gas chromatography associated with mass spectrometry detection
GSH	glutathione
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HPLC-ECD	high performance liquid chromatography coupled to electrochemical detection
HPLC-MS/MS	high performance liquid chromatography associated with tandem mass spectrometry detection
HRMS	high resolution mass spectrometry

HRMS	high-resolution mass spectra
iBu	isobutyryl-
IDMS	isotope dilute mass spectrometry
Me-Fapy-Gua	2,6-diamino-4-hydroxy-5-N-mehtylformamidopyrimidine
MLS	maximum lifespan
MN	micrococcal nuclease
MRM	multiple reaction monitoring
MS-Cl	mesitylene chloride
MSNT	1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-1*H*-triazole
NER	nucleotide excision repair
NIDDM	non-insulin-dependent diabetes mellitus
NMR	nuclear magnetic resonance
OD	optical density
PEI	polyethyleneimine
PFK	perfluorokerosene
PNPase	purine nucleoside phosphorylase
PRM	phosphoribomutase
ROS	reactive oxygen species
RP-HPLC	reversed phase high performance liquid chromatography
SCGE	single-cell gel electrophoresis
SLE	systemic lupus erythematosus
SOD	superoxide dismutase
T	thymidine

TEA	triethylamine
TEAA	triethylammonium acetate
TFA	trifloroacetic acid
Tg	thymidine glycol
THF	tetrahydrofuran
Thy	thymine
TLC	thin layer chromatography
TMS-Cl	trimethylsilyl chloride
TPase	thymidine phosphorylase
TPS-Cl	2,4,6-triisopropylbenzene-sulfonyl chloride
UV	ultraviolet spectroscopy

Chapter 1 Introduction

In the past two decades, tremendous advances have been made in our understanding of the mechanism of gene expression and of the role of reactive oxygen species (ROS) in producing DNA damage (Halliwell & Aruoma 1991). Evidence has shown that DNA oxidative damage has been implicated in several important heath issues, including cancer and ageing (Ames & Gold 1991) (Loft & Poulsen 1996) (Halliwell & Gutteridge 1999). Intensive research has been carried out to study the mechanism regarding the formation of these damages, the defence system of organisms, as well as the repair of DNA damage *in vivo*. Biomarkers are proposed as an index for cancer risk, or used to identify hazardous factors in the environment, to assess the influence of diet/life style on our health and the efficacy of certain foods and supplements in their roles as antioxidants. Development of accurate and facile analytical methods to identify and measure these biomarkers has become imperative in the studies of DNA oxidative damage.

Cellular level of oxidatively damaged bases/corresponding nucleosides and the urinary level of excreted free bases are often used as biomarkers to assess the level of DNA oxidative damage, among which 8-oxo-Gua and its corresponding nucleoside 8-oxo-dG have retained a lot of interests and have been intensively studied. Numerous analytical methods have been established to measure this

favoured biomarker, however, large discrepancies exist between the methods used and between different laboratories using the same methods, and the reported discrepancies are often over several orders of magnitude (Collins, *et al.* 1997). The lack of consensus over the level of 8-oxo-Gua/8-oxo-dG *in vivo* has triggered heated debates over the issue of the methodology of analysing oxidative DNA damage. Tremendous efforts have been made, particularly by the European Standards Committee on Oxidative DNA Damage (ESCODD), to resolve this issue (Lunec 1998). Despite achievements made, according to a recent report (Collins, *et al.* 2004), there is still a discrepancy of about ten fold, and it is believed to result from still uncontrolled oxidation in the early stages of DNA sample preparation.

One of the most promising techniques, HPLC-MS/MS, has shown great potential. It combines the versatility of GC-MS and the intrinsic sensitivity of HPLC-ECD. Its accuracy depends on the use of multiply isotopically labelled authentic internal standards. The synthesis and application of a number of multi-isotopically labelled compounds, including (M+4) 8-oxoguanosine and (M+3) guanine, as such internal standards have been published (LaFrancois, *et al.* 1998) (Nelson 1996) (Stadler, *et al.* 1994) (Hamberg & Zhang 1995). Since these compounds are monomers, they can not account for the loss or spurious gain of the target product that may occur during the early stages of sample preparation, especially during DNA isolation and hydrolysis/digestion.

In this project, we are aiming at the preparation of multiply isotopically labelled authentic internal standards in the form of DNA oligomers. We believe they can be used to develop a procedure that can make further improvement of existing

analytical procedures by adding them to the sample prior to the DNA isolation. We also hope that this approach can be used to develop a general route for establishing reliable and accurate analytical methods for the detection of other biomarkers.

A brief review of the issues, including the mechanism of the formation of oxidative DNA damage and its consequences, will be given in the following sections. The analytical methods available, their associated problems and the aim of this project will also be discussed in detail.

1.1 Oxidative Stress and DNA Damage

It is well established that Reactive Oxygen Species (ROS)* are constantly generated in living cells as by-products of normal cellular metabolism (endogenous sources) and by exogenous sources. Endogenous sources of ROS come from normal cellular metabolism through various pathways, e.g. peroxisomal metabolism, enzymatic synthesis of nitric oxide and phagocytic leukocytes (Bohr, *et al.* 2002) (Beckman & Ames 1999). They can also be generated through exogenous sources, such as ionising irradiation (Breen & Murphy 1995) and other radical inducing agents.

Oxidative stress occurs when the balance between the generation and the

* Reactive Oxygen Species: a collective term that describes both the oxygen radicals: $O_2^{\bullet-}$, $OH^{\bullet}$; peroxyl ($RO_2^{\bullet}$); alkoxyl ($RO^{\bullet}$) radicals and other non-radical derivatives: H_2O_2, HOCl, singlet O_2, ozone] {Halliwell & Aruoma 1993}

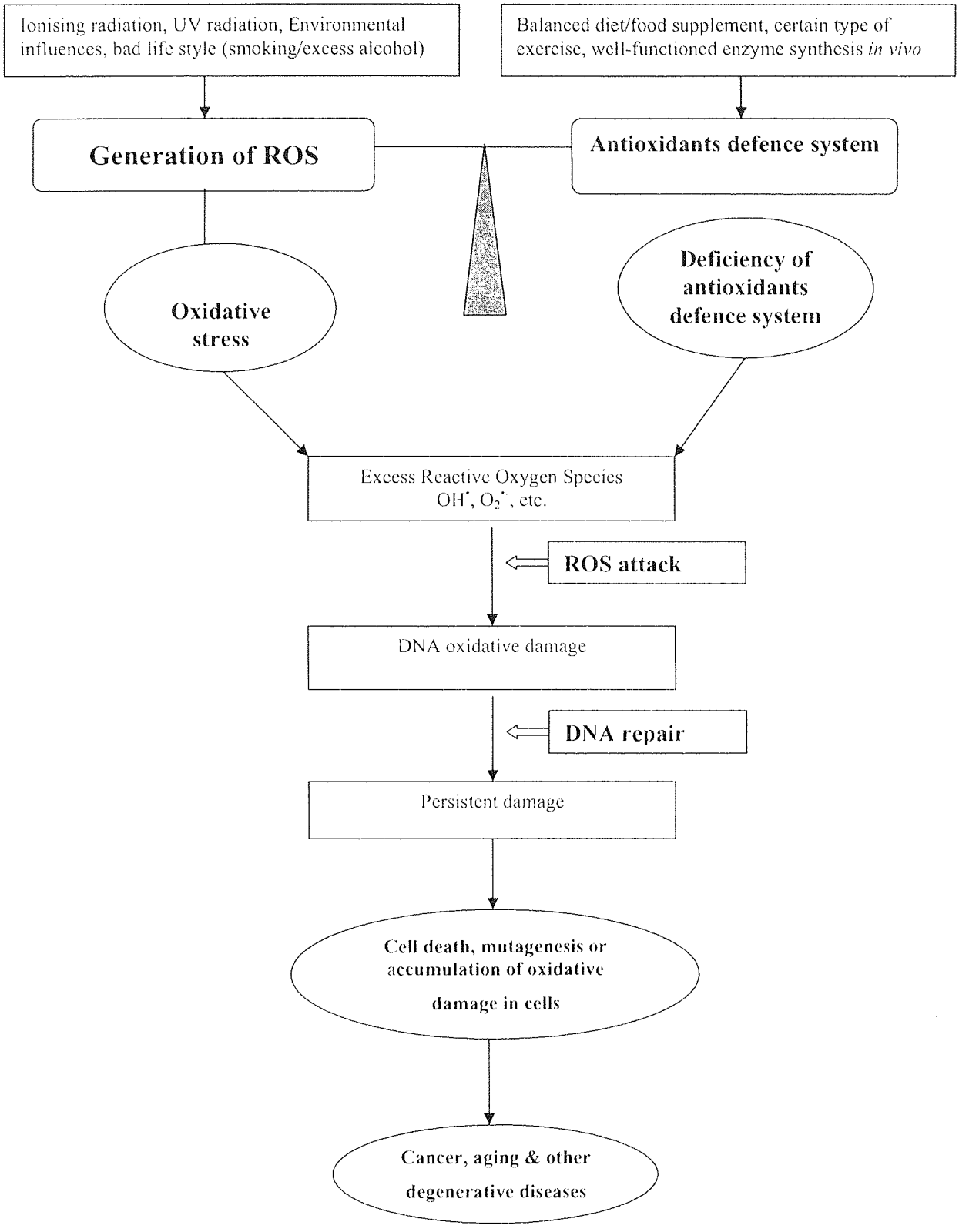


Figure 1.1. Schematic diagram of oxidative stress and its consequences.

elimination of ROS is tipped in favour of the former, e.g. when cells exposed to certain conditions, such as radiation and toxic materials or there is a deficiency of the defence system. Figure 1.1 illustrates the consequences of oxidative stress. Most tissues can tolerate mild oxidative stress, and it often leads to the induction of defence systems. These defences include enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, low-molecular-mass agents (examples being glutathione, ascorbic acid and α-tocopherol) and proteins that bind metal ions in forms that are unable to accelerate free radical reactions.

However, severe oxidative stress disrupts cell metabolism and can lead to cell death; it produces damage to DNA and other biological molecules, such as lipids and proteins.

Oxidative stress causes DNA damage by a variety of mechanisms. These damages are often referred to as DNA oxidative damage, which include: base or sugar modifications, strand breaks and DNA-protein cross links. Some of these damages can be removed through various pathways by the complex DNA repair system that exists in organisms. Oxidatively modified DNA bases are thought to be repaired mainly by the base excision repair (BER) and to a lesser degree by the nucleotide excision repair (NER), and the free modified bases are excreted into urine.

Modified bases that escaped the repair will remain in the cell reproduction cycle and lead to the induction of mutations and this is one of the critical events in carcinogenic transformation. The accumulation of macromolecular damage is implicated in the ageing process, shown by the increased amount of oxidative

damage to lipids, proteins and DNA in a variety of tissues in different species during ageing (Sohal & Weindruch 1996).

In this section, the focus will be on the following aspects: i) the mechanism of DNA oxidative damage formation that results from the ROS; ii) the human defence system against ROS; iii) the repair of some of these damages.

1.1.1 The mechanism of ROS mediated DNA damage

As mentioned earlier, there is a fine balance between the generation and elimination of ROS formation *in vivo*. Oxidative stress occurs when the ROS generation exceeds its elimination. An increase in the generation of ROS has been observed in events such as exposure to environmental pollutants (Marczynski, *et al.* 2000), certain drugs (Pagano, *et al.* 2001) (Viola, *et al.* 2000), smoking (Asami, *et al.* 1996) (Asami, *et al.* 1997), high fat diet (Loft, *et al.* 1998) and during some pathological processes such as inflammation (Ohnishi & Murata 2002). Whereas decreased elimination of ROS is the result of dysfunction of defence enzyme or nutrient deprivation (Conlon, *et al.* 2000).

The oxidative stress can also be imposed on the living cells under certain experimental conditions such as oxidising agents or ionising radiation (Fuciarelli, *et al.* 1989) (Karahalil, *et al.* 1998) (Wood, *et al.* 1990). ROS are highly reactive and can readily oxidise macromolecules in living cells, including lipids, proteins and nucleic acids, thus leading to various types of cellular dysfunction, including cell death and mutagenesis (Shigenaga, *et al.* 1994) (Ames, *et al.* 1993). Thanks to our defence system, which includes a series of enzymes and antioxidants, to

scavenge some of the ROS and, as a result, minimise the deleterious action of ROS. Further discussion with respect to the defence system and their function will be covered in the next section (1.1.2).

Among the various types of oxidative damage in cellular macromolecules, the damage to DNA is probably the most biologically significant lesion, because it affects the integrity and stability of the genome. Alteration of genetic information often leads to neoplasia and the development of degenerative diseases. Health issues associated with DNA oxidative damage will be discussed in section 1.2.

The exact mechanisms and nature of the species involved in DNA oxidative damage remain to be established (Saran, *et al.* 1998), but one of the frequently formed ROS, hydroxyl radicals ($OH^{\bullet}$) is generally regarded as the most reactive. The DNA modifications it induces, together with the resulting mutations, have been intensively studied (Breen & Murphy 1995) (Dizdaroglu, *et al.* 2002).

Hydroxyl radicals ($OH^{\bullet}$) can be generated by ionising radiation through one of the following two processes:

The first process involves the ionisation of a water molecule, which gives a hydrated electron (e^-_{aq}) and $H_2O^{\bullet+}$

$$H_2O \xrightarrow{h\nu} H_2O^{\cdot+} + e^-_{aq}$$

$H_2O^{\bullet+}$ then rapidly loses a proton to give the hydroxyl radical:

$$H_2O^{\cdot+} + H_2O \longrightarrow H_3O^{+} + HO^{\cdot}$$

The second process involves excitation of a water molecule followed by hydrolysis into $H^{\bullet}$ and a hydroxyl radical ($OH^{\bullet}$):

$$H_2O \xrightarrow{h\nu} H_2O^* \longrightarrow H^{\cdot} + HO^{\cdot}$$

$OH^{\bullet}$ can also be generated through two other frequently formed ROS: superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). Therefore, these radicals by themselves do not appear to cause any DNA damage under physiological conditions, and their toxicity is thought to result from the transition metal ion-catalysed conversion into hydroxyl radical (Fenton reaction) (Bohr, *et al.* 2002), i.e.

$$O_2^{\bullet-} + Fe(II) \rightarrow O_2 + Fe(III)$$

$$H_2O_2 + Fe(II) \rightarrow OH^{\bullet} + OH^{-} + Fe(III)$$

The hydroxyl radical is an electrophilic, highly reactive radical. It abstracts hydrogen atoms efficiently, but adds even more rapidly to double bonds. Thus, there are two main forms of modifications to the DNA: i) DNA base moiety modifications resulted from the addition of $OH^{\bullet}$ to the π–bonds of DNA bases; ii) sugar moiety modifications produced from hydrogen abstraction by $OH^{\bullet}$ from the deoxyribose sugar units in DNA (Breen & Murphy 1995). The discussion will now focus on the formation of base modifications, as these are biologically important.

Hydroxyl radicals react at diffusion controlled rates with the four free bases. They add to the π–bonds of DNA bases, at C-5 and C-6 of the pyrimidines, or C-4, C-5 and C-8 of the purines. Addition reactions yield OH-adduct radicals of DNA bases. Further reactions of these radicals yield numerous products.

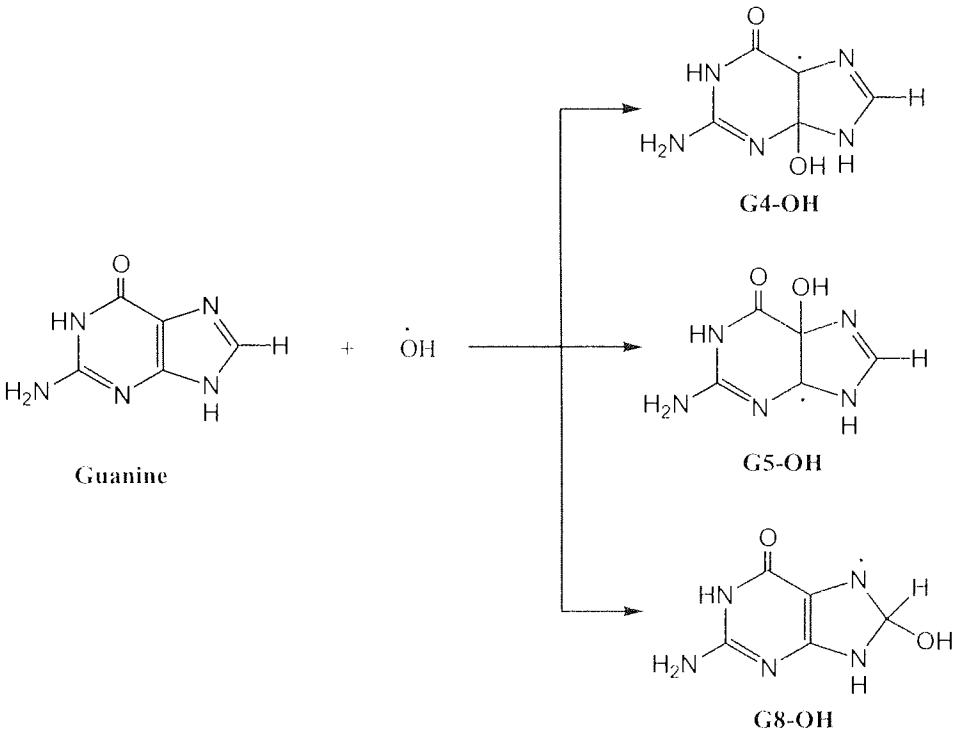


Figure 1.2. The reaction of $OH^{\bullet}$ with guanine.

As an example, Figure 1.2, Figure 1.3 and Figure 1.4 illustrate the reaction of guanine with $OH^{\bullet}$ and mechanisms of the formation of 8-hydroxyguanine (8-oxo-Gua) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua).

A hydroxyl radical adds to guanine giving rise to C4-OH-, C5-OH-, and C8-OH-adduct radicals (Figure 1.2), C4-OH- and C5-OH-adduct radicals undergo dehydration and yield oxidising Guanine (-H)$^{\bullet}$ radical, which reconstitutes guanine upon reduction (Figure 1.3).

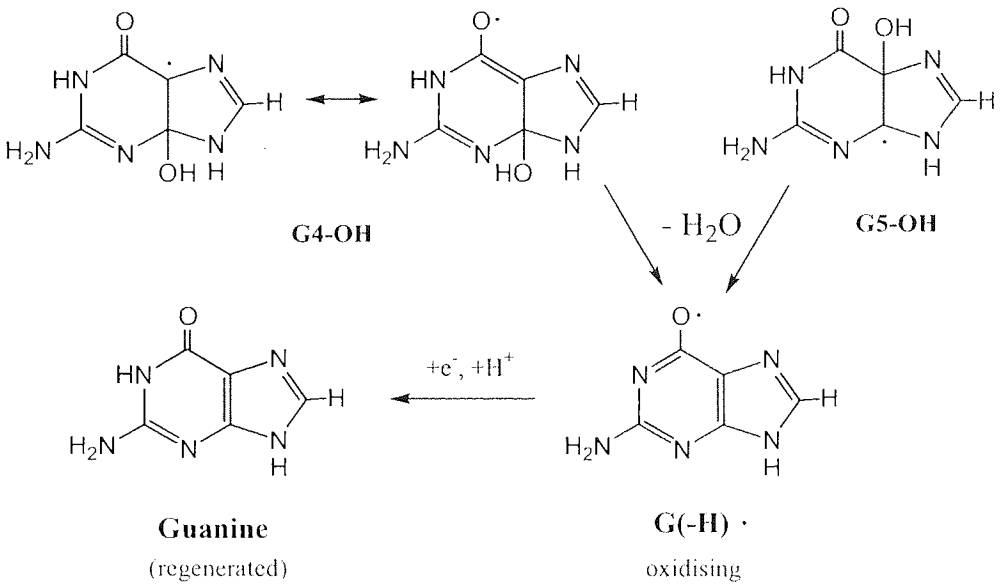


Figure 1.3. The regeneration of guanine from two of the hydroxyl radical adducts by dehydration [adapted from (Breen & Murphy 1995)].

One-electron oxidation and one-electron reduction of C8-OH-adduct radicals give rise to 8-oxo-Gua and formamidopyrimidines, respectively (Figure 1.4) (Dizdaroglu 1992) (Breen & Murphy 1995) (Dizdaroglu, *et al.* 2002). Both types of products are formed in either the absence or the presence of oxygen. Reducing agents increase the yield of formamidopyrimidines, whereas the formation of 8-oxo-purines is preferred in the presence of oxygen (Dizdaroglu 1992) (Breen & Murphy 1995). Hydroxyl radical generates multiple products in DNA by

mechanisms discussed above and others. Structures of the major modified bases generated by ROS in DNA are illustrated in Figure 1.5.

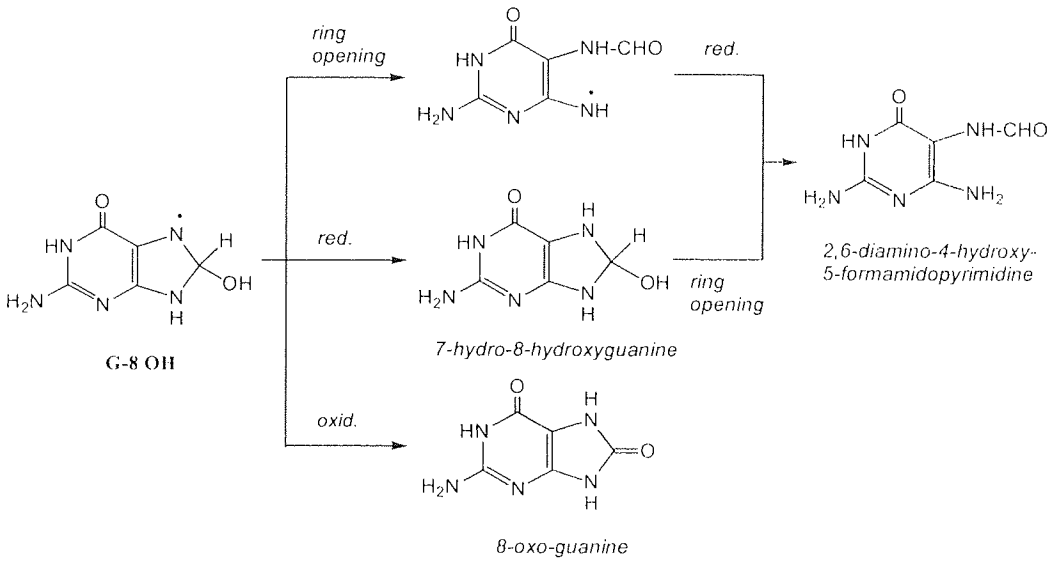


Figure 1.4. The mechanism of the formation of guanine derivatives from the C8-OH adduct radical [adapted from (Steenken 1989)].

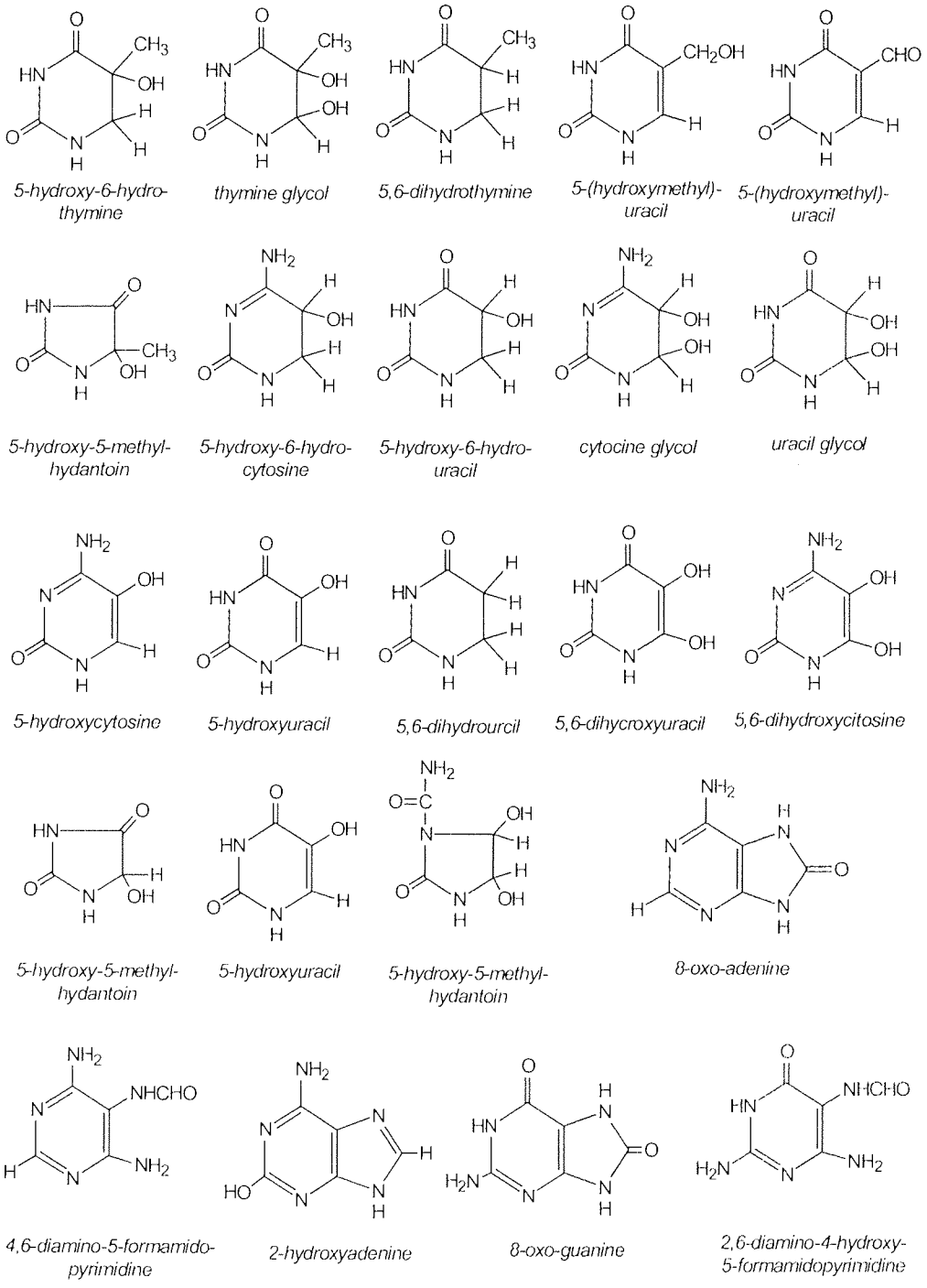


Figure 1.5. Structures of the major modified DNA bases generated by ROS [adapted from (Dizdaroglu et al. 2002)].

1.1.2 Antioxidant defence system

Cells have developed a comprehensive set of antioxidant defence mechanisms to prevent free radical formation and to limit their damaging effects. These mechanisms include enzymes to inactivate peroxides, proteins to sequester transition metals and a range of compounds to scavenge free radicals.

1.1.2.1 Enzymes that inactivate peroxides

These enzymes include superoxide dismutase, catalase and peroxidase.

Superoxide dismutases (SOD) are a group of widely distributed enzymes that remove the superoxide anion ($O_2^{\bullet -}$), with the formation of molecular oxygen and hydrogen peroxide, in the following reaction:

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2$$

The hydrogen peroxide (H_2O_2) formed is removed by the action of catalase (an enzyme found in peroxisomes), which directly catalyses the decomposition of H_2O_2 to water and ground-state O_2

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

H_2O_2 generated by several oxidase enzymes *in vivo* can also be removed by the same pathway.

Peroxidase enzymes remove H_2O_2 by using it to oxidise another substrate (SH_2)

$$SH_2+H_2O_2 \rightarrow S + 2H_2O$$

1.1.2.2 Sequestration of metal ions

Metal ions, such as iron and copper are essential in the human body for the synthesis of a huge range of enzymes and other proteins involved in biological processes, e.g. O_2 transport, and other redox reactions. However, they can also be dangerous when they act as powerful catalysts of autoxidation reactions (e.g. oxidation of adrenalin, dopamine and ascorbate), the conversion of H_2O_2 into $OH^{\bullet}$ (see 1.1.1) and the decomposition of lipid peroxides to reactive peroxyl and alkoxyl radicals.

Proteins that bind iron or copper ions will limit their pro-oxidant function by minimising their availability. Iron-binding proteins include transferrin, lactoferrin and ovotransferrin. Copper-binding proteins include caeruloplasmin, which binds 90% of total plasma copper, and albumin. Histones and some other small peptides also bind to a small percentage of copper in plasma.

1.1.2.3 Low-molecular-mass antioxidants

Low-molecular-mass antioxidants have demonstrated their ability of antioxidant defence by acting as radical scavengers through various pathways. Details of these antioxidants and their biological functions have been intensively reviewed (Halliwell & Gutteridge 1999). Only a few examples will be discussed here.

Some of the low-molecular-mass antioxidants are synthesised *in vivo*. Glutathione (GSH), for example, is synthesised *in vivo* in two steps (Meister 1995). First, the γ-glutamylcysteine synthetase catalyses dipeptide formation from L-glutamate and L-cysteine. Then the product is converted to GSH by glutathione synthetase. GSH is involved in many metabolic processes. Its antioxidant roles include i) preventing protein –SH groups from being oxidised or cross-linking, ii) scavenging reactive species (including $OH^{\bullet}$ and other free radicals), and iii) acting as chelator of copper ions and diminishing their ability to generate free radicals (Hanna & Mason 1992).

Another group of low-molecular-mass agents are derived from the diet. These include ascorbic acid (vitamin C), α-tocopherol, carotenoids and plant phenols. Ascorbic acid is one of the most studied low-molecular-mass antioxidants. At physiological pH, its mono anion is the favoured form, thus it is often referred to as ascorbate. One of the most important chemical properties of ascorbate is its ability to act as a reducing agent. The observation of its ability to inhibit the carcinogenic action of several toxic compounds fed to animals can also be partly attributed to its ability to reduce them to inactive forms. It can also reduce reactive species such as $O_2^{\bullet -}$ and $OH^{\bullet}$ and act as an radical scavenger.

Although *in vitro* experiments have demonstrated that ascorbate possesses a multiplicity of antioxidant properties, protecting various biomolecules against damage by ROS, direct evidence that ascorbate does act as an antioxidant *in vivo* is still limited (Halliwell 1996).

Only a minority of the low-molecule-mass antioxidants are synthesised by the cell itself. The majority, including ascorbic acid, α-tocopherol and carotenoids, are derived from dietary sources. Therefore, there is an intimate relationship between nutrition and antioxidant defence. The role of diet/food supplements as antioxidants in the prevention of the oxidative damage and maintaining good health have also attracted lots of research interest (see 1.2.5).

1.1.3 DNA repair system

All organisms possess a panel of highly efficient DNA repair systems to repair oxidatively damaged DNA, in order to maintain the integrity of the genome and to keep the fidelity during replication. These systems perform their repair function through various pathways and involve numerous enzymes. Failure of these systems to remove DNA damages leads to the induction of mutations, which is one of the critical events in carcinogenic transformation.

Most DNA lesions are repaired by either of the two excision repair processes, base excision repair (BER) and nucleotide excision repair (NER).

NER is one of the best studied DNA repair pathways (Van Houten 1990). It is a process consisting of DNA damage recognition, incision, excision, repair resynthesis, and DNA ligation. In this repair pathway, DNA damage is removed through incision of the damaged strand on both sides of the lesion. The patch size excised by NER is 12-13 nucleotides in prokaryotes and 24-32 nucleotides in eukaryotes including human (Sancar 1996). Many of the proteins that mediate *E.coli* nucleotide excision have been purified to homogeneity. One of the key

repair enzymes of this pathway is the uvrABC nuclease complex. The main role of NER involves the repair of bulky adducts produced by ROS or lipid peroxidation products. It also provides a back up when base excision repair of small oxidative lesions becomes saturated (Møller & Wallin 1998).

BER is thought to be the primary repair pathway to remove oxidatively damaged DNA bases (Krokan, *et al.* 1997) (Lindahl & Wood 1999). It is similar in the overall mechanisms to NER, but utilises different enzymes and removes shorter patches of 1-4 nucleotides in both prokaryotes and eukaryotes. Figure 1.6 shows the pathway of this repair. The key enzymes involved in this process are DNA glycosylases, which initiate the base excision pathway. They catalyse the cleavage of the N-glycosylic bond between the base and the sugar to release the free modified bases and leaving an apurinic/apyrimidinic (AP) site. The deoxyribose moiety that resides in the AP site after the action of the DNA glycosylase is removed by AP endonuclease enzymes (e.g., endonuclease III and endonuclease IV in *E. coli*). A DNA polymerase resynthesise the gap and a DNA ligase seals the nick in the strand. In addition to their N-glycosylic bond cleavage activity, several DNA glycosylases also display an AP lyase activity that cleaves the phosphodiester backbone 3' to the AP site generated by the glycosylase activity.

There are varieties of DNA glycosylases which have specificities for various DNA damages. For the specificities of these enzymes, see a review by Cadet *et al.* (2000).

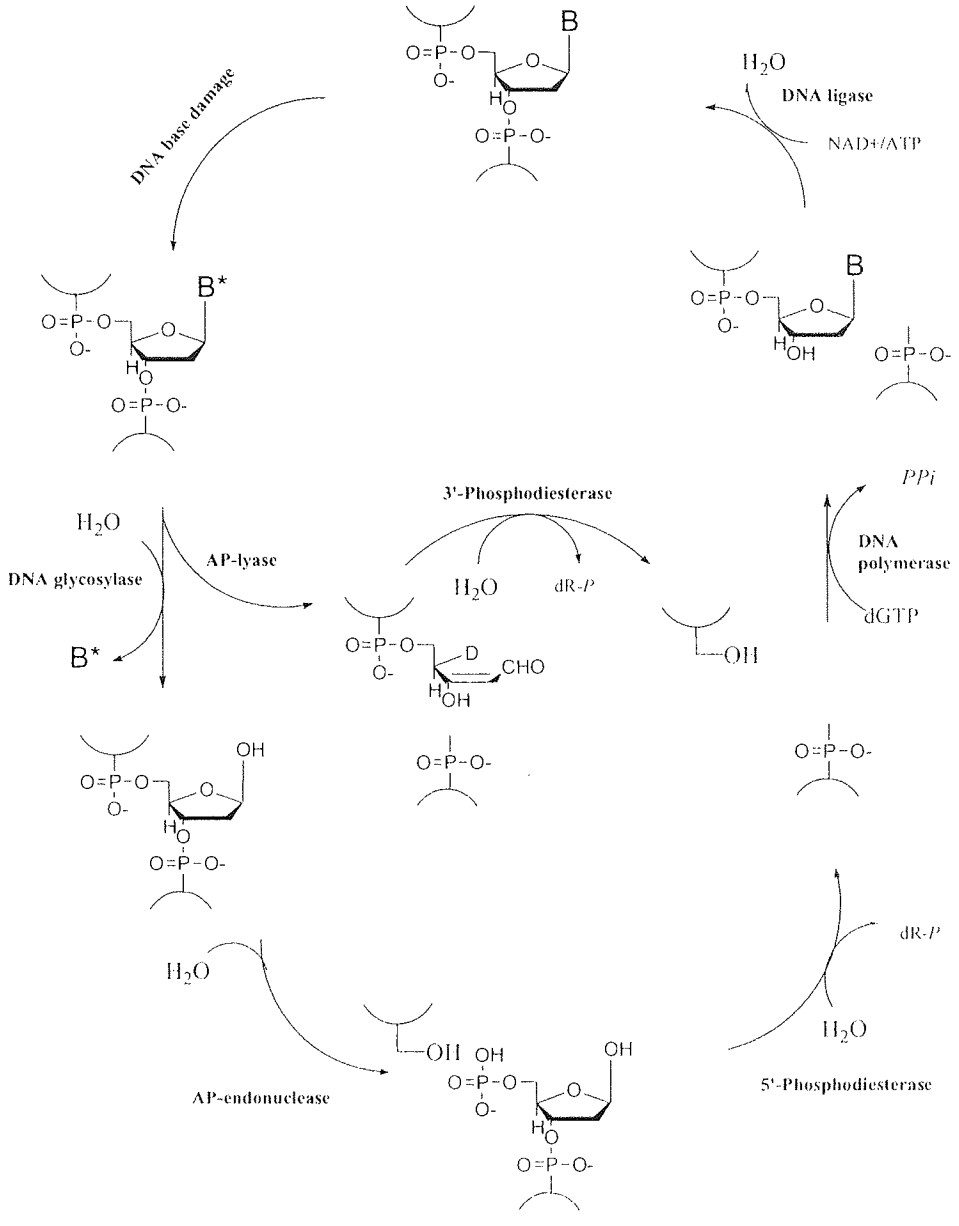


Figure 1.6. The base excision repair pathway. B = intact base; B* = modified base. [adapted from (Seeberg, *et al.* 1995)]

Formamidopyrimidine glycosylase (Fapy-DNA glycosylase or MutM protein or Fpg protein) is one of the bifunctional DNA glycosylases found in *E. coli*. As

mentioned above, in addition to its DNA glycosylase activity, Fpg protein also exhibits an AP-lyase activity, which nick DNA at the apurinic/apyrimidinic (AP) sites (Boiteux, *et al.* 1987) (Boiteux, *et al.* 1990). It excises 8-oxo-dG, its main physiological substrate, and 8-oxo-dA as well as Fapy lesions such as 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-Gua), 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine (Me-Fapy-Gua), and 4,6-diamino-5-formamidopyridine (Fapy-Ade) (Tchou, *et al.* 1991) (Boiteux, *et al.* 1992). It was also shown that 5-hydroxycytosine (5-OHC), thymine glycol (Tg) and *N*-3-(2-hydroxyisobutyric acid) urea are also recognised and excised by Fpg (Hatahet, *et al.* 1994) (Jurado, *et al.* 1998) (Purmal, *et al.* 1998).

Genes for a functional analogue of Fpg, namely Ogg1, have been cloned in yeast (Memisoglu & Samson 2000) and mammals (Lu, *et al.* 1997) (Aburatani, *et al.* 1997) as well as in human (Radicella, *et al.* 1997) (Arai, *et al.* 1997). The human counterpart of the *E. coli*, Fapy glycosylase (human mutM homologue or hMMH), also has both DNA glycosylase and AP-lyase activities (Aburatani, *et al.* 1997)

8-Oxo-dG is repaired by a variety of mechanisms involving a number of enzymes. Basic studies of the bacterium *E. coli* have identified seven different genes for DNA glycosylases, all of which have been purified and characterised in detail (Seeberg, *et al.* 1995). Two of these gene products, coded by *mutM* and *mutY*, function to correct mispairs caused by incorporation of 8-oxo-dG into DNA (Michaels, *et al.* 1992). The protein encoded by the *mutM* gene, MutM (Fpg) protein, which has been mentioned earlier, mainly involved in the process of removing C:8-oxo-dG mispair from DNA (Chung, *et al.* 1991) (Tchou, *et al.* 1991). The MutY protein has an enzymatic activity that removes an adenine base

from an A:8-oxoG mispair, as well as from an A:G mispair in DNA. There is another gene product, coded by *mutT* gene, involved in the repair of this guanine modification. It specifically hydrolyses 8-oxo-dGTP to the monophosphate form (8-oxo-dGMP), so as to prevent 8-oxo-dG:A mispairing by removing 8-oxo-dGTP from the nucleotide pool (Tajiri, *et al.* 1995).

Although it appears that BER has developed to protect the cell against the effects of endogenous DNA damage, the pathway is also essential for resistance to DNA damage inflicted by exogenous DNA-damaging agents. As indicated in several studies, the level of 8-oxo-Gua endonuclease activity in *E.coli* and mammalian cells increases when the cells are exposed to oxygen radical-producing conditions *in vitro* and *in vivo* (Asami, *et al.* 1996) (Yamaguchi, *et al.* 1996). A similar effect was also demonstrated in animal tissue under pathological conditions, such as ischemic-reperfusion (You, *et al.* 2000).

1.2 Health Issues

Oxidative damage to DNA is implicated in a number of human health issues, including cancer, ageing and other degenerative diseases.

1.2.1 Cancer

The relationship between cancer and oxidative DNA damage has been intensely studied, because of the role of oxidative damage in carcinogenesis (Floyd 1990) and mutagenesis (Wood, *et al.* 1990).

Many observations indicate a direct correlation between 8-oxo-Gua formation and carcinogenesis *in vivo* (Feig, *et al.* 1994). Elevated levels of typical free radical-induced DNA base modifications, including 8-hydroxy-guanine (8-oxo-Gua), have been found to exist in human cancerous lung tissues when compared with the cancer-free surrounding tissue (Olinski, *et al.* 1992) (Jaruga, *et al.* 1994). Similar results have also been found with breast cancer tissues, and a correlation between the clinical stage and the mean level of 8-oxo-dG in DNA of breast cancer tissues has also been observed (Matsui, *et al.* 2000). Some reports show a positive correlation between the size of a tumor and the amount of 8-oxo-Gua. This suggests that base modifications in benign tumors may be a risk factor that determines the transformation of benign tumors to malignant tumors (Foksinski, *et al.* 2000). Furthermore, oxygen-derived radicals are known to induce mutagenesis in hotspot codons of the human p53 and Ha-Ras genes (Hussain, *et al.* 1994).

A large number of papers have also been published on the formation of 8-oxo-dG in animal organ DNA after the administration of various carcinogenic chemicals, including tumor promoters (Kasai 1997) (Hodges, *et al.* 2001).

It has been proved that 8-oxo-Gua is mutagenic by mis-pairing with A and causing G:C→T:A transversion (Shibutani, *et al.* 1991). Early experimental data from studies of the association between the formation of 8-oxo-Gua and carcinogenesis treatment or protocol have shown a direct correlation between the presence of 8-oxo-Gua in DNA and conditions or protocols leading to carcinogenesis (Floyd 1990).

There is increasing evidence that cellular exposure to chronic oxidative stress may be one possible etiologic factor in the development of many cancers, including prostate cancer (DeWeese, *et al.* 2001), lung cancer (Erhola, *et al.* 1997) and breast cancer (Matsui, *et al.* 2000). ROS are involved in the development of cancer, not only by direct effects on DNA but also by affecting signal transduction, cell proliferation, cell death and intercellular communication (Halliwell 2000). However, the development of human cancer will depend on other factors such as the extent of DNA damage, antioxidant levels and DNA repair systems. The true picture will only be seen if we have reliable and sensitive techniques for the measurement of DNA damage.

1.2.2 Ageing

Ageing is another intensively studied health issue in relation to DNA oxidative damage. One hypothesis that has attempted to explain the mechanistic basis of ageing postulates that accrual of macromolecular damage, induced by reactive oxygen species, is the central causal factor promoting the ageing process. Although direct evidence establishing oxidative damage as the main causal factor in ageing has not as yet been provided, a large body of correlative evidence is consistent with the predictions of this hypothesis.

The amount of oxidative damage to various macromolecules, such as lipids, proteins and DNA, has been shown to increase exponentially during ageing in a variety of tissues in different species. The rates of mitochondrial $O_2^{\cdot}$ and H_2O_2 production increase during ageing, in parallel with increases in the amount of macromolecular oxidative damage (Sohal & Weindruch 1996).

Flight activity restricted flies were used as models to study this hypothesis (Yan, *et al.* 2000). It was found that prevention of flight activity decreased the rate of oxygen utilisation of the flies, and this has almost tripled their life span as compared to those permitted to fly. Oxidative damage to two of the mitochondrial proteins, adenine nucleotide translocase and aconitase, detected as carbonyl modifications, was also found to be attenuated, and the loss in their functional activity occurring with age was retarded in the long-lived low activity flies as compared to the short-lived high activity flies. This result has shown certain link between ROS and ageing-associated functional alterations.

Comparisons among species with differences in maximum life span (MLS) provide further indirect support for the oxidative stress hypothesis. Among species with similar metabolic potentials, MLS was found to be inversely related to rates of age-associated accrual of molecular oxidative damage (Barja & Herrero 2000).

A more recent study has shown that the ability to repair damaged DNA is lower in aged rats, and that the accumulation of oxidative DNA damage that takes place during ageing may be related to this decline in repair activity (Kaneko, *et al.* 2002).

However, antioxidants have shown few positive results in their effect on the ageing process. Although it has been demonstrated that lack of crucial low molecular weight antioxidants, such as ascorbate and α-tocopherol, can cause a variety of pathological manifestations, there is no evidence for a relationship

between additional intake of antioxidants and prolongation of life span (Herbert 1994).

1.2.3 Other diseases

In addition to the relationship between oxidative DNA damage to cancer and ageing, other disease states also appear to be associated with increased levels of oxidation. There are at least 50 diseases reported to be related to oxidative stress, including neurodegenerative conditions, such as in Parkinson's disease (Jenner 1996) and Alzheimer's disease (Spencer, *et al.* 1994); inflammatory diseases such as rheumatoid arthritis (Rall, *et al.* 2000) and systemic lupus erythematosus (SLE) (Lunec, *et al.* 1994); non-insulin-dependent diabetes mellitus (NIDDM) (Leinonen, *et al.* 1997); Down Syndrome (Jovanovic & MacLeod 1998); and heart diseases (Collins, *et al.* 1998).

A recent review by Olinski and co-workers suggests the involvement of oxidative DNA damage in atherosclerosis and acquired immunodeficiency syndrome (AIDS) (Olinski, *et al.* 2002). There is evidence that oxidative DNA damage may play a causative role in atherosclerosis (Gackowski, *et al.* 2001), and possibly lead to cell death of patients infected with human immunodeficiency virus (HIV) (Dobmeyer, *et al.* 1997) and could even influence the progression of AIDS (Ameisen & Capron 1991).

1.2.4 The contribution of environment, lifestyle/diet, etc. to oxidative damage

Reports have shown an elevated oxidative level in people exposed to certain harmful occupational environments. Tagesson *et al.* (1993) reported significantly higher levels of 8-oxo-dG in each of the exposed groups of asbestos workers, rubber workers, and azo-dye workers than in the control group. Other experiments have demonstrated that asbestos can cause significant elevation of all bases in the spent medium over a 48-hour period (Marczynski, *et al.* 2000). Results show that oxidatively damaged DNA bases are produced in response to asbestos in target cells of asbestos-induced cancers (Chen, *et al.* 1996).

A number of publications reported that lifestyle/diet, such as smoking, excessive drinking and high fat intake, also contributes to the higher level of oxidative damage. For example, high fat diet can induce oxidative DNA damage (Loft, *et al.* 1998) and sucrose-rich diet induced mutations in the rat colon (Dragsted, *et al.* 2002) have also been observed.

It was also found that cigarette smoking induced the formation of 8-oxo-dG in peripheral leucocyte DNA. Its levels correlated with the number of cigarettes smoked per day (Asami, *et al.* 1996).

It is worth mentioning that several types of medications are metabolised to free-radical intermediates that cause oxidative damage to the target tissues. Here, only a few examples are given, so that one can have an integral view of therapeutic

agents when studying oxidative damage. The drug in common use is acetaminophen, in which oxidation by peroxidases creates free radicals that are very reactive and capable of forming dimers and ultimately melanin-like polymeric products (Mason & Fischer 1986) (Hart, *et al.* 1994). Phototoxic properties of some prescription medications, including tricyclic antidepressants, such as amitriptyline and imipramine, are believed to involve free radical species production (Viola, *et al.* 2000). The metabolic activation of the antiestrogenenic drug tamoxifen has shown an overproduction of oxygen radicals in several studies (Pagano, *et al.* 2001).

1.2.5 Dietary factors and food supplements

Epidemiology has repeatedly demonstrated diet high in antioxidants is related to low incidence of cancer and cardiovascular disease (Renaud & de Lorgeril 1994). In the study of oxidative damage, dietary factors have demonstrated a positive interference in reducing the risk of cancer (Blount, *et al.* 1997) (Eguchi, *et al.* 1999). Food supplements with antioxidants such as Vitamin C, Vitamin E, carotenoid and flavinoid have been recommended to maintain good health and slow down the process of ageing (Machlin & Bendich 1987) (Machelle & Seibel 1999) (Chen, *et al.* 1999). However, results from some other studies suggest no distinguishable protection from these products, some even show negative correlation (Loft & Poulsen 1996).

The link between the dietary supplementation of antioxidant and DNA oxidative damage is yet to be fully established. Also, the controversial results have not been

properly explained, and the results from various laboratories have not always supported the hypothesis. Therefore, further investigation in this area is needed.

1.3 Quantitative Analysis of DNA Oxidative Damage

The postulated importance of oxidative DNA damage in human health, especially the linkage with cancer, ageing and other age-related degenerative processes, has prompted great interest in the development of accurate and reliable methods of its measurement in cells. These methods have helped researchers to understand the mechanisms of formation of DNA lesions and their cellular repair, and thus to ascertain the consequences of oxidative stress in certain medical conditions or identify the risk factors. Such procedures also assist in the validation of the efficacy of dietary supplements alleged to reduce the risk of cancer and maintain human health (Cooke, *et al.* 1998).

8-Oxo-guanine and its corresponding nucleoside have been generally accepted to be an important biomarker in oxidative DNA damage, and there exists a number of analytical methods to measure their level in the cells and urine. In this section, I will discuss in detail the biomarkers, the available analytical methods, and their advantages and drawbacks.

1.3.1 Biomarkers

The concept of biomarkers comes from molecular epidemiology. Free radical biologists use the term "biomarker" to describe a molecular modification in a

biological molecule that has arisen from attack by reactive oxygen, nitrogen or halide species. Information regarding formation of the modifications resulted from free radical attack, as well as the localisation of oxidative stress may be acquired from the analysis of discrete biomarkers isolated from tissues, organs or fluids (Griffiths, *et al.* 2002).

Biomarkers in DNA oxidative damage have been used for monitoring the involvement of such damage in the pathogenesis of diseases (de Zwart, *et al.* 1999), for identifying persons at risk of developing cancers, for dietary, genetic, or environmental reasons; for suggesting how the diets/lifestyle or environmental conditions of these persons could be modified to decrease that risk (Halliwell 2000), as well as for evaluating the efficacy of many antioxidants *in vitro* and *in vivo* (Griffiths, *et al.* 2002).

1.3.1.1 A promising biomarker: 8-oxo-dG

DNA base modification is one of most important lesions of DNA oxidative damage. There have been more than 20 base modifications identified (section 1.1.1, Figure 1.5), however, 8-oxo-Gua has retained a lot of attention and has been commonly used as a biomarker in the measurement of DNA oxidative damage *in vitro* and *in vivo*. There are a number of features that make 8-oxo-dG attractive to many researchers.

Firstly, 8-oxo-dG is a major product of oxidative DNA damage (Aruoma, *et al.* 1991). Its formation in DNA by several reactive species, such as singlet oxygen and hydroxyl radical, has been observed (Kasai 1997)). The level of 8-oxo-dG is

also elevated under various conditions associated with oxidant stress (Floyd 1990) and many observations have indicated a direct correlation between 8-oxo-dG formation and carcinogenesis *in vivo* (Floyd, *et al.* 1986) (Kelly, *et al.* 1992) (Ichinose, *et al.* 1997).

More importantly, its mutagenicity has been established (Shibutani, *et al.* 1991) (Cheng, *et al.* 1992) (Wood, *et al.* 1990). During DNA replication, dATP and dCTP compete to incorporate in the DNA strand opposite to 8-oxo-dG, therefore, 8-oxo-dG will often be mispaired with adenine, giving rise to G:C to T:A transversions, a common somatic mutation associated with human cancers. As shown in Figure 1.6, 8-oxo-dG may take *anti* conformation and pair with C, or it may take *syn* conformation and mispair with adenine.

Furthermore, the biological significance of 8-oxo-dG was supported by the discoveries of repair enzymes for the DNA lesions. The multiple mechanisms that have developed in biological system to remove 8-oxo-dG from DNA, or to prevent its incorporation into cellular DNA have been discussed in section 1.1.3. 8-Oxo-dG is believed to be the primary physiological substrate for a constituent glycosylase found in bacteria and mammalian cells (Tchou, *et al.* 1991).

The availability of analytical methods to quantitatively determine the level of 8-oxo-dG also facilitate the wide application of 8-oxo-dG as a biomarker, particularly after the development of HPLC-ECD (Floyd, *et al.* 1986), which enables the detection of cellular level of 8-oxo-dG *in vivo* (see section 1.3.2.1.).

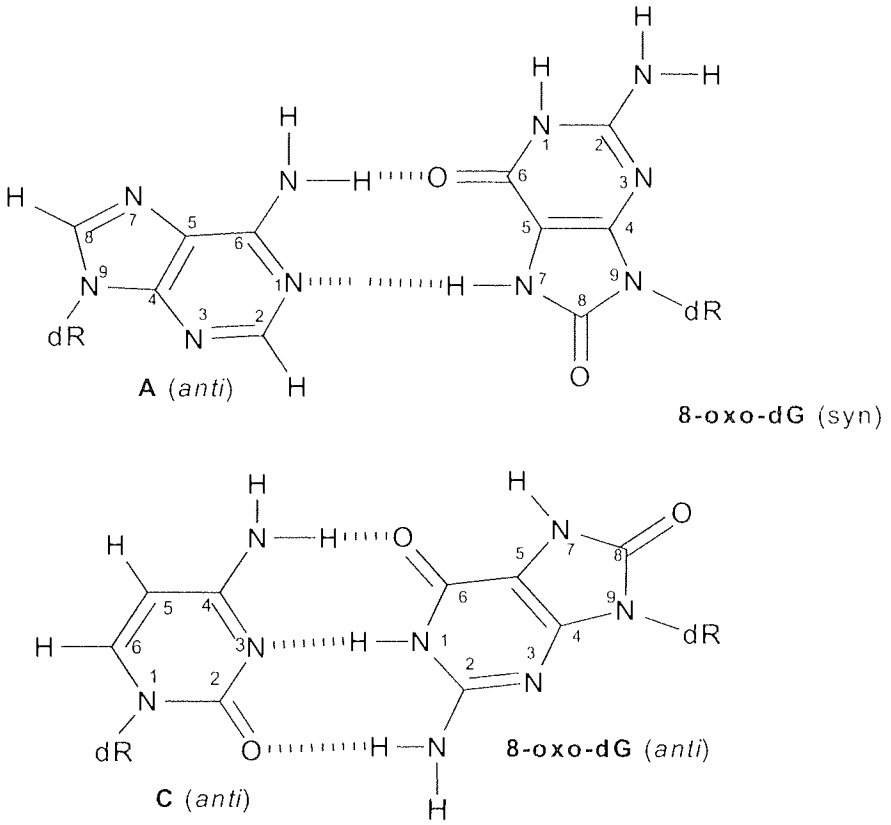


Figure 1.7. Base pairing properties of 8-oxo-dG.

1.3.1.2 Urinary 8-oxo-dG as a biomarker

DNA oxidatively damaged products are eliminated by repair enzymes and can be detected as nucleoside derivatives in urine. Urinary 8-oxo-dG is one of these repair products, and is proposed to be a sensitive biomarker of the oxidative DNA damage and repair (Shigenaga, *et al.* 1989) (Simic 1992) (Loft, *et al.* 1993).

Measurement of urinary 8-oxo-dG has become more popular in recent years to assess oxidative stress in human, because urinary 8-oxo-dG probably reflects the

level of oxidative DNA damage in the body as a whole (Loft & Poulsen 1996). Furthermore, it is found to be stable after storage in urine for an extended period and dietary 8-oxo-dG does not appear to contribute significantly to the level of 8-oxo-dG detected (Gackowski, *et al.* 2001).

However, urinary level of 8-oxo-dG as a biomarker should be carefully used. Firstly, it only indicates the repair level, not the direct endpoint of the level. For example, an agent that increases 8-oxo-dG excretion rates might be interpreted as the increase in DNA damage, but might, in fact, be the result of stimulated repair and therefore a decreased steady state 8-oxo-dG concentration in cellular DNA.

Secondly, 8-oxo-dG excreted in urine could come from sources other than oxidation of DNA, for example, oxidation of deoxyguanosine triphosphate (dGTP) in the DNA precursor pool and the apoptotic death of cells (Helbock & Ames 1999).

Finally, 8-oxo-dG is sensitive to oxidation, and may be destroyed *in vivo* before excretion, resulting in the loss of these analytes from urine.

Therefore, the measurement of urinary level of 8-oxo-dG may remain attractive, but results should be treated with caution.

1.3.1.3 Other biomarkers

Although 8-oxo-dG remains as being the most popular biomarker for many years, there is an urgent need to establish the biological significance (e.g., mutagenicity

and repair rates) of the multiple oxidised bases, other than 8-oxo-dG, that can be found in cellular DNA, and to develop sensitive and specific methods for the simultaneous measurement of multiple lesions.

A variety of free radical-producing systems produce numerous pyrimidine- and purine-derived DNA base modifications (see 1.1.1). It has been recognised that the types and yields of DNA modifications profoundly depend on the free radical-generating system, DNA-damaging agent and experimental conditions, such as the presence or absence of oxygen, and oxidative damage generates many lesions in DNA at the same time (Dizdaroglu 1992). For example, the ratio of 8-oxo-dG to Fapy-guanine is affected by the redox state of the cell, and by the presence of transition metal ions (Dizdaroglu 1998) (Fuciarelli, *et al.* 1990). In other words, the same amount of free radical attacks on DNA could give different levels of 8-oxo-dG, depending on the environment around the DNA. Therefore, the measurement of a single product such as 8-oxo-dG may be misleading.

Other base modifications, such as Fapy and thymidine glycol (Le, *et al.* 1998) (Jenner, *et al.* 1998) have also been proposed as biomarkers and simultaneous measurement of several base modifications have been reported (Podmore, *et al.* 2000) (Ravanat 1999).

1.3.2 Analytical methods available

Numerous analytical methods have been established to measure the level of 8-oxo-Gua and its corresponding nucleoside 8-oxo-dG *in vitro* and *in vivo*, including the earlier developed assays such as high performance liquid

chromatography connected to electrochemical detection (HPLC-ECD) and gas chromatography coupled with mass spectrometry (GC-MS), their improved form (HPLC-MS/MS) and ^{32}P-postlabelling assay (Cadet 1992), as well as more recently established enzyme (e.g. Fpg) based assays, such as alkaline unwinding technique (Hartwig, *et al.* 1996), alkaline elution (Epe & Hegler 1994) and comet assay (McKelvey-Martin, *et al.* 1993).

1.3.2.1 Chromatographic methods

HPLC-ECD

HPLC-ECD is probably the most popular technique in the measurement of 8-oxo-dG. It involves the separation of 8-oxo-dG with HPLC, and its identification by electrochemical detection, following DNA extraction and enzymatic digestion. The electrochemical detection system has about one thousand fold greater sensitivity than optical detection, which enables the detection of 8-oxo-dG at very low levels (subpicomoles) (Floyd, *et al.* 1986). It has received numerous applications since being established, because of its great sensitivity and simplicity of use. Initially, it was used to quantify 8-oxo-dG in DNA hydrolysates as a measure of the steady-state level of DNA oxidation, or for monitoring the action of various oxidising agents in isolated cells and tissues. Later, the development of a monoclonal antibody specific for 8-oxo-dG allowed the purification of the adducts directly from urine, blood, and tissue culture medium (Park, *et al.* 1992). It was also extended to measure a few other electroactive oxidised lesions, such as 5-hydroxy-2'-deoxycytidine, 5-hydroxy-2'-deoxyuridine (Wagner, *et al.* 1992), 8-oxo-7,8-dihydroadenine and its related 2'-deoxynucleoside (Berger, *et al.* 1990).

The types of modified bases that can be detected by this sensitive technique are restricted to electrochemically active compounds. In addition, precise quantitation is impaired by the lack of proper internal standards for controlling losses during enzymatic reactions and work-up.

GC-MS

GC-MS was first used by Dizdaroglu and his colleagues (1990) to study DNA oxidative damages, and it has been used extensively to identify and quantify a large number of oxidative base modifications and in the measurement of DNA repair, particularly for the study of the substrate specificities of DNA glycosylases, including *E.coli* Fpg and Ogg1 protein (Boiteux, *et al.* 1992) (Karahalil, *et al.* 1998). Simultaneous measurement of several bases enables the simultaneous measurement of excision rates of these lesions.

The hydrolysis of isolated DNA can be achieved by either acidic (e.g. formic acid) or enzymatic methods and the hydrolytes are converted to more volatile derivatives for the GC-MS analysis. Using MS as the detection provides unambiguous identification of DNA adducts and enables the simultaneous quantification of several base damages at the same time. It can make use of stable isotope labelled analogues as internal standards to increase the sensitivity and accuracy of the measurements. The sensitivity of the assay is significantly enhanced by applying the selective ion monitoring detection mode. This technique is called isotope dilute mass spectrometry (IDMS) and has been used successfully to study the formation of modified bases (Dizdaroglu 1993).

However, overestimates of the level of 8-oxo-Gua have been constantly reported, and it is believed this is mainly due to the oxidation of guanine during sample preparation stages, particularly during derivatisation. This drawback and the methods recommended to limit the artifactual oxidation will be discussed in section 1.3.3.1.

^{32}P-postlabelling assay

The ^{32}P-postlabelling assay is one of the most sensitive assays for detecting and measuring DNA adducts in DNA of cells and tissues. Sensitivities of 1 adduct per 10^{10} nucleotides can be reached when microgram amounts of DNA are analysed (Zeisig, *et al.* 1999).

The basic method requires enzymatic digestion of extracted DNA with a mixture of micrococcal nuclease (MN) and calf spleen phosphodiesterase (CSPD), leading to the release of deoxynucleoside 3'-monophophates. In the subsequent step, the normal and modified deoxynucleotides in the mixture are converted into 5'-^{32}P-labeled 3',5'-bishophate deoxynucleosides by using T4 polynucleotide kinase as the catalyst and [γ-^{32}P] ATP as the source of radioactive label. Then the mixture of nucleoside 3',5'-diphosphates is resolved by two-dimensional polyethyleneimine (PEI)-cellulose TLC, the separated products are detected by autoradiography and then quantitated by scintillation counting. This method has also been investigated for the analysis of 8-oxo-dG (Podmore, *et al.* 1997).

A problem encountered by all chromatographic assays is the risk of auto-oxidation from dG to 8-oxo-dG. In the case of ^{32}P-postlabelling, the labelling

agent [γ-^{32}P]ATP has been identified as an ionising radiation source to mediate the formation of 8-oxo-dG from dG (Möller & Hofer 1997). Enrichment of 8-oxo-dG by HPLC or TLC prior to postlabelling has been reported (Wilson, *et al.* 1993) (Gupta & Arif 2001). In this way, contaminating intact dG-3'-phosphate is eliminated so that the artifactual production of the oxidised material from dG-3'-phosphate during the labelling stage can be limited.

An alternative form of ^{32}P-postlabelling assay involves HPLC instead of TLC in the separation, with on-line detection of the labelled compounds. This form of assay has also been applied to measure 8-oxo-dG, where an absolute sensitivity of 1 fmol 8-oxo-dG was reported (Zeisig, *et al.* 1999).

HPLC-MS/MS

HPLC-MS/MS has emerged as a powerful tool with great potential in the analysis of DNA oxidative damage. It combines the advantages of GC-MS and HPLC-ECD, which are the two most applied methods. While possessing the great intrinsic sensitivity and simplicity of HPLC, it also has the possibility of calibration with internal standards and versatility of mass spectrometry. It has been applied in the quantitation of 8-oxo-dG in cellular DNA samples as well as in urine.

HPLC coupled with MS (HPLC-MS or LC-MS) has also been reported. Although it has a similar design to the HPLC-MS/MS, there have been only limited studies on its application. This had mainly been due to the poor sensitivity of the ionisation methods available, which remained the case until techniques such as

thermospray and electrospray presented better alternatives for the analysis of non-volatile substances, particularly when they are associated with the high specific tandem mass spectrometry method (MS/MS).

Using HPLC-MS/MS, with multiple reaction monitoring (MRM) mode, leads to a significant increase in the sensitivity, by about two orders of magnitude with respect to the HPLC-MS approach. Sensitivity is comparable to the HPLC-ECD detection method using coulometric detection (Yamaguchi, *et al.* 1996) (Ravanat, *et al.* 1998).

1.3.2.2 Intact cell (*in situ*) analysis

An alternative approach to measuring base oxidation employs the enzyme formamidopyrimidine DNA N-glycosylase (Fpg) to convert 8-oxo-Gua to strand breaks, which are then measured by alkaline unwinding, alkaline elution, or the comet assay.

An enzyme-linked immunosorbent assay (ELISA) has also been developed (Saito, *et al.* 2000).

Comet assay

Comet assay is a technique that allows for the *in situ* detection of DNA damage. It is also called single-cell gel electrophoresis (SCGE). The initial purpose of this assay was to provide a rapid, simple and sensitive means of detecting radiation or chemically induced DNA damage, including single strand breaks, double strand

breaks, and interstrand cross-links in viable cells (McKelvey-Martin, *et al.* 1993). The cells are embedded in agarose gel on microscope slides, lysed and subjected to electrophoresis. The damaged DNA, which can be denatured in an alkaline buffer, unwinds and migrates faster than the intact DNA, giving an appearance of a comet. The damage measured reflects the extent of DNA single- and double-strand breaks together with alkali-labile lesions. The quantitative determination is inferred from the measurement of the comet tail parameters, using DNA fluorescent stain.

A more targeted application of the comet assay was provided with the measurement of oxidative base damage in DNA. This was achieved by using specific repair endonucleases, such as Fpg and endonuclease III, to convert oxidised purine (Fpg) and pyrimidine (endonuclease III) bases to strand breaks (Collins, *et al.* 1993).

The comet assay is fast and can be applied to small numbers of cells, making it useful for human studies, especially large epidemiological studies, including intervention trials with putative antioxidants. Before use in this way, the comet assay needs to be validated, which can be done only by comparison with rigorous quality-controlled chemical methods applied to pure DNA isolated from the same cells. Hence, effort will still need to be paid to establish reliable methods for the determination of base oxidation products in isolated DNA (Halliwell 2000).

Alkaline unwinding

Another enzyme based technique, described by Hartwig, *et al.* (1996), combines the substrate specificity of the bacterial Fpg protein with the high sensitivity of the alkaline unwinding technique to detect DNA strand breaks. It provides a sensitive method to detect background, as well as induced oxidative DNA damage in intact mammalian cells.

Cells of interest are gently lysed with Triton X 100 and subsequently the histones are removed by high concentration salt treatment. The subsequent incubation with the Fpg protein specifically introduces DNA strand breaks at the sites of 8-oxo-dG and some other forms of ring-opened purines (Fpg sensitive sites) by the glycosylase and associated endonuclease activity. These DNA strand breaks are detected and quantified by the alkaline unwinding method: At the end of the incubation, an alkaline solution is added (pH 12.3) and DNA is allowed to unwind for a fixed time. Then the solution is neutralised, which causes unbroken DNA to renature, followed by sonification to fragment DNA. Separation of single- and double-stranded DNA fragments is performed on hydroxyapatite columns. The more breaks are present, the more DNA appears as single-stranded fragments. The whole procedure of cell lysis and alkaline unwinding needs to be calibrated with X-ray induced DNA strand breaks in cells, and the quantification of the frequency of Fpg-sensitive sites can then be achieved through the calibration curve.

The lysis, the removal of histones, and enzyme incubation take place without isolation of the DNA, which provides a fast assay that permits the analysis of

many samples in parallel. This may also help to prevent the unintended induction of oxidative DNA damage during DNA isolation procedures.

Alkaline elution

In this assay (Epe & Hegler 1994), cells are gently lysed on a microporous filter and eluted by passing an alkaline buffer through. The denatured DNA molecules pass through the pores, and the smaller the fragments are, the more quickly they manage to fall through the sieve. Thus, the rate of the elution of DNA reflects the frequency of strand breaks. In the same way as with the comet assay, calibration depends on comparison with the behaviour of DNA containing known frequencies of breaks introduced by ionising radiation.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) (Yin, *et al.* 1995) has also been developed for 8-oxo-dG determination in urine. It involves the development of specific antibody to recognise the damaged bases. Monoclonal antibody N45.1 is one of the most popular antibodies against 8-oxo-dG due to its high specificity. ELISA kits using this antibody are commercially available (Saito, *et al.* 2000).

Because it is simpler and easier to use than other chromatographic methods in aspects of equipment, analysing time, running cost and urine sample volume, the number of reports on urinary 8-oxo-dG levels determined by ELISA has been on the increase in recent years (Yoshida, *et al.* 2002).

Although N45.1 has a higher specificity than other antibodies against 8-oxo-dG, it has shown some cross-reactivity with 8-oxo-guanosine. A comparison study of ELISA and HPLC-ECD for the determination of 8-oxo-dG has shown a two fold higher level estimated by ELISA, which also suggested possible cross-reacting substances in urine (Shimoi, *et al.* 2002).

1.3.3 Limitations

1.3.3.1 Limitations associated with chromatographic methods

There are a number of sample preparation stages involved in the chromatographic analytical methods, particularly when it is used to measure cellular DNA. Artifacts which lead to overestimation of the 8-oxo-dG have been identified, and it is generally believed that this is due to oxidation of the overwhelming level of coexisting guanine during DNA isolation, hydrolysis and sample derivatisation (for GC-MS). As long as guanine is present in the sample mixture, there is a possibility that it converts to its oxidised form upon exposure to oxygen. Therefore, this limitation applies to most of the chromatographic methods. Great effort has been made to improve the analytical procedure in order to minimise the artifacts.

DNA isolation

The first problem to be considered is the possibility that DNA may be oxidatively damaged during its isolation from cells and tissues, particularly if phenol-based methods are used. It has been shown that routine phenol-based DNA purification

procedures can increase 8-oxo-dG levels 20-fold in samples that are exposed to air following the removal of phenol (Claycamp 1992) (Floyd 1990). A similar result was confirmed in a later report and an alternative phenol-free method was proposed using pronase E extraction technique (Finnegan, *et al.* 1996).

However, after evaluating various published methods for isolating DNA and investigating sources of artifacts associated with sample processing, Helbock & Ames (1999) concluded that phenol sensitisation is a real but minor contributor of artifacts and found that an alternative method, chaotropic NaI extraction [a commercial kit used by Nakae *et al.* (1995) to measure 8-oxo-dG], gives the lowest and least variable result. They also proposed a procedure to include a transition metal chelator, desferrioxamine, in the sample preparation stage, particularly in the DNA extraction and enzyme digestion buffers (Helbock, *et al.* 1998).

Although a lower level of 8-oxo-dG has been obtained with this modified extraction technique, it does not necessarily mean that it is the best method. There was a suggestion that 8-oxo-dG may decompose in the presence of NaI, therefore, its level could be underestimated (Helbock & Ames 1999) (Lenton, *et al.* 1999) (Hamilton, *et al.* 2001).

Recently, Ravanat *et al.* (2002) reported a procedure, using the ^{18}O labelled 8-oxo-dG as an internal standard, to evaluate the extent of artefactual DNA oxidation during the critical steps preceding the measurement. After the comparison of different DNA extraction protocols, it was found that artefactual DNA oxidation during the extraction step could be minimized if: (i) nuclei are

isolated after cell lysis; (ii) desferrioxamine, a transition metal chelator, is added to the different extraction buffers; and (iii) sodium iodide (or alternatively guanidine thiocyanate) is used for DNA precipitation.

Hydrolysis

Most of the chromatographic assays require either chemical hydrolysis (usually formic acid) or enzymatic digestion of the oxidised DNA subsequent to its extraction from cells or tissues.

Enzymatic hydrolysis used in HPLC-ECD analysis may underestimate the result due to the fact that 8-oxo-dG may not be fully released from the double-stranded DNA, thus enzymatic digestion of modified nucleosides or nucleotides may not be quantitative. However, it was shown from various studies, including time course experiments with increased quantities of exonuclease and comparison with acidic hydrolysis, that the nuclease P1-mediated release of 8-oxo-dG was complete (Douki, *et al.* 1997). Even so, it was recommended that real DNA samples may behave differently from the model substrates used in these experiments, and the possibility of underestimation must be borne in mind (Collins, *et al.* 1997).

Other experiments suggested that nuclease P1 incubation may lead to 8-oxo-dG overestimates, as longer incubations of DNA with nuclease at its optimal temperature (70°C) resulted in a higher ratio of 8-oxo-dG/dG, which could be the result of temperature-dependent autoxidation (Helbock, *et al.* 1998). These artifacts were reduced by limiting nuclease P1 incubation to 10 min, and by

adding desferal, a metal chelator. This suggested that the artefact was a result of transition metal contamination, involving Fenton type reactions (Cadet 1998).

Sample derivatisation in GC-MS

There are a number of publications reporting higher 8-oxo-Gua levels detected by GC-MS when compared with the value obtained from HPLC-ECD (Nagashima, *et al.* 1995) (Halliwell & Dizdaroglu 1992). It was later established that the higher value obtained by GC-MS was due to an artifactual oxidation of unmodified guanine during sample derivatisation in GC-MS. Evidence supporting this conclusion is a linear increase of the ratio 8-oxo-Gua/Gua, determined by GC-MS, with the time of derivatisation (Hamberg & Zhang 1995) (Ravanat, *et al.* 1995).

Because of the great advantage of this method, such as its specificity and versatility, tremendous efforts have been made to resolve this problem. There are generally two approaches: the first approach is to eliminate guanine prior to the derivatisation and the second approach is the prevent the occurrence of guanine oxidation by optimising the derivatisation conditions.

HPLC purification (i.e. removing intact guanine base from DNA samples by HPLC) prior to the derivatisation was recommended and has shown certain improvement (Ravanat, *et al.* 1995) (Douki, *et al.* 1996). Artifactual oxidation of normal bases other than guanine during the derivatisation was also observed, indicating that this is a general phenomenon. However, HPLC pre-purification also has limitations since the assay is restricted to compounds which are

significantly retained on the C_{18} column used for the pre-purification (Ravanat 1999), and it was regarded as a tedious procedure (Dizdaroglu 1998).

Addition of a reducing agent such as ethanethiol to DNA samples during derivatisation (trimethylsilylation) also decreased the detected levels of a number of DNA bases including 8-oxo-Gua (Jenner, *et al.* 1998). Other antioxidants have also been investigated and N-phenyl-1-naphthylamine was found to be effective in reducing the spurious oxidation during DNA sample derivatisation (Hong, *et al.* 1998).

Reducing the derivatisation temperature is another way to prevent artifactual oxidation and the technique of derivatisation at room temperature has been developed and shown to be an effective way to prevent artifactual oxidation (Hamberg & Zhang 1995). However it is only effective in certain cases.

Removal of guanine from hydrolysed DNA samples by treatment with guanase prior to the derivatisation (Herbert, *et al.* 1996) and removing air from the derivatisation reaction (e.g. carrying on GC-MS derivatisation under argon) have both demonstrated to prevent oxidation to certain degree.

Dizdaroglu (1990) (1993) (1994) and co-workers have been using GC-MS for many years and have had a lot of experience. They have suggested that emphasis should be put on the experimental procedures used, in particular the efficiency of exclusion of oxygen during the derivatisation process. Data have suggest that certain variations between laboratories appear to be the result of variations of the experimental procedures (Dizdaroglu 1998).

By comparing the results from different laboratories and reviewing literature regarding the discrepancy between GC-MS and HPLC-ECD, Lunec *et al.* (2000) concluded that for the measurement of 8-oxo-dG, the GC-MS method should be more appropriately calibrated with an isotopically labelled 8-oxo-dG standard, which is also essential for the measurement of other modified bases.

1.3.3.2 limitations associated with intact cell analysis

The great advantage of the intact cell analysis, compared to chromatographic and other assays, which usually require extensive sample preparation, is that there is little chance for spurious oxidation of guanine to occur. However, there are several potential problems:

1) Low range of detection: These enzymatic methods are intrinsically highly sensitive, however, a limited detection of high level DNA damage was often observed (Sattler, *et al.* 2000). High extent of DNA damage may not be completely recognised by enzymes such as Fpg, since higher enzyme concentration does not lead to further linear increase of the recognition factor. This is generally referred to as "saturation effect". Therefore, quantification of DNA damages using enzyme based methods is only valid when the dose response is linear, in most cases, when the level of DNA damage is moderate.

2) Indirect calibration: These methods are calibrated against the DNA breaks introduced in cells by ionising radiation, so the estimations of oxidised bases are indirect.

3) Potential systematic underestimation: When several bases are oxidised in a close proximity in the DNA, the cluster of lesions recognised by Fpg will behave as a single DNA strand break in any of the assays based on Fpg. Therefore, the strand breaks no longer represent the number of damaged bases. This can lead to the underestimation of the level of DNA damage. Such clustering of oxidised sites has been reported following X-rays irradiation of DNA samples (Ward 1995).

4) It is possible that some damage sites in the DNA are inaccessible to the enzyme due to steric hindrance. This may prevent quantitative enzyme recognition.

5) The specificity of the enzyme: Fpg is known to recognise certain ring-opened purines (mainly Fapy-Gua and Fapy-Ade), in addition to 8-oxo-Gua (see section 1.1.3) (Boiteux, *et al.* 1992) (Tchou, *et al.* 1991). Therefore, the lesions measured will include the whole range of these Fpg sensitive adducts. The numbers of 8-oxo-Gua residues in DNA may actually be overestimated.

Due to the above limitations, it was suggested that enzyme (e.g. Fpg) based methods require validation against reliable chromatographic methods.

1.4 The Aim of This Project

1.4.1 Validity

As discussed in the previous section (1.3), there are various analytical methods available to detect and quantify DNA oxidative damage, and they have been successfully applied to studies on the biological role of certain oxidative base

damage and its repair *in vivo*. However, the validity of these methods were questioned when discrepancies of up to several orders were reported since the mid-90s (Collins, *et al.* 1997). There was a lack of consensus not only between methods but also between laboratories using the same protocols. The European Standards Committee on Oxidative DNA Damage (ESCODD) has been set up to resolve the problems of methodology and to standardize assays so that the effects of oxidative stress on DNA *in vivo* can be accurately determined (Lunec 1998). This was followed by heated debates over several issues, such as which method we should use, what we should measure and where we should get the sample.

A number of factors that may account for such discrepancies have been identified and procedures standardized for all chromatographic analysis, including adding antioxidants and metal chelators during sample preparation (section 1.3.3.1).

Substantial achievements have been made over the last a few years. According to a recent ESCODD report (2003), moderate levels of 8-oxo-Gua, induced experimentally in HeLa cells, have been successfully measured with very little variation between the participating laboratories. However, estimates of the background level of DNA oxidation were still quite variable. A discrepancy in the order of ten-fold was still found, which was believed to be the result of the still uncontrolled oxidation in the early DNA sample preparation (Collins, *et al.* 2004).

Biochemical methods (section 1.3.2.2) have shown the advantage of being facile and less demanding in respect of cost, equipment and technique. However, these methods rely on the development of antibodies with high specificity or the identification, cloning and purification of specific DNA repair enzymes.

Furthermore, as they need indirect calibration, the validity of these methods should be verified against well established chemical methods.

1.4.2 Advantages of MS based methods

Among all the methods mentioned earlier (1.3.2), HPLC-ECD and GC-MS have received wide applications for the measurement of modified DNA adducts in biological samples.

HPLC-ECD is one of the most sensitive methods. However, its application is only limited to a few adducts with electrochemical activity. Moreover, the precise quantification is restricted due to the lack of proper internal standard to correct for the errors that may occur during the quantification procedures.

GC-MS has the potential advantage over all other assays applied to DNA damage of providing an almost unequivocal identification of the oxidation products. However, as mentioned earlier, GC-MS has been criticised severely for overestimating the level of oxidative damage due to the artifacts introduced from sample derivatisation. It was even regarded as unreliable by a recent report and is no longer used for the measurement of low level 8-oxo-Gua by ESCODD members (Collins, *et al.* 2004).

Nevertheless, GC-MS has continuously been used in the measurement of DNA oxidative damage. This is because that it is the only technique capable of positive identification and quantification of a large number of base products from all four DNA bases in a single DNA sample at the same time (Dizdaroglu 1998). This

versatility of GC-MS was demonstrated when five oxidative DNA lesions in human urine were simultaneously detected and quantified (Ravanat 1999).

A more recent view has emphasised the significance of the measurement of multiple lesions (Halliwell 2000) (Dizdaroglu, *et al.* 2002), as the measurement of a single product such as 8-oxo-dG may be misleading. This view is base on the following observations: i) oxidative damage generates many lesions in DNA simultaneously (Dizdaroglu 1992); ii) the product yields and ratios of product yields to one another differ depending on the type of DNA-damaging agent and experimental conditions. A good sample was given when DNA oxidative lesions were measured to establish the pro-oxidant and antioxidant role of Vitamin C *in vivo*, which clearly demonstrated the necessity of the measurement of multiple products at the same time (Podmore, *et al.* 1998).

In addition, the mass spectrometric detection makes it possible to use isotopically labelled derivatives of the molecule of interest as the internal standards, increasing the accuracy of the determination (Dizdaroglu 1993).

Furthermore, the problems associated with GC-MS lie with DNA isolation, hydrolysis and derivatisation rather than with MS itself, so that the development of LC-MS methods coupled with improved DNA hydrolysis and isolation techniques might be the answer. Indeed, a more recently developed technique, HPLC-MS/MS, has shown this great potential. It combined the versatility of GC-MS and the intrinsic sensitivity of HPLC-ECD. It should be noticed that, like all other assays employing MS, its accuracy depends on the use of multiply isotopically labelled authentic internal standards. A number of isotopically

labelled compounds have been synthesised and used as internal standards including (M+4) 8-oxo-guanosine and (M+3) guanine. However, being monomers, they cannot account for the spurious gain of the target product occurring during the prior sample preparation stages as mentioned earlier, such as DNA isolation and digestion.

1.4.3 Aims and objectives of the study

In this project, we aim to synthesise multiply isotopically labelled (M+4) purine/analogue in the form of base, nucleoside as well as DNA oligonucleotide. We believe that they can be used as internal standards to develop procedures that can make further improvements of existing analytical procedures. Once a robust and reliable method is established, it can also serve as a reference to validate other methods, such as immunoassay and enzymatic assay.

Chapter 2 The Synthesis of (M+4) Guanine and (M+4) 8-Oxo-Guanine

2.1 Introduction

Isotopically labelled DNA bases, nucleosides, oligonucleotides and their analogues are widely used in the NMR study of DNA structure, conformation and dynamics. They are also applied as internal standards for analytical studies utilising mass spectrometry methods. These isotopically labelled compounds can be obtained by an enzymatic approach, a chemical means or a combination of both.

2.1.1 Applications

2.1.1.1 NMR

Nuclear magnetic resonance (NMR) spectroscopy has become a powerful tool in the study of the structure and dynamics of macrobiomolecules including DNA (Wijmenga & van Buuren 1998). The NMR of nucleic acids makes use of atomic species ^{1}H, ^{13}C, ^{15}N, and ^{31}P. Of these, proton (^{1}H) is by far the most important, on account of its high sensitivity to NMR detection and its near 100 percent

natural isotopic abundance. The advance in the NMR technique, such as the use of 2D- and 3D- NMR (Hatanaka, *et al.* 1994), has made it possible to obtain the conformational information of DNA, which is important in the determination of the tertiary structure of DNA. However, it is very difficult to collect this information for large oligomers due to spectral overlap and line broadening. Therefore, stereoselective ^{2}H labelling can be used for simplification and sharpening of the spectrum by eliminating the large proton-proton dipolar and scalar interactions. For example, the incorporation of deuterated 2'-deoxyribonucleoside blocks into DNA oligonucleotides has simplified the overcrowding of the ^{1}H NMR spectra and selectively enhanced the resolution and sensitivity (Földesi, *et al.* 1998).

^{13}C, ^{15}N NMR spectra are also important for the structure elucidation of DNA. However, ^{13}C, ^{15}N are much less sensitive than ^{1}H, due to the much lower natural abundance (only 0.4% for ^{15}N and 1% for ^{13}C). These difficulties can be overcome by the synthesis of ^{13}C, ^{15}N labelled DNA oligonucleotide building blocks. Therefore, with the help of specific ^{15}N and ^{13}C labelling, key information on local interactions such as hydrogen bonding (Goswamib, *et al.* 1993), protonation (Wang, *et al.* 1991), hydration (Gaffney, *et al.* 1992), ligand interactions (Rhee, *et al.* 1993) and stacking (Zhang, *et al.* 1997) on large oligomers can be provided.

Furthermore, oligonucleotides containing isotopically labelled modified bases should prove invaluable in examining the structures of damaged base pairs by NMR spectroscopy (LaFrancois, *et al.* 1998).

2.1.1.2 MS

In addition to their utility in NMR studies, isotopically labelled DNA bases, nucleosides and oligomers are important for the analytical studies which employ mass spectrometry methods. They are normally used as internal standards in the analysis of DNA oxidative damages.

In analytical procedures employing MS, such as GC-MS, quantification of analytes is achieved by adding a suitable internal standard to DNA samples at an early stage of the analysis such as prior to the hydrolysis of DNA. In mass spectrometry, a stable isotope-labelled analogue of an analyte can be used as an internal standard. This procedure is often called isotope-dilution mass spectrometry (IDMS) (Crain 1990). The use of stable isotope-labelled analogues permits compensation for possible losses of the analyte during the sample preparation and GC-MS analysis, because the analyte and its analogue have essentially the same chemical and physical properties.

2.1.2 Synthetic approaches

The labelled oligomers can be obtained by either a chemical or enzymatic approach, or a combination of both.

2.1.2.1 Enzymatic approach

The enzymatic approach relies on the availability of uniformly labelled ^{13}C or/and ^{15}N-deoxynucleotide triphosphates (dNTPs), which are generally prepared from

the DNA components extracted from a suitable microorganism grown in an isotopically enriched media (Werner, *et al.* 2001). A general procedure of this approach is shown in Figure 2.1.

Firstly, the microorganism is allowed to grow in a media containing [^{15}N]ammonium chloride for ^{15}N labelling and [^{13}C]glucose for ^{13}C labelling. Then the labelled DNA was extracted and digested. The deoxynucleotide monophosphates obtained are separated and purified by chromatography. The individually purified dNMPs are converted to their corresponding dNTPs by enzymatic phosphorylation. Fully labelled DNA oligomer can thus be synthesised using DNA polymerases.

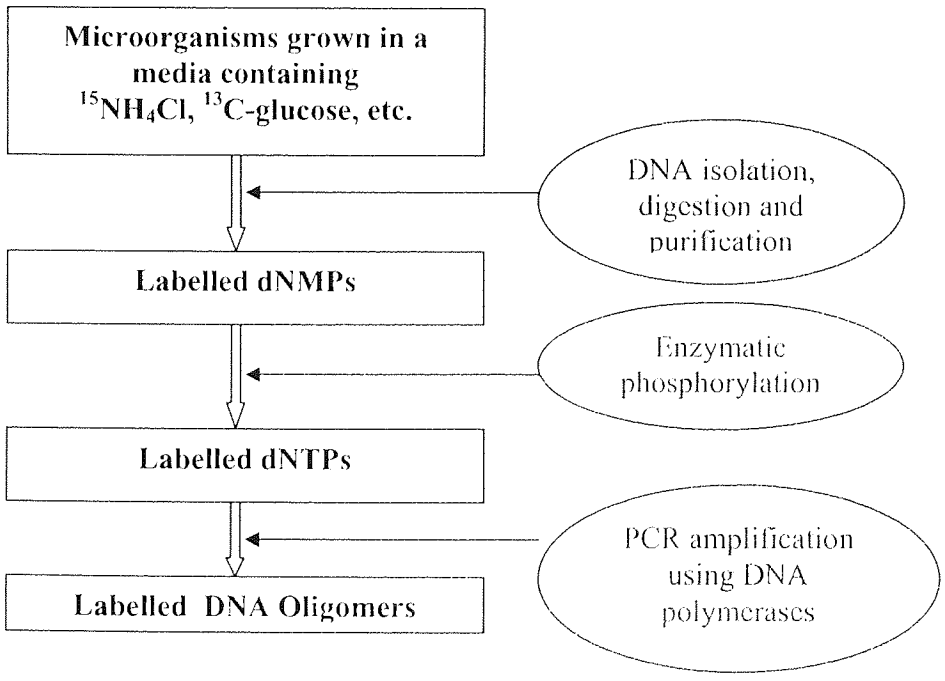


Figure 2.1. Procedures for the enzymatic preparation of ^{13}C, ^{15}N –labelled oligodeoxyribonucleotides.

This method is suitable for preparing fully labelled or nucleoside specifically labelled oligomers and can be used to prepare a longer DNA than the chemical synthetic approach. However, the yield and purity of the labelled oligomers are sequence dependent.

2.1.2.2 Chemical approach

Compared to the enzymatic approach, chemical synthesis of DNA oligomers are more technically demanding, requiring practical expertise in organic chemistry. However, this approach is much more flexible and allows us to prepare the monomer units having regio-selective labels with ^{2}H, ^{13}C, ^{15}N at any desired sites. It can also be used to obtain labelled modified nucleosides and oligonucleotides (Kojima, *et al.* 2001). Furthermore, the yield and purity of the labelled oligomer are almost independent of the sequence.

The most crucial step in the synthesis of site-specifically labelled oligomers is the efficient synthetic routes to isotopically labelled nucleoside phosphoamidites, which ideally should only involve the least expensive labelled materials and have a satisfactory overall yields.

For NMR measurements, a wide variety of labelled samples is required, thus the synthetic routes are also expected to be applicable for the preparation of fully labelled, atom-specifically labelled and stereoselectively labelled nucleosides.

For MS internal standards, particularly for the studies of DNA base modifications, atom-specific labelling is not required, but multiple labelling is essential. M+4 is

generally required to minimise interference from its natural occurrence. The labelling is preferred to be at the level of bases as our main interests are in the base modifications.

For labelled purine synthesis, triaminopyrimidine is often chosen as a pivotal intermediate. The key step of this approach is the formation of the imidazole ring, where ^{13}C at C-8 can be introduced. Therefore, dichlorohydroxypyrimidine may serve as an ideal starting material for the preparation of multiply labelled guanine and adenine derivatives (Stadler, *et al.* 1994) (Figure 2.2).

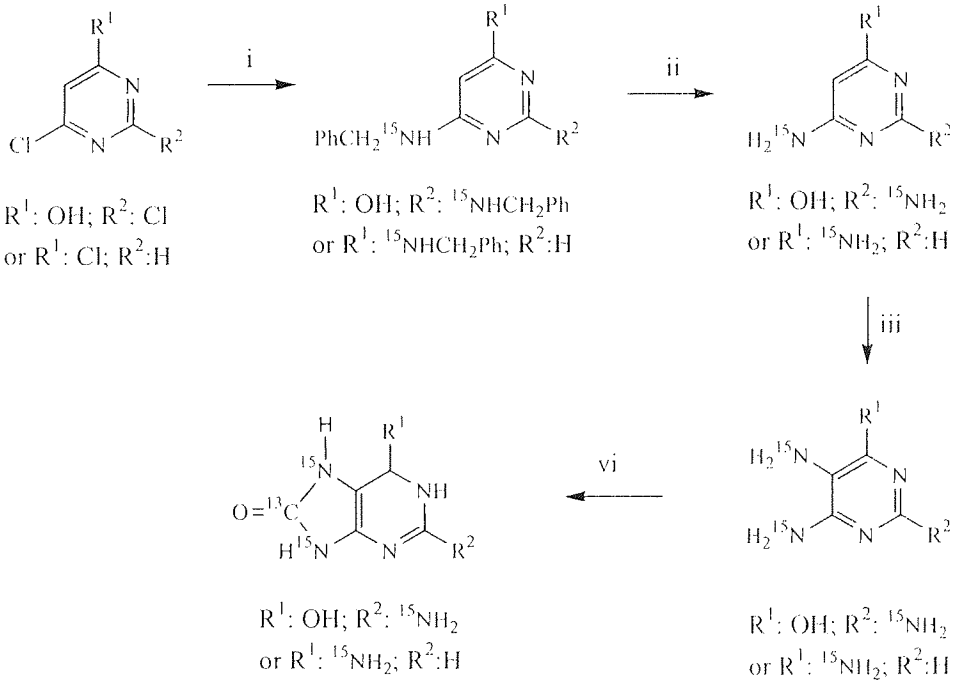


Figure 2.2. The outline of the synthesis of multiply isotopically labelled purine analogues starting from dichlorohydroxypyrimidine. i) [^{15}N]-benzylamine; ii) Pd-C/H_2 or AlC_{13}; benzene; iii) a. [^{15}N]-Na NO_2, b) $Na_2S_2O_4$; iv) [^{13}C]-urea.

The common starting materials for labelled pyrimidine derivatives are ^{15}N enriched malononitrile and thiourea. Purines can also be prepared by this approach, and again with triaminopyrimidine as the intermediate. Thus, condensation of [$^{15}N_2$]-malononitrile and [$^{15}N_2$]-thiourea yields [$^{15}N_4$]-4,6-diamino-2-mercaptopyrimidine, which can be desulfurized and converted to [$^{15}N_4$]-4,5,6-triaminopyrimidine. Subsequent ring closure with dimethylethylacetate (DMEA) yields adenine (Laxer, *et al.* 2001) (Figure 2.3).

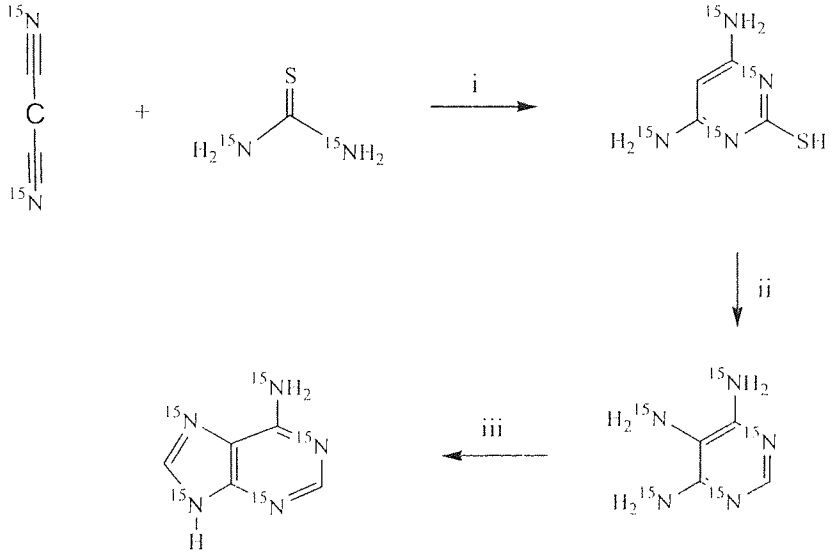


Figure 2.3. The outline of the synthesis of multiply isotopically labelled adenine with malononitrile and thiourea as starting materials. i) EtONa, EtOH, reflux 3h; ii) a. $Na^{15}NO_2/H_2O$, 3h; b. RaNi, DMF, overnight; iii) DMEA.

Other starting materials are ^{15}N and/or ^{13}C enriched guanidine hydrochloride and ethyl cyanoacetate. Reaction of these compounds, followed by nitrosation and reduction, produces the intermediate - labelled triaminohydroxy pyrimidine, from which guanine, adenine and their analogues can be prepared. A number of isotope

analogues of oxidatively modified bases have been synthesised using this approach, including (M+4) 8-oxo guanine and (M+4) Fapy guanine (Nelson 1996) (Figure 2.4).

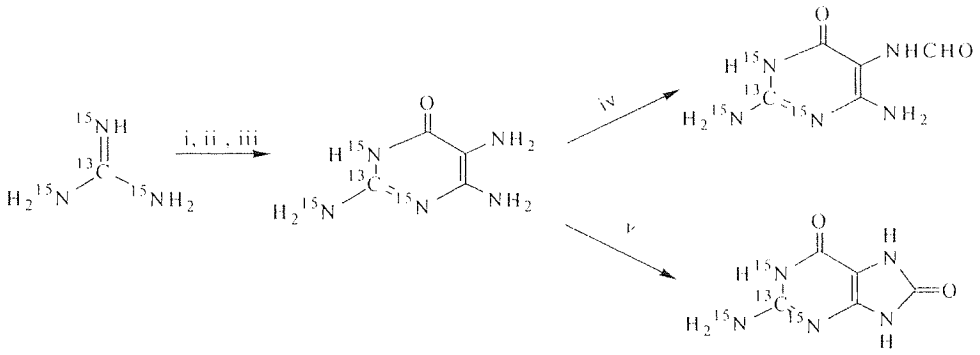


Figure 2.4 The outline of the synthesis of multiple labelled DNA base and analogues from multi-labelled guanidine. i) $EtOOCCH_2CN$; ii) $NaNO_2$; iii) $Na_2S_2O_4$; iv) $HCOOH$; v) $(H_2N)_2CO$.

Due to the high costs of labelled materials, when considering a synthetic route for the synthesis of a multiply labelled compound, a number of rules need to be followed: i) minimal number of synthetic steps; ii) an high overall yield based on the labelled materials used; and iii) using the least expensive commercially available labelled materials.

2.2 Experimental

2.2.1 Materials and methods

2.2.1.1 Chemicals

All chemicals were from Aldrich, unless otherwise specified. Solvents used for reactions were purchased from Fisher and used without further purification. [^{15}N]benzamide, [^{15}N]benzylamine, [^{15}N]sodium nitrite, [^{13}C]urea, all at 98+ % isotopic abundance, were purchased from Aldrich or Cambridge Isotope Laboratories.

2.2.1.2 Apparatus

Melting points were taken on a heated stage microscope melting point apparatus, uncorrected. Proton NMR spectra were recorded at 250 MHz on a Bruker AC-250 instrument. Mass spectra were obtained on an HP G1034C GC/LC-MS Chemstation using atmospheric chemical ionisation (ACPI) method. High-resolution mass spectra (HRMS) data were collected on VG Prospec mass spectrometer [electron impact mode (EI)] using perfluorokerosene (PFK) as the reference. Thin-layer-chromatography (TLC) was carried out using aluminium backed Merck Silica Gel 60 F_{254} plates and viewed under UV light at 254nm.

2.2.2 Experimental procedures

[^{15}N] Benzylamine. This compound was synthesised essentially according to a published procedure (Reich, *et al.* 2001). Thus, $LiAlH_4$ (1.4 g, 36.8 mmol) was suspended in 70 ml of dry THF and cooled to 0°C. [^{15}N]Benzamide (1.5 g, 12.4 mmol) in 25 ml dry THF was added dropwise to the above suspension under stirring. The mixture was then refluxed at 70-75 °C for 4h and slowly cooled down to 0°C. The reaction was terminated by careful addition of 1 ml water, 2 ml 15% NaOH and then 3 ml water. After being stirred for a few minutes, the reaction mixture was filtered and washed with 4 x 20 ml THF. The clear filtrate was dried over Na_2SO_4 anhydrous and evaporated *in vacuo* at room temperature to afford 0.96 g (76%) of [^{15}N]benzylamine, which was employed directly without further purification. Rf: 0.25 [ethyl acetate: methanol, 2: 1 (v/v)]. ^{1}H NMR spectrum (DMSO-d_6): 7.20-7.34 (m, 5H, Ar); 3.80 (s, 2H, NH_2); 1.6 (s, 2H, CH_2).

4-Hydroxy-2,6-dichloropyrimidine (2). This compound was synthesised by selective base hydrolysis of 2,4,6-trichloropyrimidine (**1**) according to a reported method (Hübsch & Pfleiderer 1989). To a solution of 9.18 g (0.05 mol) of 2,4,6-trichloropyrimidine in 150 ml of dioxane, 5 g (0.125 mol) of NaOH in 40 ml of H_2O was added with vigorous stirring. A precipitate separated out gradually, and after 4h, the mixture was concentrated *in vacuo* to a small volume, forming a thick paste. H_2O was added until a clear solution was obtained on boiling (~80 ml). On cooling and standing in the ice-bath, colourless crystals (**2'**, Isomer of **2**) were separated. The filtrate was then evaporated to 25 ml, a further amount of **2'** was separated.

The filtrate of the preceding step was cooled with ice, then gently acidified by 5N HCl to pH 2. The obtained precipitate was washed with a little cold H_2O, and dried in a vacuum desiccator over KOH. Recrystallization from H_2O gives colourless crystals of **2** (2.54 g, Yield: 40.9%). Rf: 0.48 [$CHCl_3$: CH_3OH, 2: 1 (v/v)]. mp 169-171°C [Lit: 170-171°C (Hübsch & Pfleiderer 1989)]. ^1H NMR spectrum (DMSO-d_6): (s, 1H, 6.18). MS: m/z 165 $[M+H]^+$.

4-Hydroxy-[2,6-$^{15}N_2$]bis(benzylamino)pyrimidine (3). Free base of [^{15}N]benzylamine (0.96 g, 9 mmol) was added to dried **2** (0.3 g, 1.8 mmol) and the mixture heated in a glass vial at 145°C for 30 min. After reaching room temperature, the white solid was suspended in hot water (5 ml, ~60°C) and filtered, and the residue was washed with hot water (20 ml). The product **3** was recrystallized from 95% ethanol, affording 0.48 g of **3**. Yield: 86%. Rf: 0.52 [$CHCl_3$: CH_3OH, 8: 1 (v/v)]. mp 191°C. ^1H NMR spectrum* (DMSO-d_6): 9.77 (s, 1H, OH); 7.28-7.13 (m, 11H, 10H-phenyl, 1H-NH); 6.68 (s, 1H, NH); 4.43 (s, 2H, CH_2); 4.40 (s, 1H, CH heterocyclic); 4.28 (s, 2H, CH_2). HRMS on the molecular ion calcd for $C_{18}H_{18}N_2{}^{15}N_2O$, m/z 308.1421, found 308.1426 (for HRMS spectrum, see the appendix).

4-Hydroxy-[2,6-$^{15}N_2$]diaminopyrimidine (4). A 10% palladium-carbon catalyst (0.8 g, wet~*50% water*) was added to a solution of **3** (0.35 g, 1.14 mmol) in 10 ml of ethanol/water/1M hydrochloric acid, (15: 5: 2 v/v). The reaction mixture was hydrogenated (>1atm) at 48-50°C for ~96h under rapid stirring. The reaction was monitored by TLC. The catalyst was removed by filtration and the clear filtrate

* ^1H NMR data obtained from unlabelled compound

was evaporated to dryness *in vacuo* (~40°C) to afford the hydrochloride salt of **4** as a white powder (98 mg, yield: 53%). Rf: 0.34 [$CHCl_3$: CH_3OH, 2: 1 (v/v)]. HRMS on the molecular ion calcd for $C_4H_6N_2{}^{15}N_2O$, m/z 128.0482, found 128.0476 (for HRMS spectrum, see the appendix).

4-Hydroxy-[2,5,6-$^{15}N_3$]triaminopyrimidine (5). The hydrochloride salt of **4** (0.26 g, 1.58 mmol) was dissolved in 10 ml of 10% acetic acid. The solution was kept in ice bath under stirring and 0.4 g (4.5 mmol) [^{15}N]-labelled sodium nitrite (dissolved in 1 ml of ice-old water) was added portionwise. After 2 h, the pink precipitate that had formed was separated by centrifugation (*Beckman J21 centrifuge*, 14,000 rpm, 2 min), washed with cold water (10 ml), and suspended in 8 ml of hot water (~ 60°C) under stirring. Sodium dithionite (0.6 g) was added in portions and the mixture filtered. The filtrate was kept on ice and the resulting cream-like precipitate was then washed with cold water and acidified with 2 N sulfuric acid. A final wash with cold water and dring *in vacuo* afforded 156 mg (yield: 41.4%) of the sulfate of **5** as a white powder. mp >300°C. HRMS on the molecular ion calcd for $C_4H_7N_2{}^{15}N_3O$, m/z 144.0562, found 144.0567 (for HRMS spectrum, see the appendix).

[2-amino, 7,9-$^{15}N_3$, 8-^{13}C]8-oxo-guanine (6). [^{13}C]urea (80 mg, 1.32 mmol) was added to solid **5** (80 mg, 0.33 mmol). The mixture was heated at 160°C in a thermostated heater block for 25 min. After reaching room temperature, the yellow residue was dissolved in 4 ml of warm 1N KOH solution. Charcoal (~0.1 g) was added and the mixture was stirred for 15 min. After filtration, the clear filtrate was acidified to pH 5.5 with 1N HCl, and the solution was left at 4°C for

3h, resulting in a white precipitate, which was washed first with water and then with methanol, affording compound **6** 45 mg, (yield: 66.8%). mp >300°C. HRMS on the molecular ion calcd for $C_4{}^{13}CH_5N_2{}^{15}N_3O_2$, m/z 171.0388, found 171.0384 (for HRMS spectrum, see the appendix).

[2-amino,7,9-$^{15}N_3$,^{13}C]Guanine (7). Dried sulfate of 4-hydroxy-[2,5,6-^{15}N]triaminopyrimidine (**5**) (100 mg, 0.42 mmol) was dissolved into 1 ml of [^{13}C]formamide at 200°C. The solution was heated for 25 min and light brown precipitate gradually formed. After reaching room temperature, water (1 ml) was added, and the mixture was allowed to stand at 4°C for 48h and then filtered, washed with water, methanol and ether, and dried *in vacuo* overnight, affording compound **7** 57.7 mg (yield: 90%). HRMS on the molecular ion calcd for $C_4{}^{13}CH_5N_2{}^{15}N_3O$, m/z 155.0439, found 155.0442 (for HRMS spectrum, see the appendix).

2.3 Results and Discussion

The synthetic route to (M+4) 8-oxo-guanine (**6**) and (M+4) guanine (**7**) is summarised in Figure 2.5. There were a number of available routes for the synthesis of the desired compounds (**6** and **7**) as discussed earlier (section 2.1.2). Using [$^{15}N_3$, ^{13}C]guanidine hydrochloride as the starting material (Figure 2.4) was ruled out because of its significantly higher cost and a much lower overall yield, based on the labelled compound (2%) (Nelson 1996). Another possible route reported in the literature (Hamberg & Zhang 1995) used the same approach as outlined in Figure 2.4, but using unlabelled guanidine with other labelled reagents,

include [^{15}N] KCN. The yield of this approach was claimed to be comparable, but again the starting labelled materials were more expensive and it involved the use of highly toxic KCN. Therefore, it was also ruled out.

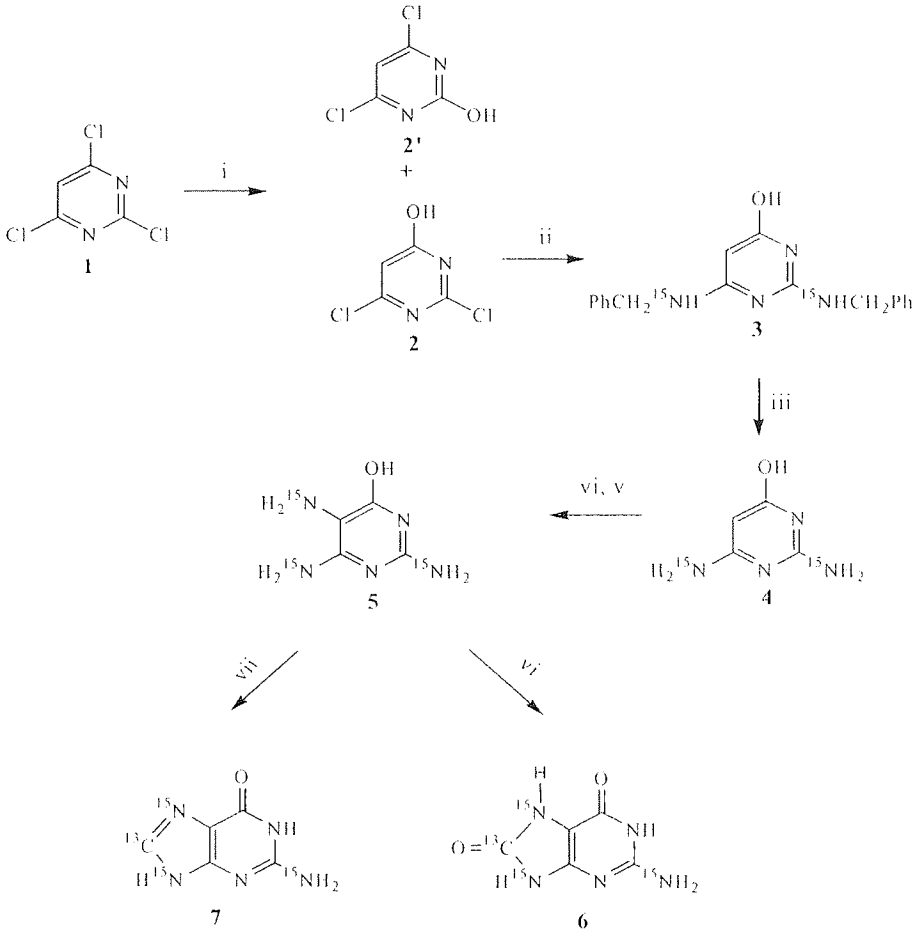


Figure 2.5. The synthesis of (M+4) guanine and (M+4) 8-oxo-guanine. i) $NaOH/H_2O$; ii) [^{15}N] Benzylamine, 145°C; iii) 10 % Pd-C/H_2, 45-50°C; iv) $Na^{15}NO_2$; v) $Na_2S_2O_2$; vi) [^{13}C]urea; vii) [^{13}C]formamide.

A dry run was performed based on the previously described procedure (Stadler, *et al.* 1994), with some modifications, before the isotope reagents were used. (M+4) 8-oxo-guanine was synthesised from 2,4,6-trichloropyrimidine, a commonly used intermediate for the synthesis of purine and pyrimidine derivatives. Selective

base hydrolysis produced 4-hydroxy-2,6-dichloropyrimindine (**2**). By displacing the two chlorine atoms of **2** with [^{15}N]benzylamine, which is commercially available, two ^{15}N were introduced simultaneously (**3**). Catalytic hydrogenolysis of **3** removed both benzylic groups to give 4-hydroxy-[2,6-^{15}N]-diaminopyrimidine (**4**). A third ^{15}N was introduced by the treatment of **4** with [^{15}N]-labelled sodium nitrite, followed by the treatment with solid sodium dithionite to afford 4-hydroxy-[2,5,6-^{15}N] triaminopyrimidine (**5**). Fusion of the triply-labelled triaminopyrimidine **5** with [^{13}C]urea gave (M+4) 8-oxo-guanine (**6**). (M+4) guanine (**7**) was synthesised by the treatment of **5** with [^{13}C]formamide.

2.3.1 Amination

In step 2, the conditions for halogen displacement of **2** with [^{15}N]-benzylamine were optimised in the reported procedure and the optimal conditions were applied in our experiment. However, when a dry run was carried out with unlabelled materials, the first few attempts failed to produce the desired compound. The properties of the reaction mixture were very much different from those described in the literature, e.g. it could not be suspended in hot water, instead, it turned into a sticky paste; it was not soluble in ethanol on boiling, and the TLC showed several impurities.

More experiments were carried out under various reaction conditions, i.e. various temperatures, prolonged or shortened duration of the reaction and different molar ratios of the reactants. None of these trials produced isolable target compound. In our desperation, fresh benzylamine was purchased as the last hope to solve the

problem. Fortunately, the result was fully satisfactory: NMR spectra, melting point and MS calculated mass all confirmed that the resulting compound was indeed 4-hydroxy-bis(benzylamino)pyrimidine. Therefore, it was concluded that, apart from the reaction conditions, complete amination was also dependent on the quality of benzylamine. For the first few attempts of the dry run, old benzylamine was used , this suggest that the impurities that formed during storage might be the main cause of the failure of the experiments.

However, the same problem occurred when we tried to synthesise the labelled compound **3**, with [^{15}N]-benzylamine newly purchased from Aldrich. Considering the relative expense of the isotopically labelled reagent, and that TLC indicated a reasonable amount of **3** (see Figure 2.3), we managed to separate out pure **3** by column chromatography. However, the yield was very low, 40% instead of 90% as reported.

The result was much more satisfactory following the use of [^{15}N]-benzylamine purchased from Cambridge Isotope Laboratories. This again confirmed the importance of the purity of the reagent in this reaction.

Unfortunately, it appeared that there were some variations of the qualities of the commercially available [^{15}N]-benzylamine from CIL. The same problem occurred as described earlier, with the second batch of [^{15}N]-benzylamine purchased from Cambridge Isotope Laboratories (CIL). The HPLC chromatogram (Figure 2.7) of the high purity benzylamine was compared with that of the "bad" batch of [^{15}N]-benzylamine from CIL. An additional intense peak was observed from the "bad"

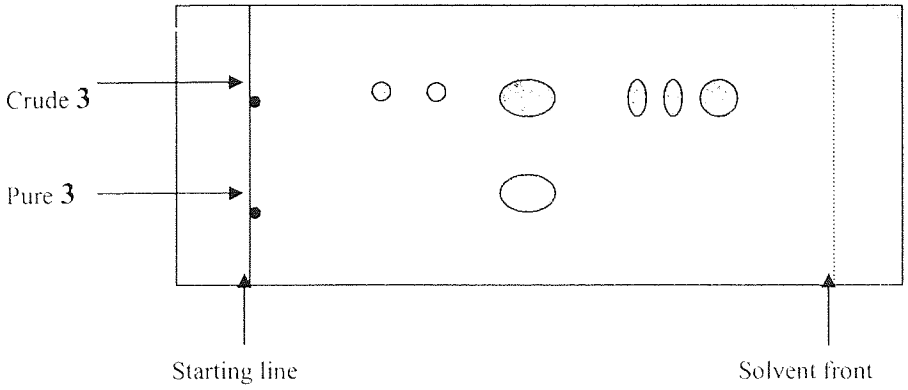


Figure 2.6. TCL analysis of the crude compound **3**.

batch, indicating the existence of an impurity. This issue was raised with the supplier (CIL) and a replacement was then received and subsequently used in our experiment. No improvement over the previous batch was evident and the same level of impurity was again identified by HPLC. Eventually, a refund was arranged as the supplier failed to provide the required quality of [^{15}N]-benzylamine.

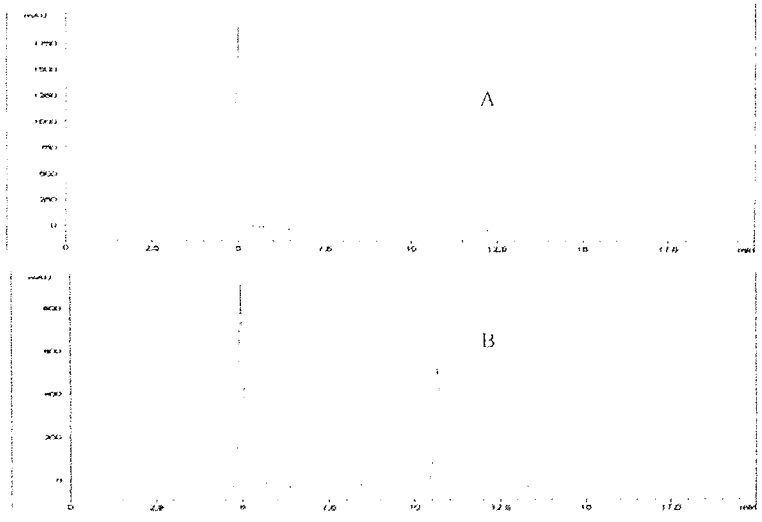


Figure 2.7. HPLC chromatograms of benzylamine. A) pure unlabelled benzylamine; B) [^{15}N] labelled benzylamine purchased from CIL.

Having been frustrated with the commercially available [^{15}N]-benzylamine, I decided to prepared this reagent by myself. The [^{15}N]-benzylamine was then successfully prepared from [^{15}N]-benzamide according to the following scheme (Figure 2.8.), based on a published procedure (Reich, *et al.* 2001).

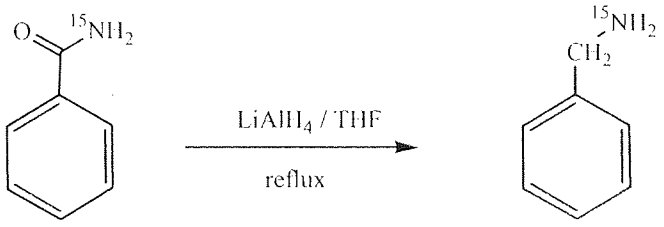


Figure 2.8. The preparation of [^{15}N]-benzylamine.

2.3.2 Hydrogenolysis

Hydrogenolysis of compound **3** to produce **4** presented a great challenge. In the initial attempts, a self-designed simple equipment was used, which consisted of a three-layer balloon with a three-way stopcock. The reaction took up to 9 days to complete and impurities were produced during this long reaction time, as detected by TLC. It appeared to us that the reason might be the insufficient pressure of hydrogen provided by the balloon, as other key conditions for debenzylation had been applied as optimized by Huegi *et al.* (1982). A commercially available hydrogenator was subsequently purchased by the Medicinal Chemistry group, and used in our new trials. However, little improvement was made even with increased pressure up to 50psi. Further attempts were made with various reaction

conditions, including elevated temperature up to 80°C, and different ratios of reactant/catalyst. It was found that the elevated temperature did increase the reaction speed, but any temperature above 45°C did not seem to improve the rate any further. Adding extra catalyst during the reaction eventually enabled the completion of the reaction within 4 days, but resulted in a very low yield. This was considered as unacceptable due to the high costs of isotope labelled reactants.

One of the reasons for these unsuccessful attempts using the commercial hydrogenator may be inefficient stirring. Therefore, the reaction was resumed with our simple, home-made equipment. Realising that atmospheric gas, especially oxygen, can diffuse through a balloon skin at a surprising rate, even with three layers, the previous procedure was modified by replacing hydrogen every 5 to 10 hours instead of leaving it for up to 24 hours. This greatly increased the reaction speed, and a substantially increased yield was also achieved.

2.3.3 Cyclization

The cyclization of 4-hydroxy-2,5,6 triaminopyrimidine sulfate with formamide to yield guanine was traditionally carried out by refluxing, or boiling for a period of time (Massefski & Redfield 1990). As formamide functions as both the reagent as well as the solvent in this reaction, an excess amount is normally used, quite often up to 100-fold molar excess (Hamberg & Zhang 1995). It was also noticed that after the reflux, the crude product was darkly coloured and required complicated purification. During the experiment, it was found that 4-hydroxy-2,5,6 triaminopyrimidine sulfate dissolved well in formamide at 190-200°C, just below its boiling point (210°C). Based on this finding, the procedure was modified

slightly. Instead of being refluxed, the mixture was heated at 190-200°C for 25 min. As a result, pure guanine was obtained through simple work-up. Better yield was achieved by avoiding complicated purification. This modification also enabled us to reduce the amount of formamide to 50-fold molar excess, which increased the efficient use of isotopically labelled reagent.

The cyclization of 4-hydroxy-2,5,6 triaminopyrimidine sulfate with urea to yield 8-oxo-guanine was carried out smoothly by heating the mixture of starting material **5** and the reagent at 160°C in a thermostated heater block. As there was no solvent involved, thorough mixing of the 4-hydroxy-2,5,6 triaminopyrimidine sulfate and urea before heating the mixture is important.

Chapter 3 Preparation of Deoxynucleosides of (M+4) Guanine and 8-Oxo-guanine

3.1 Introduction

Following the successful synthesis of (M+4) 8-oxo-gunaine and (M+4) guanine, the next step was the synthesis of their deoxynucleoside adducts by glycosylation, and eventually oligonucleotides containing these multiply labelled bases. The glycosylation of purine and analogues can be achieved chemically or enzymatically.

Chemical synthesis of nucleosides and their analogues have been studied intensively due to their enormous importance as potential antiviral (Griengl, *et al.* 1985) and anticancer agents. Chemical glycosylation of a suitable sugar derivative with a desired heterocyclic base has been used to synthesise a great number of natural or modified deoxynucleosides. It is a versatile approach and can provide authentic standards for comparison with biologically derived materials and reagents for the preparation of modified oligonucleotides. However, there are certain limitations associated with the chemical approach, particularly with 2'-deoxynucleosides (Vorbrüggen & Ruh-Pohlenz 2001). Firstly, the preparation of certain classes of deoxynucleoside adducts is difficult because of the instability of intermediates under the synthetic conditions, for example, purine

deoxyribosides are labile to acids. Secondly, the general yields of this approach are often low, partly due to the tedious protection and deprotection procedures. Furthermore, the ratios of the desired natural β anomer to the undesired α anomer are often difficult to control and to reproduce.

Although the enzymatic approach is limited by the ability of the enzymes to recognise substrates, it has tremendous potential for the stereocontrolled synthesis of 2'-deoxy- and 2'3'-dideoxynucleosides (Carson & Wasson 1988) (Krenitsky, *et al.* 1983), particularly when isotopically labelled materials are used, and thus high yields become a paramount issue. A number of isotopically labelled nucleosides, such as β-2'-deoxy [9-^{15}N]- (Orji & Silks III 1996), [7-^{15}N]-labelled deoxyadenosine and deoxyguanosine (Gaffney, *et al.* 1990) were synthesised, adopting the enzymatic approach (Massefski & Redfield 1990) (Roy, *et al.* 1986).

Two types of enzymes have been used as catalysts in the synthesis of nucleosides of purines and their analogues: *trans-N*-deoxyribosylases (Carson & Wasson 1988) and nucleoside phosphorylases (Krenitsky, *et al.* 1981). The *trans-N*-deoxyribosylases seem to be limited in their distribution to the bacterial *genus Lactobacillus,* and also in their catalytic use for the synthesis of 2'-deoxyribonucleosides. In contrast, the phosphorylases are widely distributed in nature and have been used for the synthesis of both ribonucleosides and 2'-deoxyribonucleosides, and therefore are more versatile.

There are two approaches for the synthesis of purine deoxynucleosides involving a purine nucleosides phosphorylase (PNPase) as the catalyst. The first approach involves the displacement of phosphate from deoxyribose 1-phosphate by purines

to give purine deoxynucleosides. This is a straightforward approach, however, although deoxyribose 1-phosphate is commercially available, it is extremely expensive. The alternative is to generate it from deoxyribose 5-phosphate. The transfer of the phosphate, from 5-positon to 1-position of the deoxyribose, is catalyzed by phosphoribomutase (PRM) (Roy, *et al.* 1986) (Ouwerkerk, *et al.* 2002). This approach is illustrated in Figure 3.1.

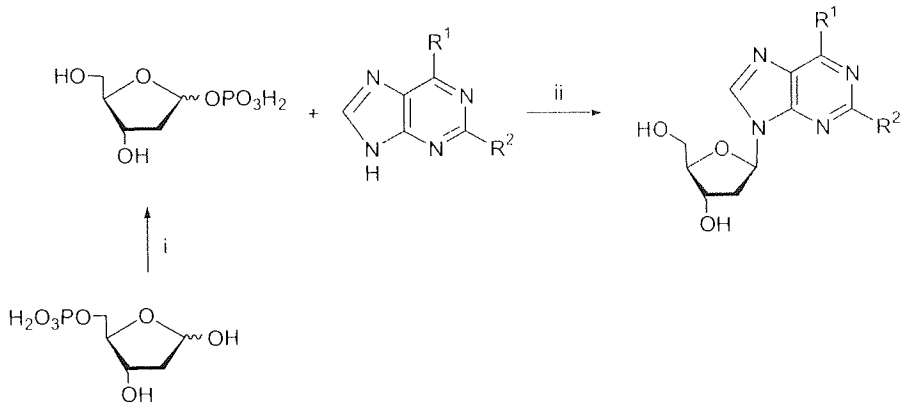


Figure 3.1. The synthesis of purine deoxynucleosides by direct enzymatic coupling (catalyzed by PNPase). i) phosphoribomutase; ii) purine nucleoside phosphorylase (PNPase).

Deoxyribose-1-phosphate can also be generated *in situ* by phosphorolysis of thymidine, catalyzed by thymidine phosphorylase (TPase). Thymidine phosphorylase degrades thymidine in the presence of phosphate to thymine and deoxyribose-1-phophates, which are transformed *in situ* by adding purines and purine nucleoside phosphorylase to the corresponding purine deoxynucleosides. Thus, pyrimidine nucleosides can serve as pentosyl donors and purines as the acceptors, as shown in Figure 3.2.

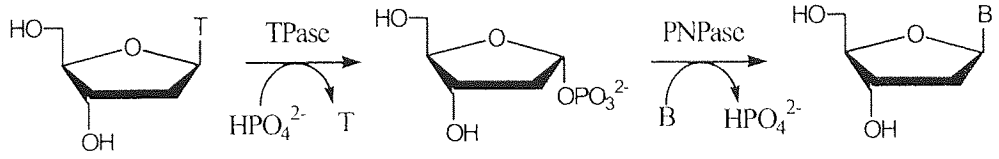


Figure 3.2 The synthesis of purine deoxynucleosides by enzymatic transglycosylation. B = purine or purine analogues. [adapted from (Chapeau & Marnett 1991)].

We have explored both of these approaches in the attempt to synthesise our target deoxynucleosides, and the second method, using thymidine as pentosyl donor, was eventually adopted.

3.2 Experimental

3.2.1 Materials and methods

3.2.1.1 Chemicals

Thymidine, thymidine phosphorylase and purine nucleoside phosphorylase were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were purchased from commercial companies as described in section 2.2.1.1.

3.2.1.2 HPLC methods

Reverse phase HPLC was carried out on a *Hewlett Packard Series 1100* instrument with a UV detector.

Method 1.

This method was used for the analysis of both dG and 8-oxo-dG.

HPLC column: Techsphere ODS 5μm (250 x 4.6 mm).

Mobile phase: A = 2% acetonitrile in 0.1M TEAA (pH 7.0), B = 70% acetonitrile in 0.1M TEAA (pH 7.0).

Gradient elution was performed at a flow rate of 1 ml/min and monitored at 295 nm for 8-oxo-dG and 260 nm for dG. The gradient was formed with 0.5% of the mobile phase B for the first 8min, then increased to 14% of B over the following 17 min.

The retention times, t_R (min), were: [2-amino,7,9-$^{15}N_3$,8-^{13}C]guanine, 5.4; thymine, 7.3; [2-amino,7,9-$^{15}N_3$,8-^{13}C]2'- deoxyguanosine, 13.5; thymidine, 15.5; [2-amino, 7,9-$^{15}N_3$, 8-^{13}C]8-oxo-guanine, 5.5; [2-amino,7,9-$^{15}N_3$,8-^{13}C]8-oxo-2'-deoxyguanosine, 17.0.

Method 2.

This method was used for the semi-preparation of 8-oxo-dG.

HPLC column: SphereClone ODS 5μm (10 x 250 mm).

Mobile phase: 10% MeOH, pH 4.0 (adjusted with acetic acid).

Isocratic elution was performed at a flow rate of 3 ml/min and monitored at 295 nm.

The retention times, t_R (min), were: [2-amino, 7,9-$^{15}N_3$, 8-^{13}C]8-oxo-guanine, 7.0; thymidine, 8.9; thymidine, 15.5; [2-amino, 7,9-$^{15}N_3$, 8-^{13}C]8-oxo-2'-deoxyguanosine, 18.8.

Method 3.

This method was used for the isolation of dG on a semi-preparative scale.

HPLC column: SphereClone ODS 5μm (10 x 250 mm).

Mobile phase: A = 2% acetonitrile (aq.), B = 70% acetonitrile (aq.).

Gradient elution was performed at a flow rate of 3 ml/min and monitored at 260 nm. A linear gradient was run from 0% to 10% of B over 20 min.

The retention times, t_R (min), were: [2-amino,7,9-$^{15}N_3$,8-^{13}C]guanine, 8.0; thymine, 11.0; [2-amino,7,9-$^{15}N_3$,8-^{13}C]2'- deoxyguanosine, 14.7; thymidine, 17.0.

3.2.1.3 Gel filtration for preparative separation of dG:

The reaction mixture was concentrated to 1/5 of its original volume, and filtered (0.45 μm); and 5 ml aliquots were applied to a Sephadex G10 column, 1.5 cm in diameter and 100 cm in height, and pre-equilibrated with 10% methanol. The column was eluted with the same solvent at a flow rate of 20 ml/hour and fractions of ~7 ml each were collected. The fractions containing the desired product, identified by UV (260 nm) and HPLC, were then combined, concentrated *in vacuo*, and lyophilized.

3.2.2 Experimental procedures

[2-amino, 7,9-$^{15}N_3$, 8-^{13}C]8-oxo-2'-deoxyguanosine (8). [2-Amino, 7,9-$^{15}N_3$, 8-^{13}C]8-oxo-guanine (**6**) (1 mg, 5.85 μmol) was dissolved in 1M NaOH (~0.1 ml) and 2 ml water was added, immediately followed by 20 ml hot (~80°C) solution of 50 mM KH_2PO_4 and a few drops of 1M NaOH, and the pH was adjusted to 8.0 by 1M NaOH. The resulting clear solution was added to 7 ml hot DMSO under stirring. After cooling down to 38°C the pH was adjusted to 7.9 by dropwise addition of 1M HCl. Thymidine (40 mg, 165 μmol), thymidine phosphorylase

(200 µl, 200 units), purine nucleoside phosphorylase (13 mg, 200 units), and sodium azide (15 mg, 0.05%) were added. The mixture was shaken at 38°C for various durations and the reaction was monitored by HPLC (method 1, Figure 3.6). After reaching equilibrium, the reaction was terminated by boiling the mixture at 100°C for 1 min. The solution was filtered through a 0.45µm filter and the 0.8 ml aliquots of the filtrate were injected onto a semi-preparative RP-HPLC column using method 2. The desired peak was collected and lyophilized, affording pure [2-amino,7,9-$^{15}N_3$,^{13}C]8-oxo-2'-deoxyguanosine 1.2 mg (yield: 71.5%), as a white fluffy solid. HRMS on the [MNa] ion calcd for $C_9{}^{13}CH_{13}N_2{}^{14}N_3O_5Na$, m/z 310.0759, found 310.0762 (for HRMS spectrum, see the appendix).

[2-amino,7,9-$^{15}N_3$,8-^{13}C]2'-deoxyguanosine (9). Compound **7** (3.1 mg, 0.02 mmol) was dissolved in 2ml 2N HCl and thymidine (14.5 mg, 0.06 mmol) in 20 ml of 20 mM potassium phosphate buffer was added. The pH was then adjusted to 7.4 by 1N NaOH. Thymidine phosphorylase (TPase, 3 units) and purine nucleoside phosphorylase (PNPase, 3 units) were subsequently added. The solution was shaken at 37°C and the reaction was monitored by HPLC (method 1, Figure 3.5). After reaching equilibrium, the reaction was terminated by boiling the mixture at 100°C for 1 min. The solution was concentrated to ~5 ml and filtered through a 0.45µm filter. 0.2 ml aliquots of the filtrate were injected onto a semi-preparative RP-HPLC column using method 3. The desired peak was collected and lyophilized, affording pure [2-amino,7,9-$^{15}N_3$, 8-^{13}C]2'-deoxyguanosine 3.9 mg (yield: 72%) as a white fluffy solid. HRMS: HRMS on

the [M+Na] ion calcd for $C_9{}^{13}CH_{13}N_2{}^{14}N_3O_4Na$, m/z 294.0810, found 294.0801 (for HRMS spectrum, see the appendix).

[scale up preparation of **9**, 54.2 mg (0.35 mmol) of compound **7** was used in the above reaction, pure product was obtained by gel filtration, affording 79 mg (yield: 84%)].

[direct enzymatic coupling: unlabelled guanine (3 mg, 0.02 mmol) was dissolved in 2ml 2N HCl and 10 ml 0.2 M Tris solution was added and then back titrated to pH 7.1 with 1N NaOH. 2-deoxyrbose 1-phosphate (4.5mg, 0.02mmol) and 3units (~0.25mg) of NPase in ~1 ml 0.1M Tris-Cl buffer (pH = 7.3) were added. The reaction was monitored by uv absorption at 305nm].

3.3 Results and Discussion

3.3.1 Enzymatic coupling

There are two enzymatic approaches for the synthesis of purine nucleosides and their analogues (see section 3.1), both approaches were explored in this project. The first approach is the direct coupling of 2'-deoxyribose1-phosphate with purine bases catalyzed by PNPase. Unlabelled 2'-deoxyguanosine was first synthesised by this approach according to a reported procedure (Roy, *et al.* 1986) with some modifications (see Figure 3.3).

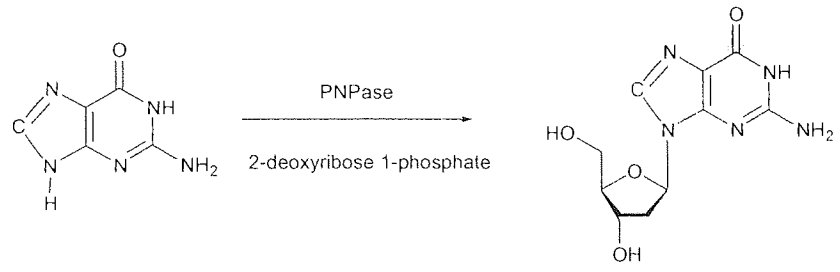


Figure 3.3. Enzymatic synthesis of dG by direct coupling of 2'-deoxyribose 1-phosphate with guanine.

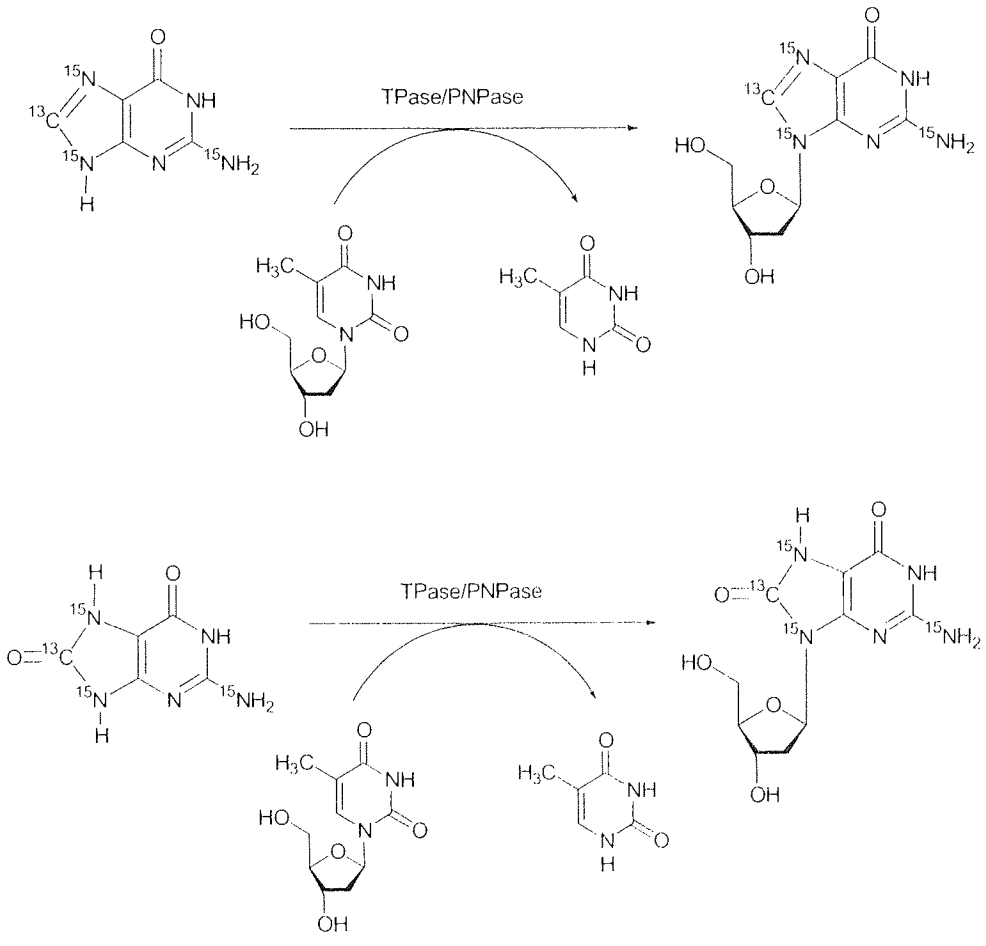


Figure 3.4. Enzymatic synthesis of (M+4) dG and (M+4) 8-oxo-dG by coupling of 2'-deoxyribose 1-phosphate, generated *in situ*, with purine bases.

Because the 2'-deoxyribose 1-phosphorate is extremely expensive, the preparation of (M+4) 8-oxo-2'-deoxyguanosine and (M+4) 2'-deoxyguanosine was eventually carried out using a published procedure (Chapeau & Marnett 1991) with some modifications, in which the 2'-deoxyribose 1-phosphate was enzymatically generated by TPase *in situ* from thymidine. Similar to the first approach, enzymatic coupling of the corresponding bases to 2'-deoxyribose-1-phosphate was catalysed by purine nucleoside phosphorylase (PNPase) (Figure 3.4).

The formation of (M+4) dG and (M+4) 8-oxo-dG under the incubation conditions was monitored by RP HPLC using HPLC method 1. The enzymatic coupling of guanine with 2'-deoxyribose 1-phosphate to form dG was fast. Equilibrium was reached in 4-5 hours (Figure 3.5), and it required very low enzyme concentration.

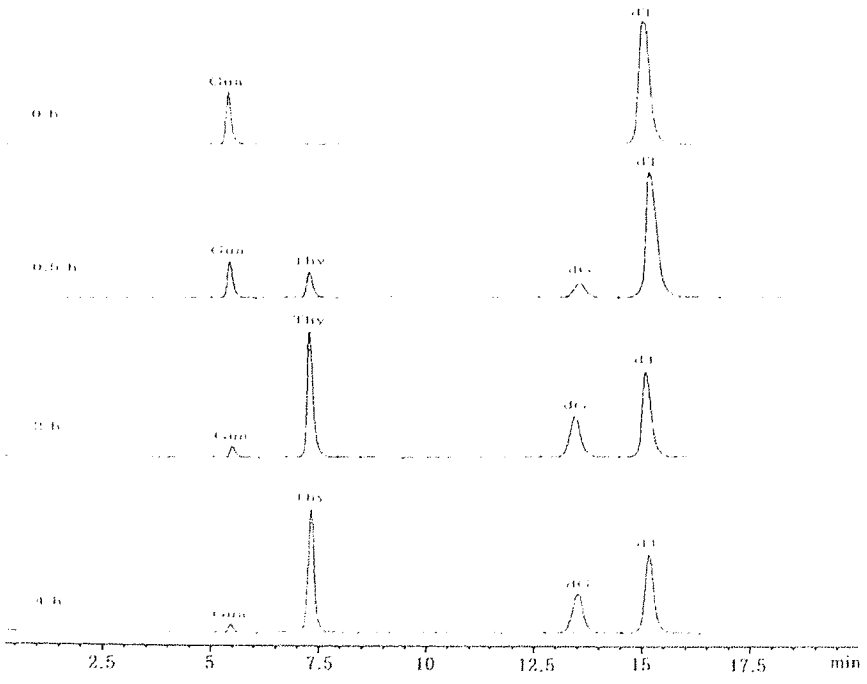


Figure 3.5. Reaction course of the formation of (M+4) dG in the enzymatic coupling reaction monitored by HPLC (method 1).

Due to the low solubility of 8-oxo-dG in aqueous solutions at the pH value (7.4) for the reaction, DMSO was added to the reaction mixture. Therefore a much higher enzyme concentration (~200 times that used for dG coupling) and longer incubation was required for the coupling of 8-oxo-dG, as shown in Figure 3.6.

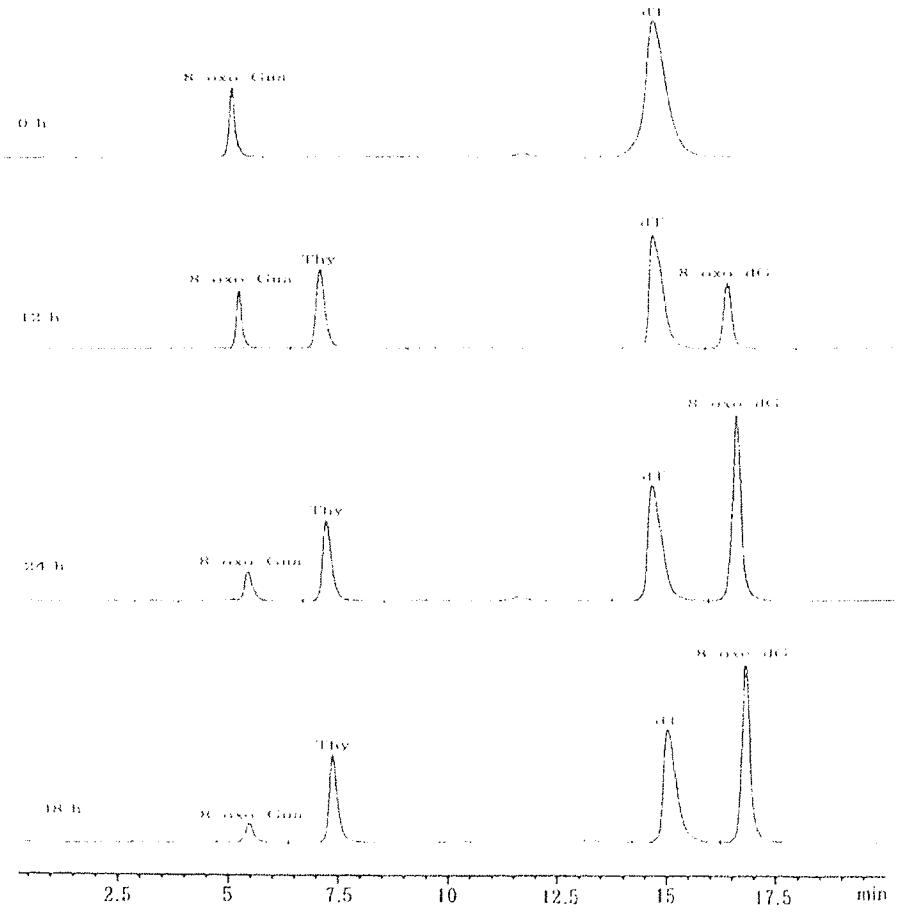


Figure 3.6. Reaction course of the formation of (M+4) 8-oxo-dG in the enzymatic coupling reaction monitored by HPLC (method 1).

3.3.2 Purification of (M+4) 2'-deoxyguanosine and (M+4) 8-oxo-2'-deoxyguanosine

Normal column chromatographic purification methods involving silica gel are not suitable for the separation of dG or 8-oxo-dG because of the low solubilities of these compounds in organic solvents. RP-HPLC is a method of choice. We successfully established the programmes for the purification of both 8-oxo-dG (HPLC method 2) and dG (HPLC method 3), using a semi-preparative column. However, only about 1 mg can be obtained after a dozen of separations of the compounds, and the purity was not constant.

In order to obtain enough material (at least 60 mg of the deoxynucleoside) for carrying out the synthesis over the next three steps of preparing a phosphoramidite building block, a gel filtration procedure for the separation of dG was developed, using Sephadex G10. It did not require sophisticated equipment, the packed column was reusable and the separation was excellent. A typical separation result is shown in Figure 3.7.

This approach (gel filtration) combined the separation and desalting into a single step. The collected fractions were checked by measuring the conductivity and UV absorbance at 260 nm. The fractions containing dG were pooled and checked by HPLC for purity. Quantification of the pure dG can also be carried out at this stage (see below). Only one peak corresponding to the retention time of the authentic dG was observed (Figure 3.8).

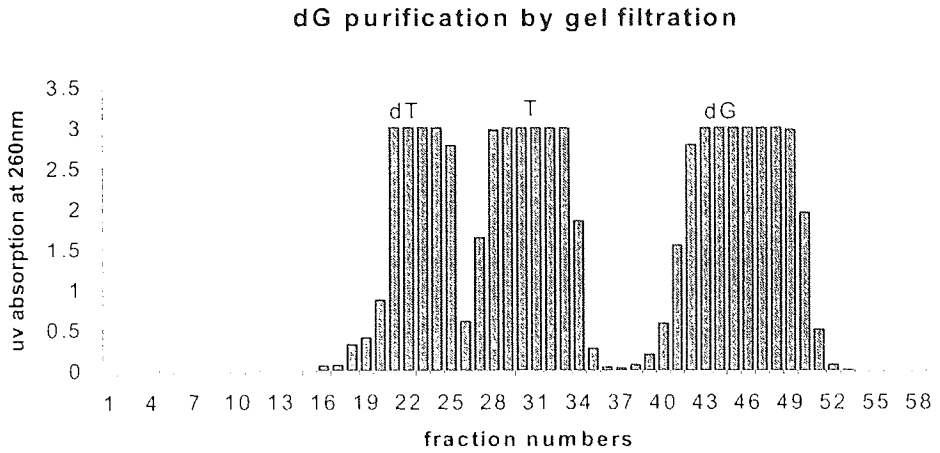


Figure 3.7. UV absorbance of the fractions collected form gel filtration.

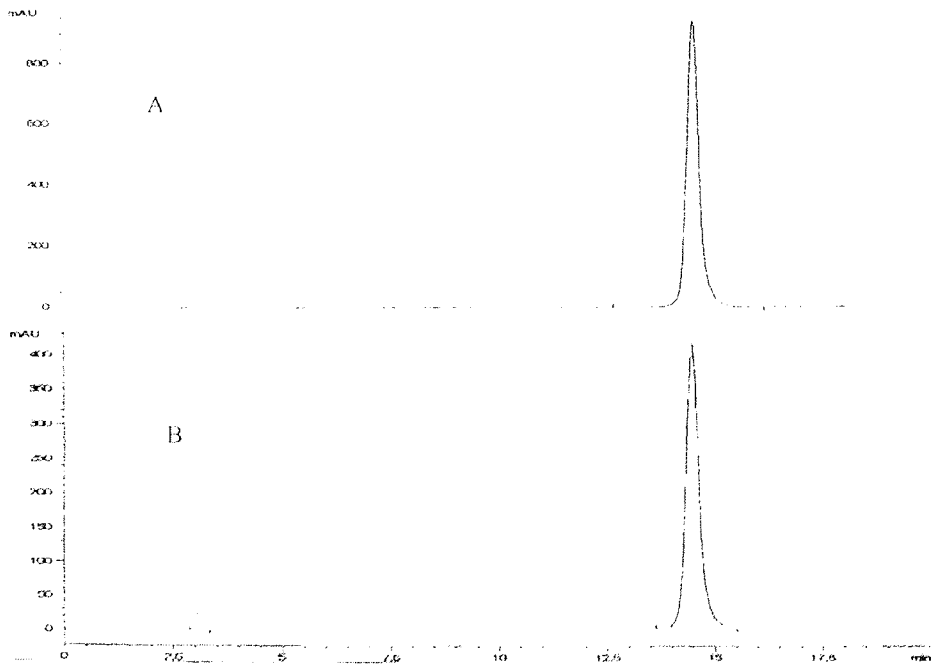


Figure 3.8. The HPLC chromatograms of the authentic dG (A) and that of the separated dG (B).

3.3.3 Quantification of dG

From the UV absorbance, the amount of dG contained in the combined fractions can be quantified. A series of standard dG solutions with a gradient concentration were prepared and the UV absorbance of each of these solutions recorded. Thus, a calibration curve and an equation (Figure 3.9) of dG concentration verse UV absorbance can be obtained. From which the concentration and the amount of the dG were calculated.

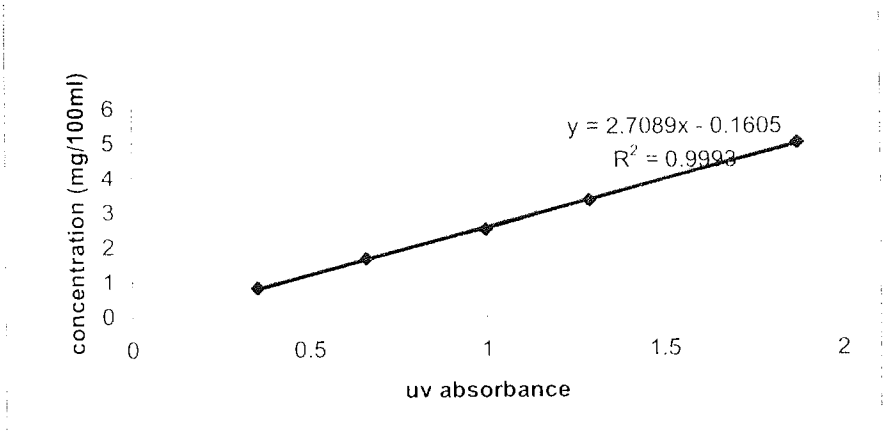


Figure 3.9. The calibration curve of the concentration of dG against UV absorbance.

Chapter 4 Synthesis of (M+4) 2'-Deoxyguanosine Phosphoramidite and its Incorporation into DNA Oligonucleotides

4.1 Introduction

The chemical synthesis of oligonucleotides is of increasing importance because of the advances in nucleic acid sciences in recent years, particularly the development of therapeutic agents in the form of oligonucleotide. In the synthesis of oligonucleotides, a fundamental process is internucleotide-bond formation. This can be achieved through various approaches, which include phosphodiester approach, phosphotriester approach, phosphoramidite approach and H-phosphonate approach. Among these approaches, the phosphoramidite method has gained the most popularity because of its higher overall yield, its reliability in the synthesis of longer oligonucleotides beyond 50 residues and its easy application to the automated solid-phase synthesis.

4.1.1 Phosphodiester approach

Although phosphotriester method was the first to be reported for the chemical synthesis of dinucleoside phosphate (Michelson & Todd 1955), it was not fully investigated until the mid-1960s (see below). During that period of time, the whole field of oligonucleotide synthesis was dominated by phosphodiester approach, first developed by H. G. Khorana and his co-workers (1956). In this approach, the internucleotide phosphodiester linkages were left unprotected throughout the coupling reaction. An example of the application of this approach is illustrated in Figure 4.1: A nucleoside of base B^1, with free 3'-OH and 5'-OH protected by 4,4,- dimethoxytrityl (DMTr) was allowed to react with a nucleoside 5'-phosphate of base B^2, where 3'-OH protected by an acyl group, using mesitylene (MS-Cl) or 2,4,6-triisopropylbenzene-sulfonyl chlorides (TPS-Cl) as a coupling reagent.

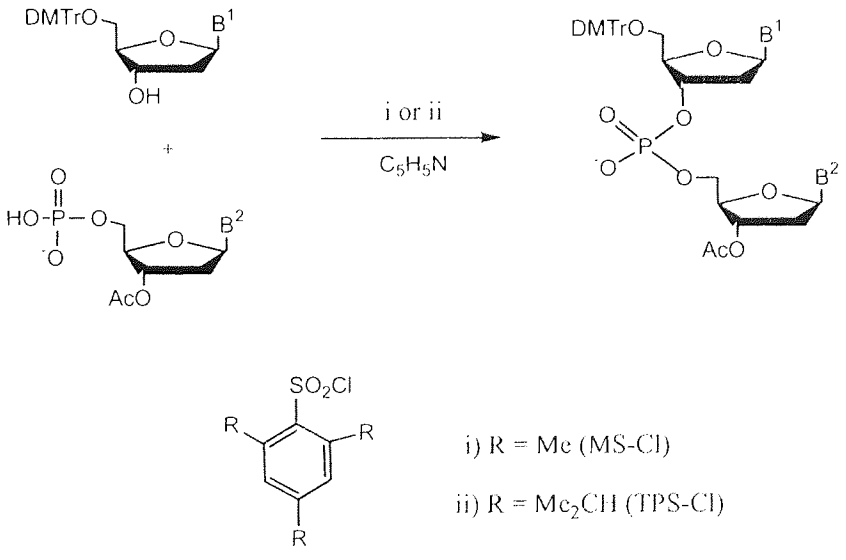


Figure 4.1. Phosphodiester approach for the synthesis of oligonucleotides.

The phosphodiester approach was adapted both to the stepwise (Jacob & Khorano 1965) and to the block synthesis (Kössel & Khorana 1967) of moderately high molecular weight oligodeoxyribonucleotides. Moderately good yields were obtained in stepwise coupling reactions if a large excess of a monomer was used. However, yields were generally lower in block coupling reactions (Kössel & Khorana 1967). It gradually became evident that the accumulation of charged phosphodiester internucleotide linkages led to side-reactions and therefore the yields were generally low (Kössel, *et al.* 1967). Furthermore, the purification of the intermediate blocks and the final products by chromatography on DEAE-cellulose was both painstaking and time-consuming. It was partly for this reason, that the successful application of the phosphodiester approach became dependent on the experimental skill of a research individual involved. It may take several months to synthesise an oligonucleotide of 10-15 residues even by a skilled individual.

4.1.2 Phosphotriester approach

To overcome the problems encountered by the phosphodiester approach, the internucleotide linkages have to be protected. The phosphotriester approach then started to attract a lot of attention. For the development of this approach, a matter of crucial importance is the choice of the protecting group for the internucleotide linkages. The protecting group has to be relatively easy to introduce, it should be stable under the reaction conditions and it should be removable at the end of the synthesis, under conditions that the desired product is stable. Since the mid-1960s, a reinvestigation of the phosphotriester approach was undertaken in several

laboratories and various protecting groups, including 2,2,2-trichloroethyl group (Eckstein & Rizk 1967a) (Eckstein & Rizk 1967b), 2-cyanoethyl group (Letsinger & Mahadevan 1965) (Letsinger & Ogilvie 1967) and phenyl group (Reese & Saffhill 1968), were used to protect the internucleotide linkages. Although the 2-cyanoethy group proved to be too labile for use in the solution phase oligonucleotide synthesis, it later became the protecting group of choice in solid-phase synthesis (see below).

Another important issue for the phosphotriester approach was to develop an appropriate coupling reagent. Many coupling reagents have been investigated and 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-1*H*-triazole (MSNT) became established as the coupling reagent of choice both in solution and in solid-phase synthesis for the phosphotriester approach.

The basic chemistry of phosphotriester approach is shown in Figure 4.2, where R^1 is the protecting group of choice and R^2 can be a protecting group or part of a growing chain. Thus, a 5'-dimethoxytrityldeoxynucleoside phosphate is added at the 5'-position of the growing chain in the present of the coupling reagent MSNT. Phosphotriester approach revolutionised the preparation of oligonucleotides by protecting the internucleotide linkages. With this approach, high yields and short reaction times were achieved, and rapid chain assembly became possible.

However, as discussed above, the phosphotriester method of oligonucleotide synthesis requires coupling of one appropriately protected monomer unit containing a 3'-phosphate with another containing a 5'-hydroxy group and separation of products and unreacted starting materials on a column of silica gel.

Each coupling step requires a chromatographic purification and, although a skilled person can do this fairly quickly, adequate resolution of long chains is difficult and the method is labour intensive. It is therefore best applied to situations where large quantities (>50 mg) of oligonucleotide are required (Gait 1984).

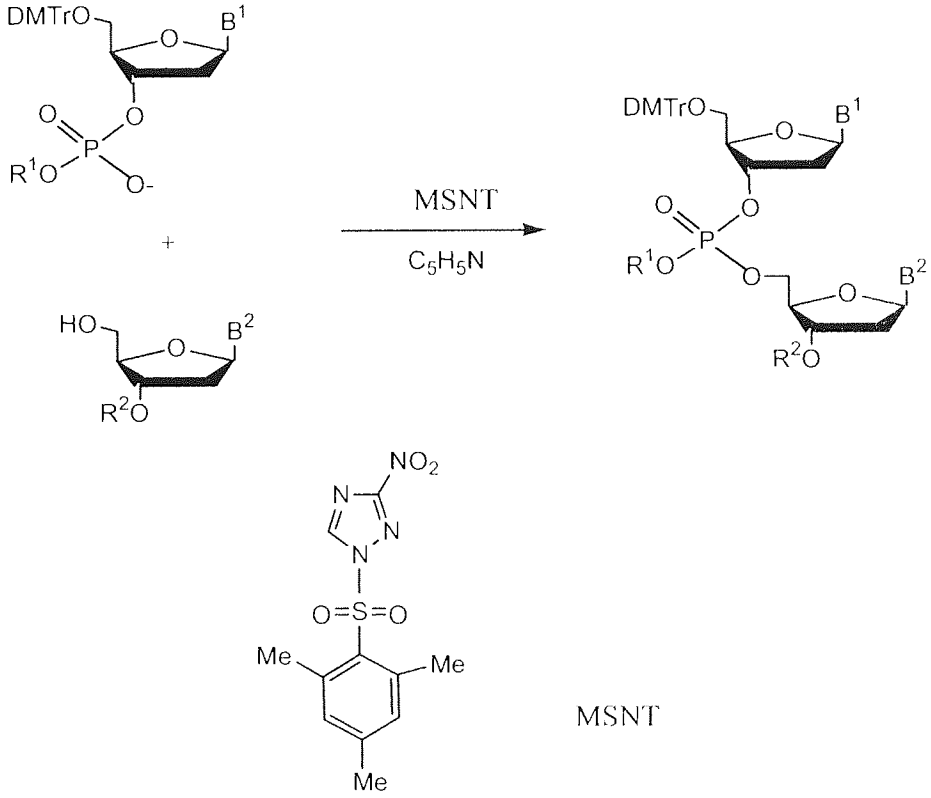


Figure 4.2. Phosphotriester approach for the synthesis of oligonucleotides.

4.1.3 Phosphoramidite approach

In 1976, Letsinger and Lunsford (1976) introduced an innovative approach in phosphorylation methodology – the phosphite triester approach. It was observed

that P(III) were considerably more reactive than the corresponding P(V) acylating agents. Therefore, 2-chlorophenyl phosphorodichloridite (Figure 4.3) reacted rapidly with the 5'-protected thymidine derivative at –78°C. The putative intermediate phophorochloridite obtained was then allowed to react with the 3'-portected thymidine derivative to give a fully protected dinucleoside phosphate. However, the use of a very reactive bifunctional phosphorodichloridite inevitably led to a mixture of the desired product with a 3'→5'– internucleotide linkage and two possible symmetrical products with 3'→3'- and 5'→5'- internucleotide linkages. Another serious limitation of this methodology was the instability of the reactive intermediates (nucleoside phosphomonochloridites) towards hydrolysis and air oxidation. Nevertheless, the phosphite triester approach set the basis for the development of the phosphoramidite approach.

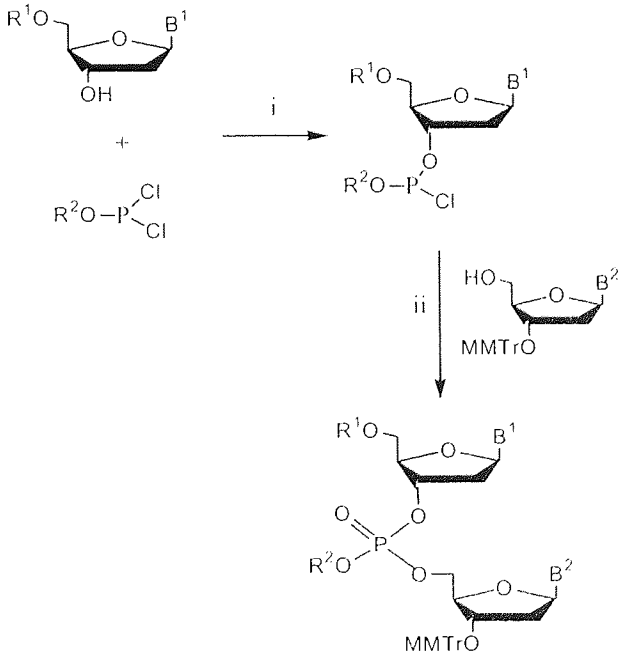


Figure 4.3. Phosphite triester approach for the synthesis of oligonucleotides.
i) 2,6-lutidine, THF; ii) 2,6-lutidine, I_2, THF, H_2O.

In 1981, monofunctional phosphitylating agents, e.g. chloro-N,N-dimethylaminomethoxyphosphine [$CH_3OP(Cl)N(CH_3)_2$], was introduced by Beaucage and Caruthers (1981) and a new class of nucleoside phosphoramidites was also developed. By then, the full advantages of the P(III) approach to oligonucleotide synthesis became clear. In their original study by Beaucage and Caruthers, a DMT protected 2'-deoxynucleoside was treated with chloro-N,N-dimethylaminomethoxyphosphine to give the corresponding protected nucleoside phosphoramidite. This product was isolated as colourless powder in high yield (90% or greater). The reaction between this phosphoramidite and 3'-O-levulinoylthymidine, under the catalysis of 1*H*-tetrazole, was revealed to be fast, and a very high yield of the expected fully-protected dinucleoside phosphite was achieved (Figure 4.4.).

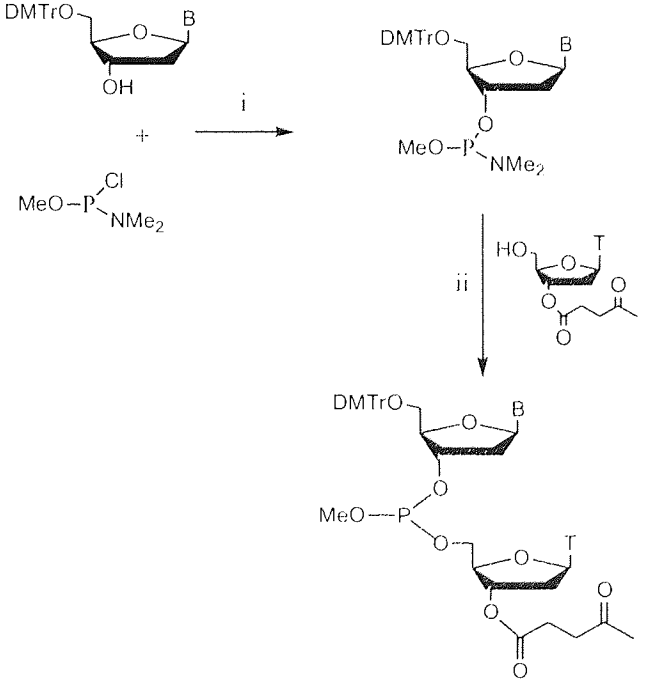


Figure 4.4. Phosphoramidite approach for the synthesis of oligonucleotides.
i) Pr^i_2NEt, $CHCl_3$; ii) 1*H*-tetrazole, CH_3CN.

It was later shown that N,N-diisopropylphosphoramidites were considerably more stable than N,N-dimethylphosphoramidites (Adams, *et al.* 1983) (McBride & Garuthers 1983). In another important study, Köster and his co-workers (Sinha, *et al.* 1983) found that 2-cyanoethyl phosphoramidites were generally more suitable building blocks than the corresponding methyl esters. 2-Cyanoethyl N,N-di-isopropylphosphoramidites have since been used virtually exclusively in phosphoramidite-based solid-phase oligonucleotide synthesis.

4.1.4 H-phosphonate approach

Like the phosphotriester approach, the H-phosphonate approach to oligonucleotide synthesis was first reported from Todd's Cambridge laboratories in the 1950s (Hall, *et al.* 1957). However, the true synthetic potential of the approach was not realised for almost 30 years. The real significance of H-phosphonate approach only became apparent in 1986 when two group of workers, Frochler & Matteucci (1986) (1986) and Garegg *et al.* (1986), applied it to solid-phase oligonucleotide synthesis. Using pivaloyl chloride or adamantane-1-carbonyl chloride as a coupling reagent (Figure 4.5), the coupling reaction became very fast and high molecular weight oligodeoxyribonucleotides were able to be prepared.

Another advantage of H-phosphonate approach is that although the building blocks required for the approach are commercially available, they can also be easily prepared in a laboratory (Frochler 1993) (Figure 4.6) and isolated as pure stable solids.

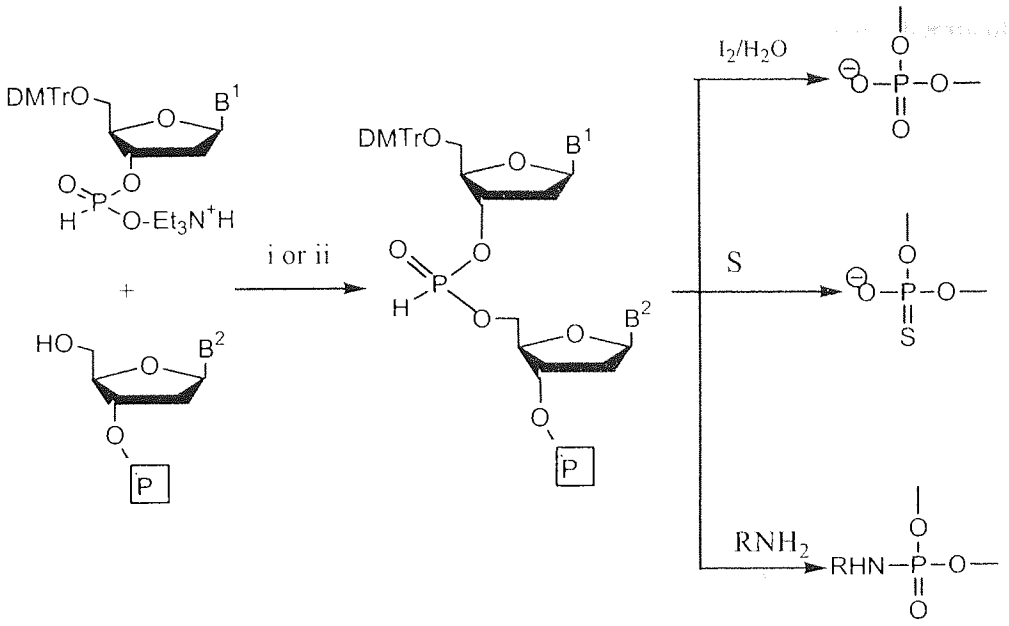


Figure 4.5. H-phosphonate approach for the synthesis of oligonucleotides and their conversion to phosphate analogues. i) pivaloyl chloride; ii) adamantolyl chloride.

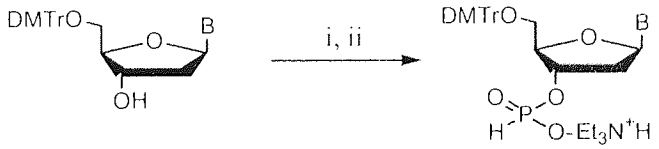


Figure 4.6. Synthesis of building blocks for H-phosphonate approach. i) PCl_3, 1,2,3-1*H*-triazole and Et_3N, CH_2Cl_2; ii) aqueous triethylammonium bicarbonate.

Furthermore, the generated diester H-phosphonates from the coupling reaction (Figure 4.5) can be oxidised to the phosphates, thiated to the phosphorothioates, or aminated to give phosphoramidates as required (Brown 1993). This property enables the application of H-phosphonate approach to be extended to the synthesis of oligonucleotide phosphorothioates analogues, which, at present, constitute the

main class of oligonucleotide analogues intended for use as chemotherapeutic agents. For example, Vitravene, the world's first antisense drug, a 21-mer oligodeoxyribonucleotide phosphorothioate sequence, that has been approved by the US Food and Drug Administration (FDA) for the treatment of cytomegalovirus (CMV)-induced retinitis has been prepared by means of the modified H-phosphonate approach in solution (Sanghvi 2004).

4.1.5 Solid-phase method

Solid-phase synthesis has become the method of choice in oligonucleotide synthesis in many laboratories. It has clear advantages of being readily applicable to automation and its remarkably short time synthetic cycle. The yield and the quality of the oligomers are no longer dependent on the expertise of the individuals.

Solid-phase synthesis of oligonucleotides was developed from the idea of automated solid-phase synthesis of peptides during 1980s. When a DNA oligonucleotide is synthesised in this way, the first deoxyribonucleoside is attached through its 3'-hydroxyl group to an inert insoluble support, such as controlled-pore glass (CPG) and highly crosslinked polystyrene. It then involves the addition of one nucleotide residue at a time to this immobilised protected nucleoside or oligonucleotide. By simple washing, excess reagents are easily removed from the reaction product, therefore, no purification steps are required until the fully assembled sequence is released from the solid support.

The phosphotriester approach was successfully applied to the solid-phase synthesis, but it has now been largely superseded by phosphoramidite-based solid-phase synthesis. There are a number of reasons that may account for the domination of the phosphoramidite approach. First and most importantly, the coupling reaction is very rapid and is usually completed within a few minutes and irreversible side-reactions can generally be avoided. Secondly, fully protected phosphoramidite building blocks, such as benzoyl-protected cytidine and adenine, isobutyryl- protected guanine phosphoramidite, are commercially available at reasonable prices. Using these building blocks, with 1*H*-tetrazole as the activating reagent, average coupling yields of 98% (or greater) can be achieved. Furthermore, the commercial availability of easy-to-operate automatic DNA synthesisers, which accommodate phosphoramidite building blocks and reagents, have also promoted the popularity of this approach. Automated solid-phase synthesis of DNA oligonucleotide, using such instruments is illustrated in Figure 4.7 (Urbina, *et al.* 1998).

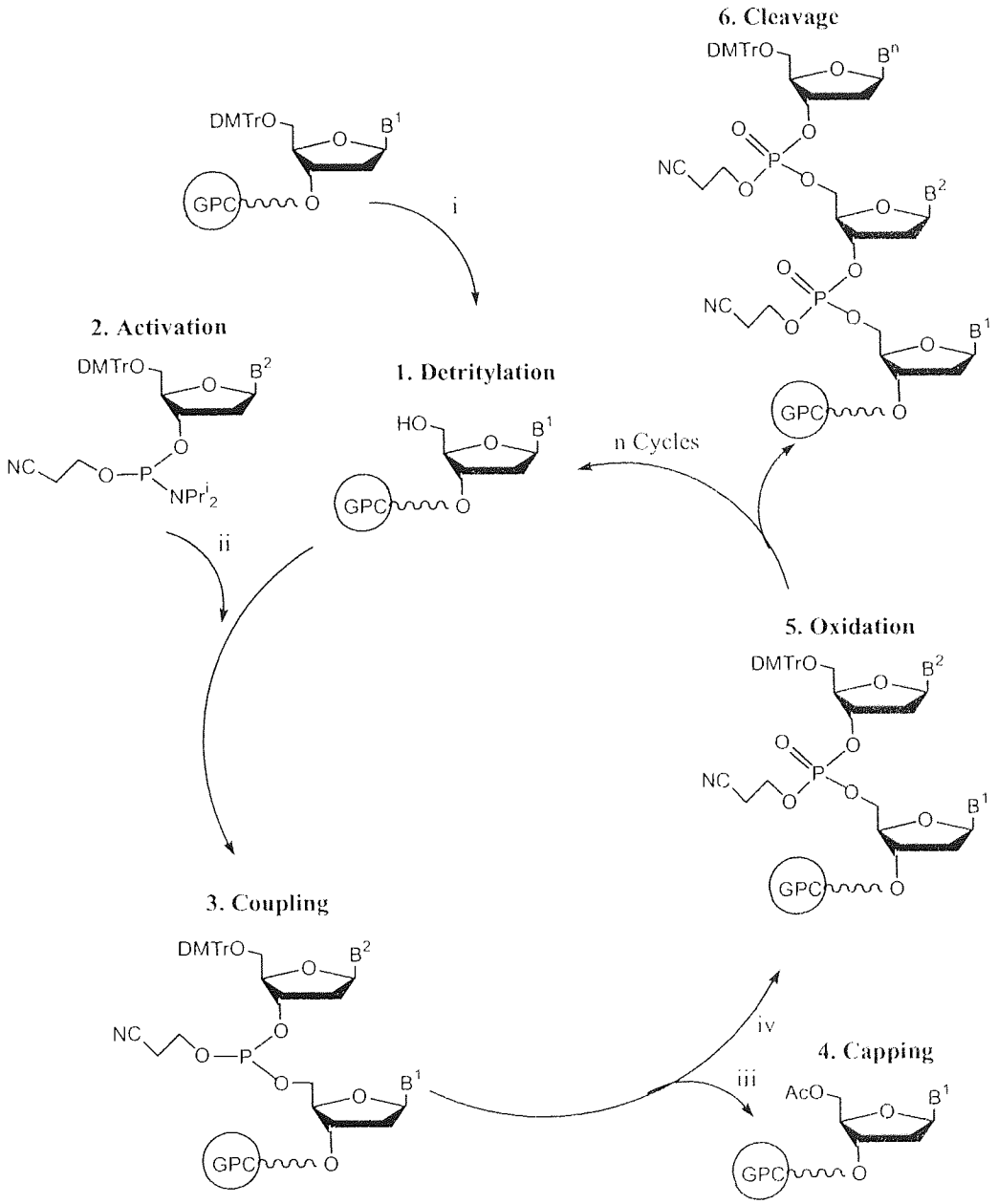


Figure 4.7. Automated solid-phase phosphoramidite approach for DNA synthesis [Adapted from (Urbina *et al.* 1998)]. i) 97% dichloromethane, 3% trichloroacetic acid; ii) 97% acetonitrile, 3% tetrazole; iiia) 80% THF; 10% acetic anhydride, 10% lutidine; iiib) 93 THF, 7% 1-methylimidazole; iv) 93% THF, 3% iodine, 2% water, 2% pyridine.

4.2 Experimental

4.2.1 Materials and methods

4.2.1.1 Chemicals and instruments

All chemicals used were purchased from Aldrich unless stated otherwise. Dry solvents were purchased from Aldrich prior to use. Standard 2'-deoxyribonucleoside phosphoramidites, reagents for solid-phase DNA synthesis and DNA columns were purchased from Glen Research (Cambio, Cambridge, UK).

^{31}P NMR spectra were recorded on a Bruker AC-250 instrument using 85% H_3PO_4 as an external reference. Mass spectra were obtained on an HP G1034C GC/LC-MS Chemstation using electrospray ionisation. Other instruments have been described in section 2.2.1.1.

4.2.1.2 HPLC method

Reverse phase HPLC was carried out on a *Hewlett Packard Series 1100* instrument with a UV detector set at 260 nm.

<u>Method 4</u>

This method was used for the analysis of DNA oligomers.

HPLC column: Techsphere ODS 5µm (250 x 4.6 mm).

Mobile phase: A = 2% acetonitrile in 0.1M TEAA (pH 7.0), B = 70% acetonitrile in 0.1M TEAA (pH 7.0).

Gradient elution was performed at a flow rate of 1 ml/min and monitored at 260 nm. A linear gradient was run from 0% to 30% B over 20 min.

The retention time of the synthesised oligomer was 16.4 min.

4.2.1.3 Deprotection and purification of DNA oligonucleotides

Deprotection: The oligonucleotides were cleaved from the support by a standard procedure [concentrated ammonia (28%) overnight at 55°C].

Purification: Purification of the oligomers was carried out on NENSORB columns (purchased form Du-Pont), following essentially the manufacturer's instructions. The cartridge was firstly activated with 10 ml CH_3OH, then pre-equilibrated with 5 ml 0.1M TEAA. The sample solution, i.e. DNA oligomers in ammonium hydroxide, was loaded directly after the deprotection. 10 ml 0.1M TEAA (pH 7.0) was used to wash the cartridge, followed by 10 ml 10% CH_3CN in 0.1M TEAA, 25 ml 0.5% TFA and 10 ml 0.1M TEAA (pH 7.0). The oligomers were finally eluted with 35% methanol. Fractions of 1 ml each were collected with Eppendorf tubes. 50ul of each fraction was diluted to 1 ml and the UV absorbance at 260 nm were measured. Fractions containing oligomers were subjected to HPLC analysis for purity check, then pooled, lyophilized and stored at -20°C.

4.2.2 Experimental procedures

N^2-isobutyryl-[2-amino,7,9-$^{15}N_3$, 8-^{13}C] 2'-deoxyguanosine (10). [2-Amino,7,9-$^{15}N_3$, 8-^{13}C] 2'-deoxyguanosine (**9**) (77 mg, 0.25 mmol) was co-evaporated with dry pyridine (5 x 1.5 ml) and then suspended in dry pyridine (1.5 ml). The suspension was cooled in an ice bath. Trimethylsilyl chloride (0.16 ml, 1.25 mmol) was added via a disposable syringe and the reaction was stirred for 10 min. The ice bath was removed and stirring was continued for another 50 min at room temperature. Isobutyric anhydride (0.22 ml, 1.25 mmol) was added via measuring pipette and the reaction was allowed to continue at room temperature for a further 2 hours. The reaction vessel was cooled in an ice bath and water (0.5 ml) was added with stirring. After 15 minutes concentrated ammonium hydroxide (28%, 0.4 ml) was added and reaction mixture was stirred for an additional 15 minutes. The reaction mixture was evaporated under vacuum to a creamy paste and dissolved in 2.5 ml water, washed with ether (3 ml). The aqueous layer was separated immediately and crystallization occurred within 5-10 min. Crystals were collected by filtration, washed with cold water and dried *in vacuo* overnight, affording N^2-isobutyryl-[2-amino,7,9-$^{15}N_3$, 8-^{13}C] 2'-deoxyguanosine 60 mg, yield: 71%. ^{1}H NMR* (DMSO-*d6*): 12.06 (s, 1H, N2); 11.69 (s, 1H, N1), 8.23 (s, 1H, H-C8); 6.19 (t, 1H, H1'); 5.32 (d, 1H, 3'OH); 4,97 (t, 1H, 5'OH); 4.36 (m, 1H, H3'); 3.52 (m, 2H, H5'); 2.74 (sept, 1H, CH-^{i}Bu); 2.2~2.4 (m, 2H, H2'); 1.1 (d, 6H, 2xCH_3-^{i}Bu). HRMS: HRMS on the [M+Na] ion calcd for $C_{12}{}^{13}CH_{19}N_2{}^{14}N_3O_5Na$, m/z 364.1228, found 364.1226 (for HRMS spectrum, see the appendix).

* H1 NMR data obtained from unlabelled compound

[2-Amino,7,9-$^{15}N_3$,8-^{13}C]5'-O-(4,4'-dimethoxytrityl)-N^2-isobutyryl-2'-deoxyguanosine (11) N^2-isobutyryl-[2-amino,7,9-$^{15}N_3$, 8-^{13}C] 2'-deoxyguanosine (**10**) (60 mg, 0.17 mmol) was co-evaporated with dry pyridine (4 x 2 ml) and then dissolved in dry pyridine (1.5 ml). The solution was cooled in an ice bath and 4,4'-dimethoxytrityl chloride (0.2 mmol, 70 mg) was added to the solution. The reaction mixture was stirred at 5°C and monitored by TLC (chloroform: methanol, 8:1, v/v). When the reaction was completed, methanol (0.25 ml) was added and the reaction mixture was stirred for 20 min. The solvent was removed under vacuum and the residue was dissolved in ethyl acetate (5 ml), washed with 5% $NaHCO_3$ (2 x 2 ml), water (1 x 5 ml). The organic layer was separated, dried over anhydrous sodium sulfate and evaporated to dryness. The crude product was purified by silica gel column chromatography using a 0-3% methanol/chloroform gradient to yield pure compound 90 mg, yield: 81%. ^{1}H NMR* (DMSO-*d6*): 12.07 (s, 1H, N2); 11.65 (s, 1H, N1); 8.10 (s, 1H, H-C8); 7.15-7.30 (m, 9H, Ar-DMTr); 6.21-6.26 (m, 4H, Ar-DMTr); 6.24 (t, 1H, H1'); 5.34 (d, 1H, 3'OH); 4.38 (m, 1H, H3'); 3.93 (s, 1H, H4'); 3.88 (m, 2H, H5'); 3.69 (s, 6H, 2xCH_3O); 2.74 (m, 1H, CH-^{i}Bu); 2.2~2.4 (m, 2H, H2'); 1.1 (d, 6H, 2xCH_3-^{i}Bu). HRMS: HRMS on the [M+Na] ion calcd for $C_{12}{}^{13}CH_{37}N_2{}^{14}N_3O_7Na$, m/z 666.2535, found 666.2529 (for HRMS spectrum, see the appendix).

[2-Amino,7,9-$^{15}N_3$,8-^{13}C]5'-O-(4,4'-dimethoxytrityl)-N^2-isobutyryl-2'-deoxyguanosine cyanoethyl-diisopropylamine phosphoramidite (12). [2-Amino,7,9-^{15}N,8-^{13}C]5'-O-(4,4'-dimethoxytrityl)-N^2-isobutyryl-2'-deoxyguanosine (**11**) (64 mg, 0.1 mmol) was coevaporated with dry pyridine and

* 1H NMR data obtained from unlabelled compound

dry dichloromethane (2 x 0.5 ml each) and dissolved in 0.5 ml dry dichloromethane. 5-Ethylthiotetrazole (7 mg, 0.05 mmol) and diisopropylamine (9 µl, 0.05 mmol) dissolved in 0.25 ml dry dichloromethane was added, followed by dropwise addition of 2-cyanoethyl N,N,N',N'-tetraisopropylphosphorodiamidite (40 µl, 0.12 mmol). The reaction mixture was stirred at room temperature and monitored by TLC. When the reaction was completed (about 2h), the reaction mixture was evaporated to almost dry in vacuum at room temperature, the residue was dissolved in 5 ml argon saturated ethyl acetate and washed with ice cold 10% Na_2CO_3 (2 x 5 ml). The organic layer was separated and dried over anhydrous sodium sulfate and evaporated to dryness at room temperature under vacuum. The dried crude compound was purified by silica gel column chromatography, using a mixture of hexane/chloroform/ triethylamine (5/94/1, v/v/v) as the eluting solvent. The purified compound was dissolved in 2 ml dry benzene and lyophilized to yield dry pure compound (**12**) 40 mg, yield: 48%. ^{31}P NMR (DMSO-*d6*): 149.2, 148.4. HRMS: HRMS on the [M+Na] ion calcd for $C_{43}{}^{13}CH_{54}N_2{}^{14}N_3O_8NaP$, m/z 866.3614, found 866.3626 (for HRMS spectrum, see the appendix).

Synthesis of DNA oligonucleotides containing (M+4) guanine.

Automated solid-phase DNA oligonucleotide synthesis was carried out on a 1µmol scale using a *Beckman Oligo 1000* DNA synthesiser following the standard protocol recommended by the manufacturer.

The above synthesised (M+4) monomer (**12**) was incorporated into oligonucleotides by DNA synthesiser using the manufacturer's program except

that **12** (10 mg in 100 µl acetonitrile anhydrous and 100 µl activator) was injected manually with 250 µl dry gas tight syringe with coupling time of 3 min. When synthesis completed, the column was removed and dried in vacuo for 10-15 min. CPG support, bearing the synthesised 18 mer [(5'-GTA TGA CXA TCG CGC CAT-3', in which "X" is (M+4) dG], was transferred to a 1.5 ml Eppendorf tube. The oligomer was deprotected with 1 ml concentrated ammonia at 55°C for 12-16h (overnight) and purified using NENSORB columns, following the manufacture's procedure, to remove most of the failed sequences and obtain the 5'-detritylated oligomer. The oligonucleotide-containing fractions from this purification step were collected, combined and the quantity of purified oligomer estimated using absorbance at 260 nm as 26 OD. The purity was checked by HPLC (Method 4), only one peak corresponding to the retention time of the standard oligomer with the same sequence was observed. MS: MS for $C_{174}{}^{13}CH_{222}N_{65}{}^{14}N_3O_{106}P_{17}$, calcd 5501.95, found: 5502.23.

4.3 Results and Discussion

The key element in the synthesis of DNA oligonucleotides is the preparation of appropriate deoxynucleotide monomer building blocks. It was discussed earlier in section 4.1 that the most popular method is the automated solid-phase synthesis with phosphoramidite chemistry. To prepare the phosphoramidite monomer from (M+4) deoxyguanosine, the exocyclic amino group of the guanine base needs to be protected and so does the 5'-OH group. The protected nucleoside was then treated by a proper phosphitylating reagent to give the desired (M+4) guanine phosphoramidite. The synthesis route is outlined in Figure 4.8.

A dry run was performed with unlabelled deoxyguanosine and the procedures were optimized. NMR data presented were obtained from the unlabelled compounds. All the labelled compounds appeared homogeneous when co-eluting with unlabelled compounds by TLC. The identities of the labelled compounds were further verified by high resolution mass spectrometry (HRMS).

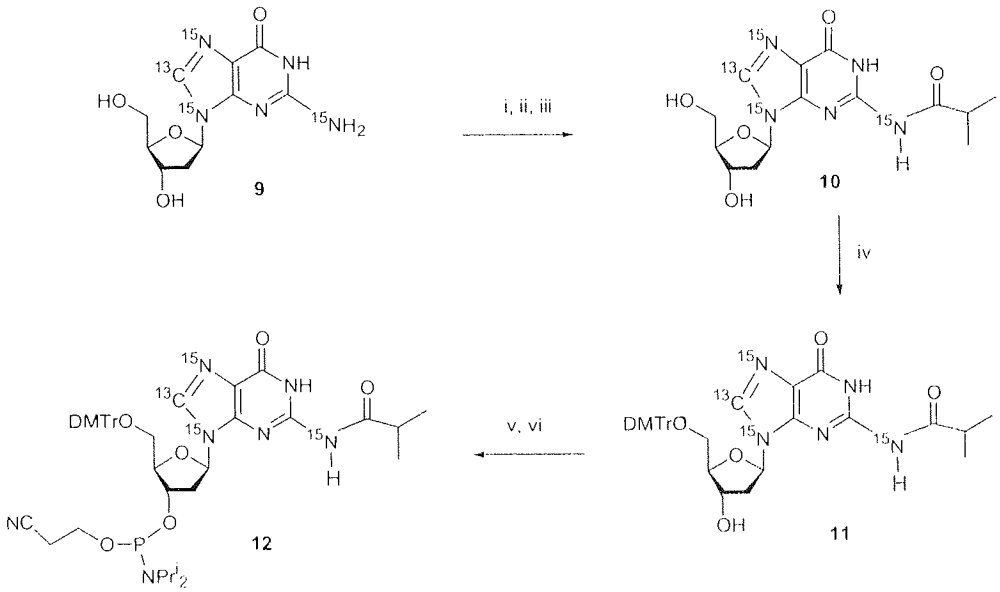


Figure 4.8. The preparation of (M+4) isobutyryl-deoxyguanosine phosphoramidite. i) TMS-Cl/pyridine; ii) isobutyric anhydride; iii) H_2O/NH_2OH; iv) DMTr-Cl/pyridine; v) diisopropylamine, 5-ethyl-tetrazole/pyridine, dichloromethane; vi) 2-cyanoethyl (N,N,N',N')-tetraisopropylphosphorodiamidite.

4.3.1 Protection of 2-NH_2

Several protection groups have been used to protect the exocyclic amino group of guanine, including phenoxyacetyl group and isobutyryl group. Phenoxyacetyl

group was introduced by Schulhof *et al.* (1987a) to protect the exocyclic amino group of guanine and adenine. Because it can be cleaved in aqueous ammonia in a shorter time at lower temperature, it is most suitable in the synthesis of modified oligonucleotides containing fragile bases.

We compared both of these protecting groups, and isobutyryl group was chosen for the protection of (M+4) dG, because the yield of isobutyryl protected dG was higher than that of the phenoxyacetyl dG (75% compared to 55%). Also for the present work, all the monomers involved in the DNA synthesis were stable under the standard deprotection condition, therefore the extra sensitivity of the protection group was not required.

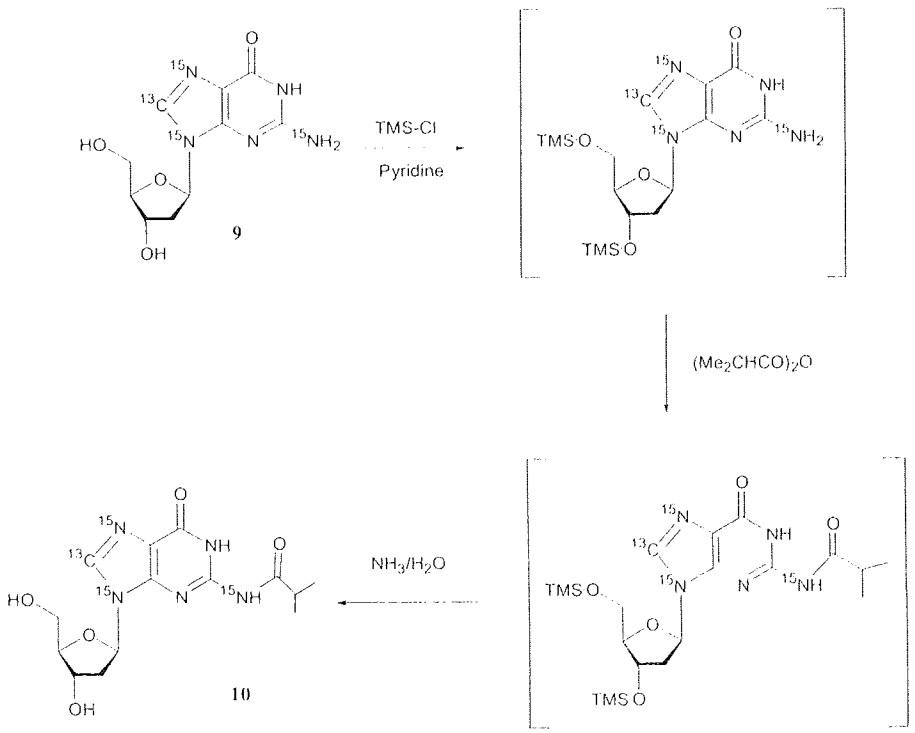


Figure 4.9. Transient protection of exocyclic $-NH_2$ group of (M+4) deoxyguanosine.

Thus isobutyryl group was introduced using transient protection method developed by Jones *et al* (Ti, *et al.* 1982) to give compound **10**. The three-step reaction was conducted in one flask without separating the intermediates as shown in Figure 4.9.

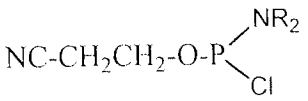
4.3.2 Protection of 5'-OH group

5'- Hydroxyl group was protected by 4,4'-dimethoxyltrityl (DMT), which is a standard protecting group introduced firstly by Khorana and his co-workers (Schaller, *et al.* 1963). This group was introduced to the N-protected (M+4) deoxyguanosine by the reaction with 4,4'-dimehoxyltrityl chloride in the presence of pyridine which acted as a basic catalyst as well as the solvent. The reaction is regioselective for the primary 5'-hydroxyl group due to the bulk of the DMT group. DMT is acid labile so it can be removed by the treatment with an acid, such as trichloroacetic acid.

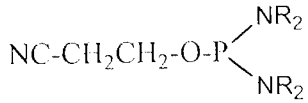
4.3.3 Phosphitylation

Deoxynucleoside phosphoramidite building blocks, used in solid-phase DNA synthesis, can be prepared from protected deoxynucleosides using one of the two types of phosphitylating reagents shown in Figure 4.10.

2-Cyanoethyl diisopropylchlorophosphoramidite (Figure 4.10 a. R = Me_2CH) is a commonly used phosphitylating reagent and is commercially available.

a) 

a ß-cyanoethyl monochlorophosphoramidite
or chloro(dialkylamino)-ß-cyanoethoxyphosphine

b) 

a ß-cyanoethyl bis-dialkylphosphoramidites
or bis-dialkylamino-ß-cyanoethoxyphosphine

Figure 4.10. Structures of the commonly used phosphitylating reagents.

Another group (Figure 4.10, b) of phosphitylating reagents are called bis-dialkylaminophosphines. They were originally developed by Barone *et al.* (1984) and used for a strategy involving *in situ* generation of deoxynucleoside phosphoramidites for deoxyoligonucleotide synthesis on a solid support. Compared to the chlorophosphines mentioned above, this type of phosphitylating reagents are easier to prepare and less prone to hydrolysis and oxidation. Although there is no need to prepare them now, as the most commonly used bis-dialkylaminophosphines are commercially available, it is still more advantageous over its counterpart chlorophosphine in respect of being more tolerant of trace amounts of water that may present in the reaction mixture.

It has also been demonstrated that deoxynucleoside phosphoramidites can be prepared using this type of phosphitylating reagent in good yields. One key issue

is the use of tetrazole amine salt as catalyst at 0.5 equivalent to the protected nucleoside to ensure a rapid and selective reaction.

In this project, a bis-diisopropylaminophosphine, 2-cyanoethyl (N,N,N',N')-tetraisopropylphosphorodiamidite (Figure 4.10, b. R = Me_2CH), was chosen as the phosphitylating reagent and 5'-OH, N^2-NH_2 protected deoxyguanosine phosphoramidite was prepared based on a procedure described previously (Schulhof, *et al.* 1987b) with some modifications.

4.3.4 Oligonucleotide synthesis, cleavage and purification

The oligonucleotides containing (M+4) guanine were synthesised by an automated solid-phase DNA synthesiser with slight modification of the supplier's program. Due to the high cost of isotopically labelled phosphoramidite, the amount available was limited. Therefore, the coupling was carried out manually during the isotopically labelled monomer cycle. The automatic synthesis was paused just after detritylation, and resumed after the activated (M+4) 2'-deoxyguanosine phosphoramidite solution was injected manually through the support in about 3 min.

The deprotection was straightforward using a standard procedure, i.e. concentrated ammonium hydroxide overnight at 55°C. Cleaved oligonucleotides were purified using NENSORB column. According to the manufacturer's instructions, the column needs to be activated by methanol and equilibrated with 0.1M TEAA, (pH 7.0). The flow rate should not exceed 10 ml/min in order to get the best result. After the crude oligomer was loaded, the column was eluted with

10% CH_3CN in 0.1M TEAA to remove the failure sequences, which had no DMT group attached, and other by products that were more polar than the still tritylated oligomer. This was followed by 0.5% TFA to remove the trityl group of the desired oligomers. After the acid was washed off with 0.1M TEAA (pH 7.0), the column was then eluted by 35% methanol to retrieve the purified oligomers. Fractions of 1 ml each were collected and monitored by UV absorbance at 260 nm. The result is shown in Figure 4.11.

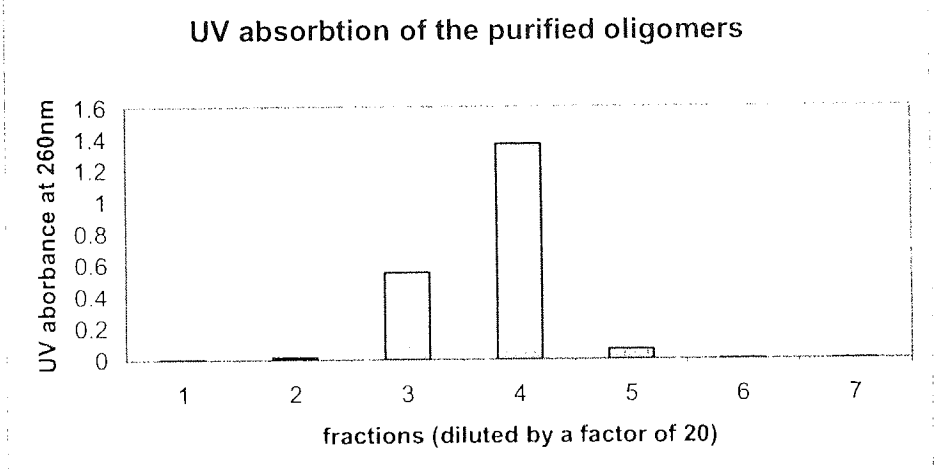


Figure 4.11. UV absorption of the fractions collected from NENSORB column purification.

Fractions containing the desired oligomer were combined and the purity was checked by HPLC. As shown in Figure 4.12, only one peak was observed, corresponding to the standard oligomer with the same sequence .

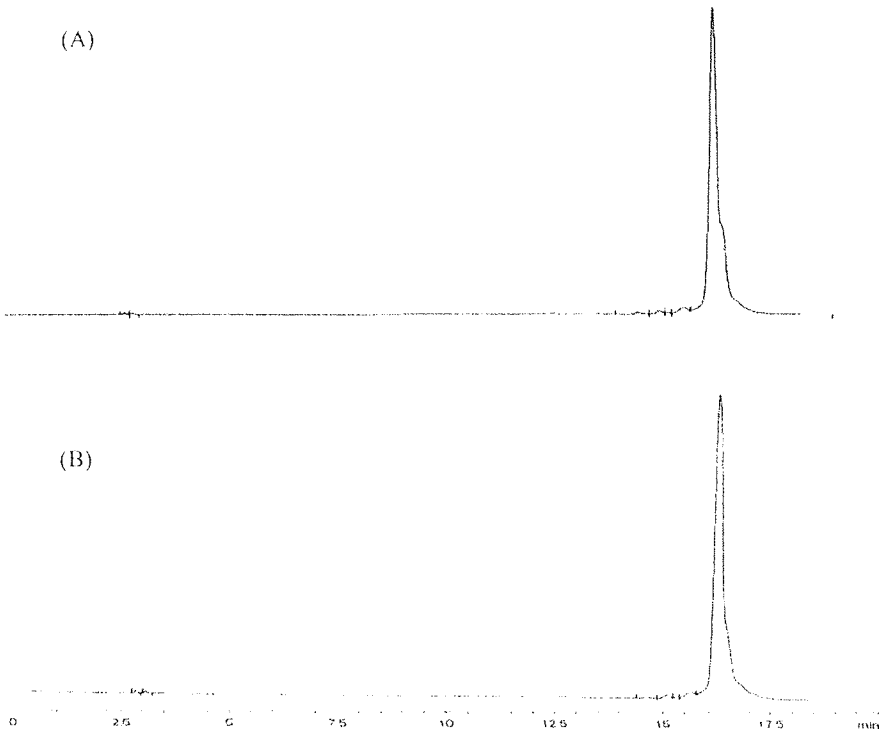


Figure 4.12. The HPLC chromatogram of the DNA oligomer containing (M+4) 2'-deoxyguanosine (A) and that of the co-elution of the DNA oligomer containing (M+4) 2'-deoxyguanosine with the normal DNA oligomer having the same base sequence (B).

Oligomers with the sequence 5'-GTA TGA CXA TCG CGC CAT-3' were prepared with various sources of monomer X, various amounts of X and two different synthetic protocols for the coupling of X. The yields of these oligomers are compared in Table 4.1.

Oligomer	Source of monomer X	Synthesis method in cycle "x"	Quantity (mg)	Yield (O.D.)
1	Commercial dG	Automatic	N/A	60
2	Self prepared unlabelled	Manual	5 mg	9
3	Self prepared unlabelled	Manual	10 mg	40
4	Self prepared labelled	Manual	10 mg	26

Table 4.1. Comparison of the yields of oligomers prepared by various methods with various sources of monomer X.

The yields of the oligomer prepared using self prepared labelled (M+4) monomer and injected manually had a lower yield. This may be explained by a number of factors:

Coupling conditions in the manual step such as coupling time, monomer concentration, etc, was not optimized as the dry run has provided acceptable coupling efficiency.

The phosphoramidite synthesis procedure adopted in this project is standard and includes aqueous work-up. However, lower coupling efficiency was noticed by Bodepudi *et al.* (1991) when this standard procedure was used in the preparation of 8-oxo-dG phosphoramidite. They also reported that the problem could be resolved by preparing the phosphoramidite building blocks under completely anhydrous conditions.

Conclusion

The measurement of oxidative base damage, such as 8-oxo-dG, in cellular DNA remains an analytical challenge, particularly establishing a baseline or an absolute level *in vivo*. There appears to be certain limitations in almost every assay so far developed for the determination of levels of 8-oxo-dG.

The Fpg based methods, which are still in an early stage of development, are limited by a number of factors, such as the substrate specificity, indirect calibration and lower range of detection. Therefore, these need to be validated against a robust chemical method.

Chemical methods, including the popular HPLC-ECD, GC-MS, and the more recent HPLC-MS/MS, are constantly hampered by the issue of spurious oxidation of guanine during sample preparation stages. Although a number of the problems have been identified, and some of them have been tackled by using optimized protocols, the errors that may occur during early stages of the sample preparation remain to be eradicated.

Among these analytical methods, those combine the use of chromatographic and mass spectrometric techniques, particularly LC-MS/MS, have great potential advantages over all other assays that had been applied to DNA damage measurements, because they can not only provide an almost unequivocal identification of the oxidation products, but also simultaneously measure numerous products. The simultaneous measurement of multiple products in the

same DNA sample has been recognised as an important issue, as it can avoid misleading conclusions drawn from the measurement of a single product as discussed in section 1.4.2.

With proper internal standards, levels of modified bases in cellular DNA can be accurately measured. However, the currently employed internal standards are all in the form of a monomer, therefore are not able to account for the errors that may arise in the early stages of DNA sample preparation, particularly DNA isolation and DNA polymer breakdown (hydrolysis). One of the keys to a solution will be the available of internal standards in the form of DNA oligonucleotides.

Furthermore, in addition to the accurate detection of 8-oxo-dG, it is crucial that intact dG is measured correctly, since the content of 8-oxo-dG is expressed relative to dG.

In this report, a synthetic route was developed to prepare multi-isotopically labelled (M+4) 2'-deoxyguanosine, which has been successfully incorporated into a DNA oligonucleotide. This is intended to be used as an internal standard in the MS based assays for the determination of DNA oxidative damage.

(M+4) 8-oxo-deoxyguanosine has also been prepared and our next objective is to incorporate this (M+4) modified nucleoside into an oligonucleotide, and to use these oligomers as internal standards to validate MS-based assays for the measurement of levels of oxidative DNA damage.

We believe that these oligomers can be used to develop procedures to make further improvements on the existing analytical methods, by adding them to the biological samples prior to the DNA isolation.

Future work

As mentioned earlier, following the successful incorporation of (M+4) 2'-deoxyguanosine into DNA oligomer, the next objective of the group will be the incorporation of (M+4) 8-oxo-2'-deoxyguanosine into DNA oligomers. This can be achieved in a similar fashion as its unmodified counterpart. Therefore, the first challenge is to obtain a sufficient amount of purified (M+4) 8-oxo-2'-deoxyguanosine. Gel filtration can be considered first, however, due to the lower solubility of (M+4) 8-oxo-2'-deoxyguanosine in aqueous solution, the elute solvent needs to be adjusted.

(M+4) 8-oxo-2'-deoxyguanosine phosphoramidite can be synthesised according to the method described in literature (Massefski & Redfield 1990)(Bodepudi *et al*, 1991). Dry runs should be conducted to validate the synthetic route and optimise the reaction conditions.

Once (M+4) 8-oxo-2'-deoxyguanosine phosphoramidite is obtained, its incorporation into the oligonucleotide can be readily synthesised by an automated solid-phase DNA synthesiser with minor modifications to the supplier's program, as described in section 4.3.4.

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Appendix

High Resolution Mass Spectra of the Isotopically Labelled Compounds

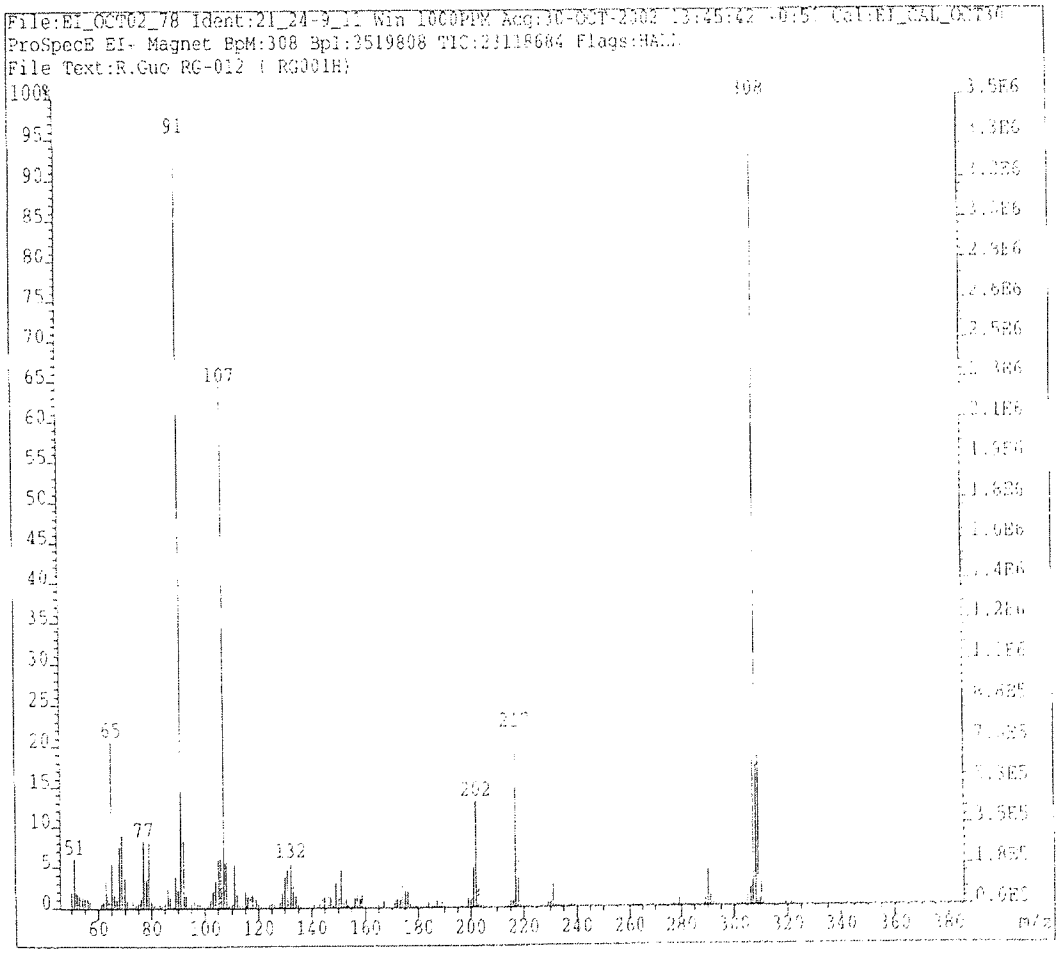
Compound 3: 4-Hydroxy-[2,6-$^{15}N_2$]bis(benzylamino)pyrimidine

Elemental Composition Date : 30-OCT-2002

File:EI_OCT02_78 Ident:36 Acq:30-OCT-2002 13:45:42 +.:21 Cal:EI_CAL_OCT30
ProSpecE EI+ Magnet BpI:222043 TIC:1791908 Flags:HAL.
File Text:R.Guo RG-012 (RG001H)
Heteroatom Max: 20 Ion: Both Even and Odd
Limits:

			-0.5	18	18	2	2	1
308.142578	100.0		20.0	18	18	2	2	1

Mass	mDa	PPM	Calc. Mass	DBE	C	H	N	15N	O
308.142578	-0.4	-1.4	308.142131	12.0	18	18	2	2	1



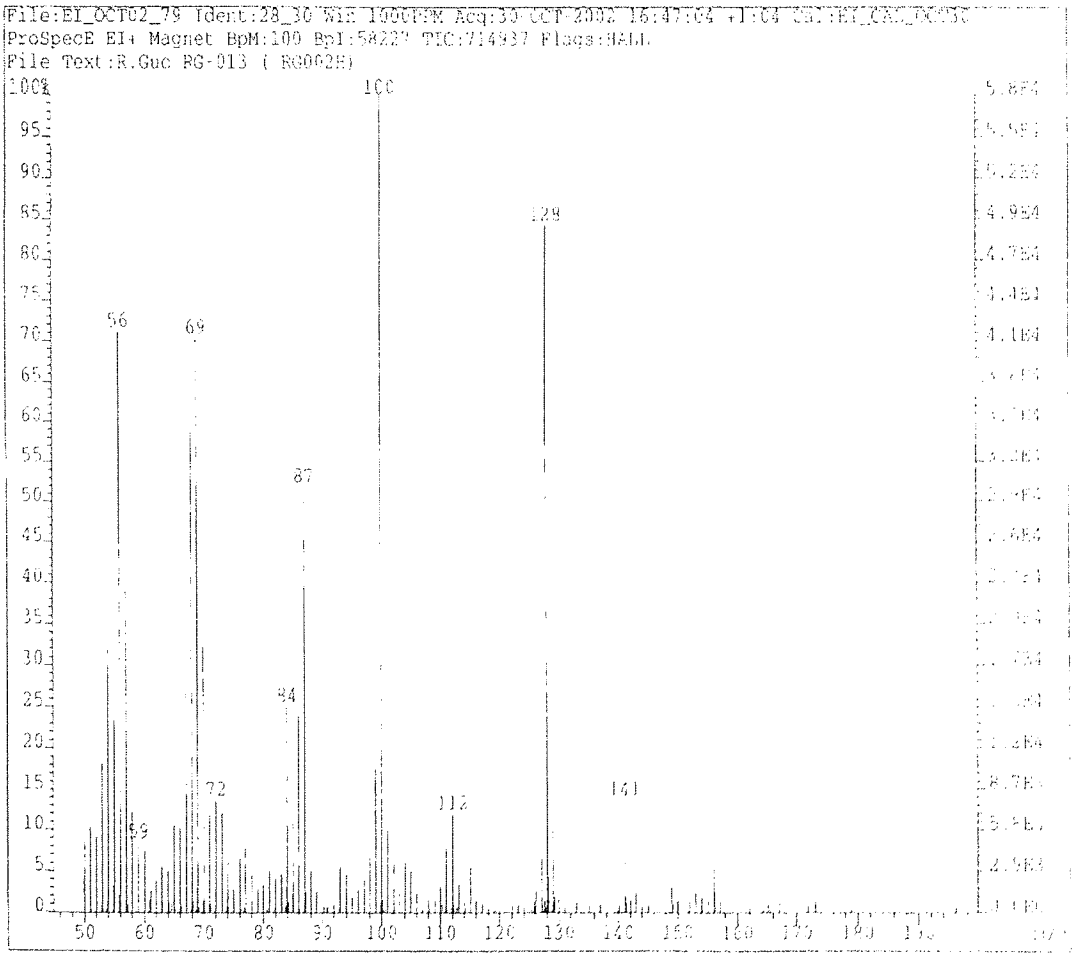
Compound 4: 4-Hydroxy-[2,6-$^{15}N_2$]diaminopyrimidine

Elemental Composition Date : 30-OCT-2002

File:EI_OCT02_79 Ident:28_29 Win 1000PPM Acq:30-OCT-2002 16:47:04 +1:03
ProSpecE EI+ Magnet BpM:100 BpI:72880 TIC:859657 Flags:HALL
File Text:R.Guo RG-013 (RG002H)
Heteroatom Max: 20 Ion: Both Even and Odd
Limits:

			-0.5	4	6	2	2	1
128.047610	100.0		20.0	4	6	2	2	1

Mass	mDa	PPM	Calc. Mass	DBE	C	H	N	15N	O
128.047610	0.6	4.8	128.048231	4.0	4	6	2	2	1



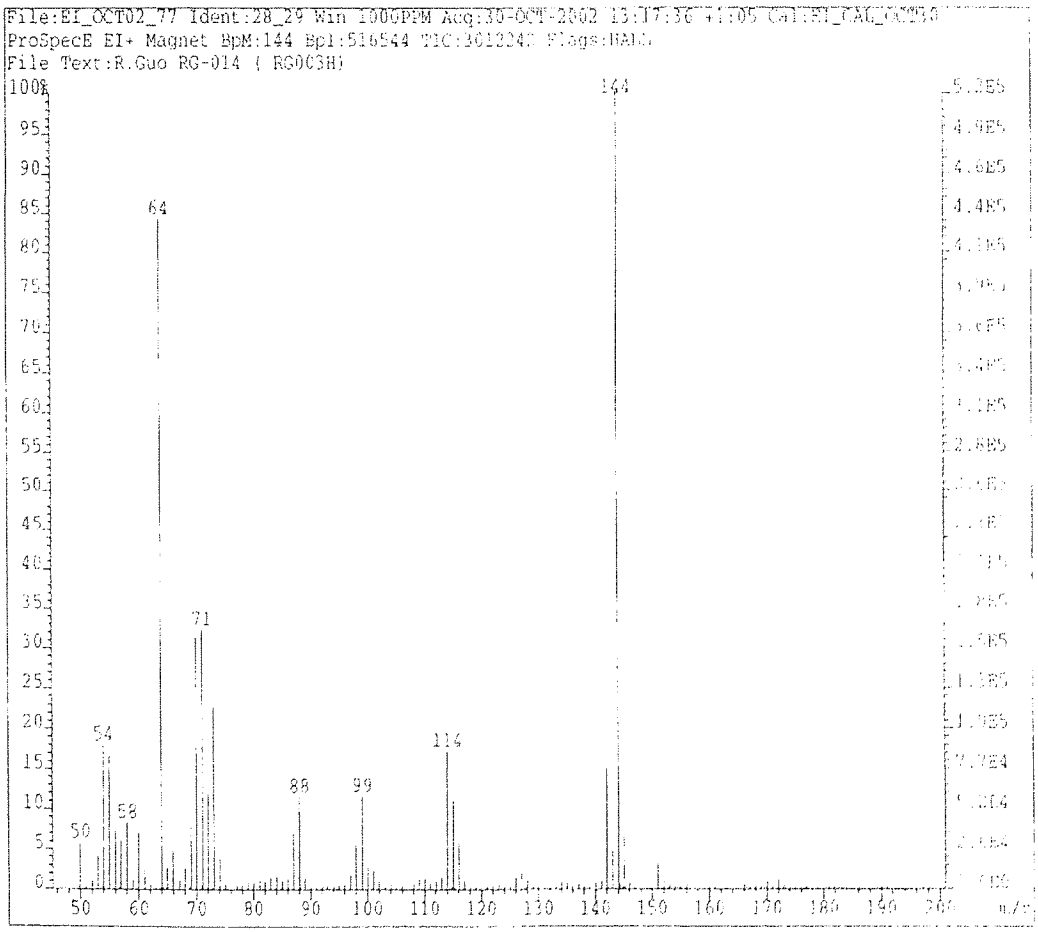
Compound 5: 4-Hydroxy-[2,5,6-$^{15}N_3$]triaminopyrimidine

Elemental Composition Date : 30-OCT-2002

File:EI_OCT02_80 Ident:31_43 Win 1000PPM Acq:30-OCT-2002 17:07:01 +1:23
ProSpecE EI+ Magnet BpM:64 BpI:16283 TIC:184154 Flags:HALL
File Text:R.Guo RG-014 (RG002H)
Heteroatom Max: 20 Ion: Both Even and Odd
Limits:

				-0.5	4	7	2	3	1
144.056658		100.0		20.0	4	7	2	3	1

Mass	mDa	PPM	Calc. Mass	DBE	C	H	N	15N	O
144.056658	-0.5	-3.4	144.056165	4.0	4	7	2	3	1



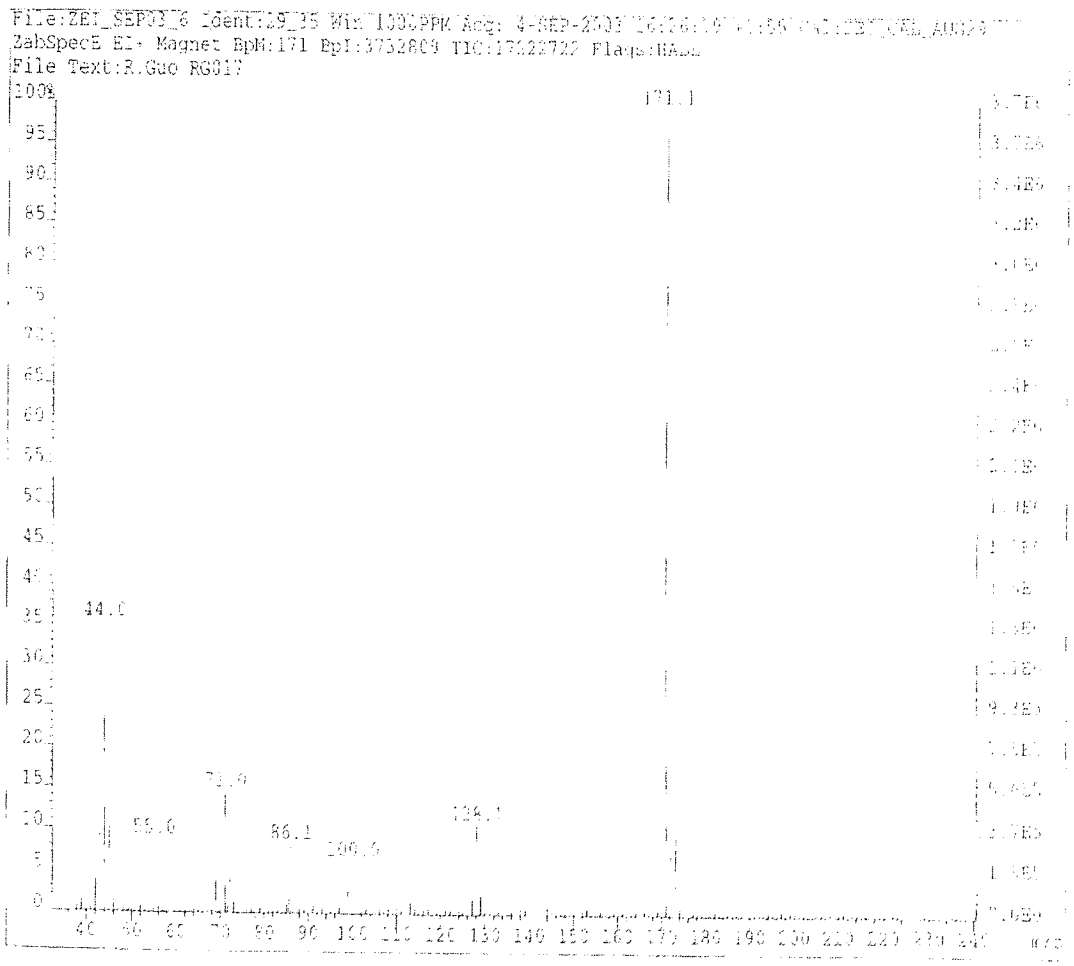
Compound 6: [2-amino, 7,9-$^{15}N_3$, 8-^{13}C]8-oxo-guanine

Elemental Composition Date : 4-SEP-2003

File:ZEI_SEP03_3 Ident:28_33 SMO(1,3) PKD(7,3,7,0.20%,0.0,0.00%,F,F)
ZabSpecE EI+ Voltage BpI:481024 TIC:734416256 Flags:NORM
File Text:R.Guo RG 017
Heteroatom Max: 20 Ion: Both Even and Odd
Limits:

				-0.5	4	1	5	2	3	2
171.038365	10.0			20.0	4	1	5	2	3	2

Mass	mDa	PPM	Calc. Mass	DBE	C	13C	H	N	15N	O
171.038365	0.4	2.5	171.038784	6.0	4	1	5	2	3	2



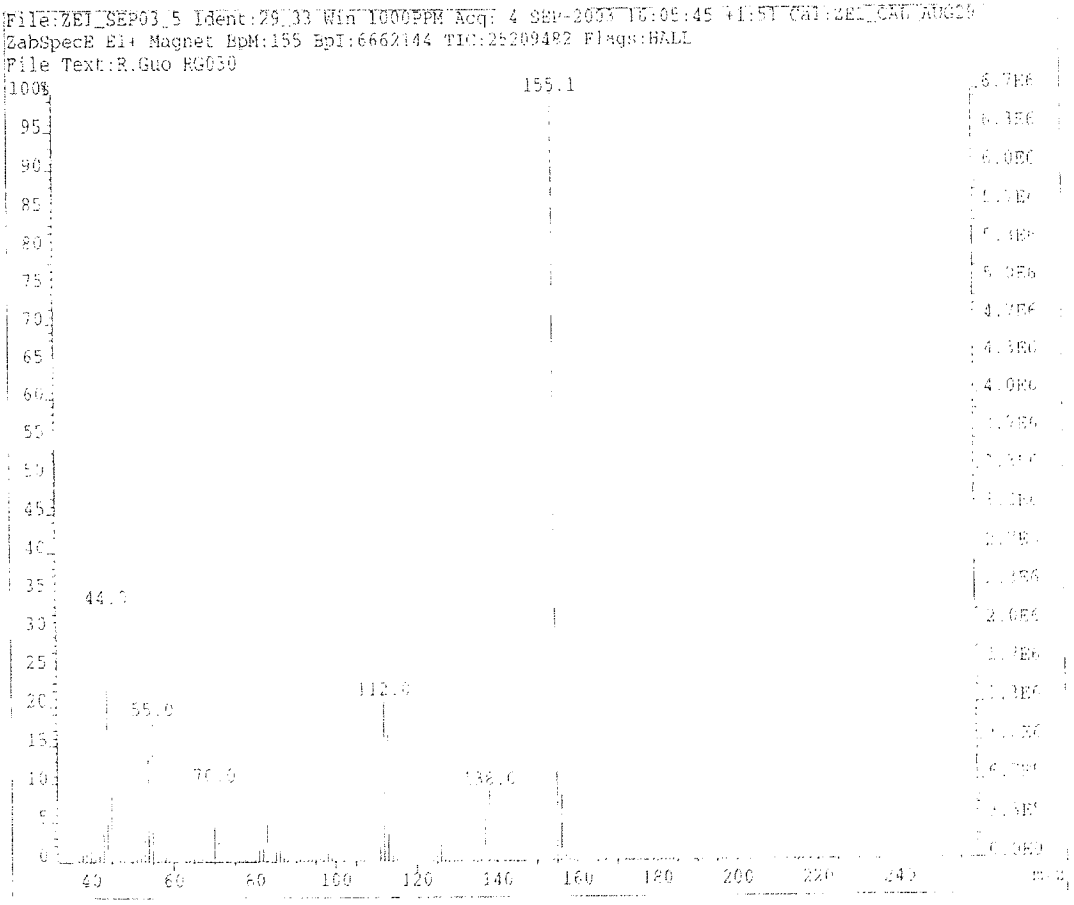
Compound 7: [2-amino,7,9-$^{15}N_3$,^{13}C]Guanine

Elemental Composition Date : 4-SEP-2003

File:ZEI_SEP03_4 Ident:51_52 PKD(11,3,11,0.20%,0.0,0.00%,F,F) SPEC(Heights,Top)
ZabSpecE EI+ Voltage BpI:261440 TIC:262146688 Flags:NORM
File Text:R.Guo RG 030
Heteroatom Max: 20 Ion: Both Even and Odd
Limits:

				-0.5	4	1	5	2	3	1
155.044226	10.0			20.0	4	1	5	2	3	1

Mass	mDa	PPM	Calc. Mass	DBE	C	13C	H	N	15N	O
155.044226	-0.4	-2.3	155.043870	6.0	4	1	5	2	3	1



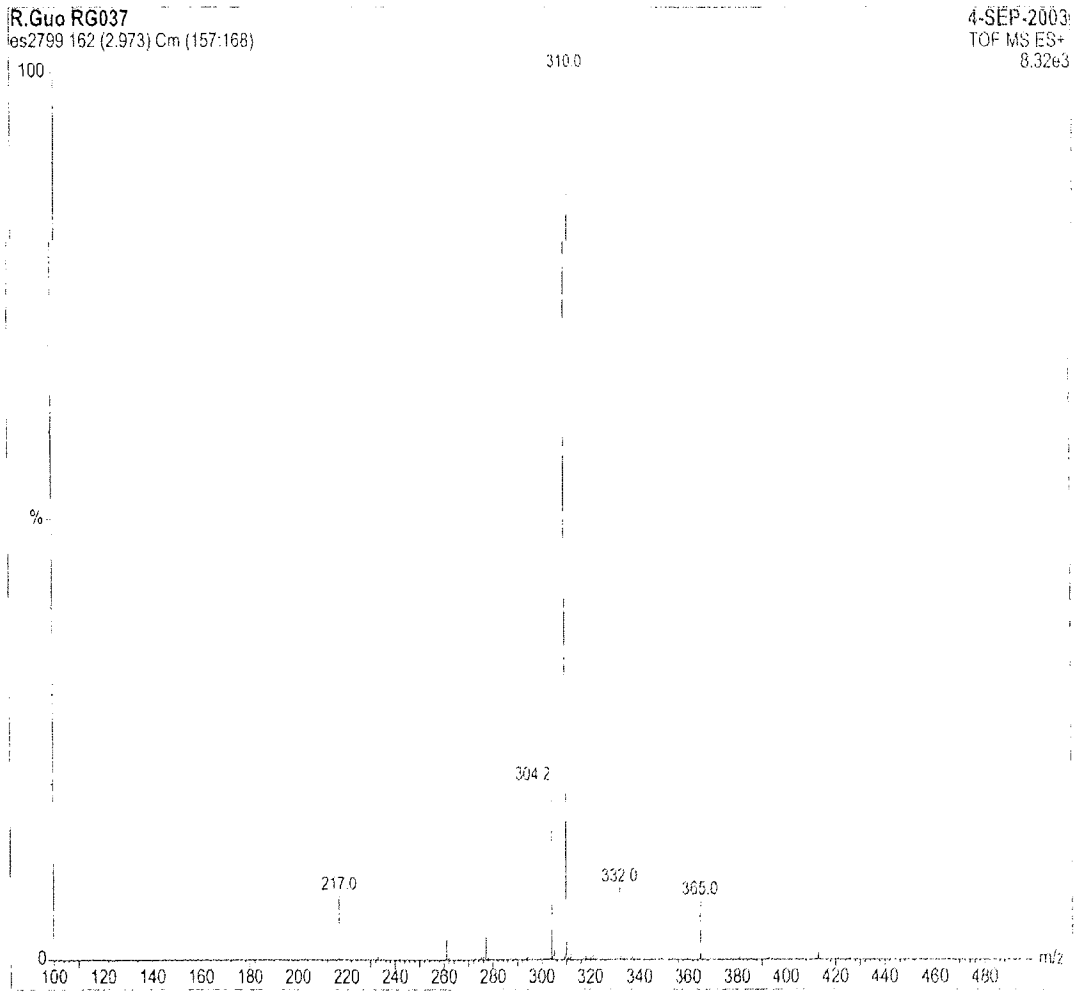
Compound 8: [2-amino, 7,9-$^{15}N_3$, 8-^{13}C]8-oxo-2'-deoxyguanosine

Elemental Composition Report **R. Guo RG 037**

Single Mass Analysis
Tolerance = 100.0 PPM / DBE: min = -0.5, max = 80.0

Monoisotopic Mass, Odd and Even Electron Ions
21 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

Minimum:				-0.5	
Maximum:		2000.0	100.0	80.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
310.0762	310.0759	0.3	1.0	6.5	12C9 13C H13 14N2 15N3 O5 Na



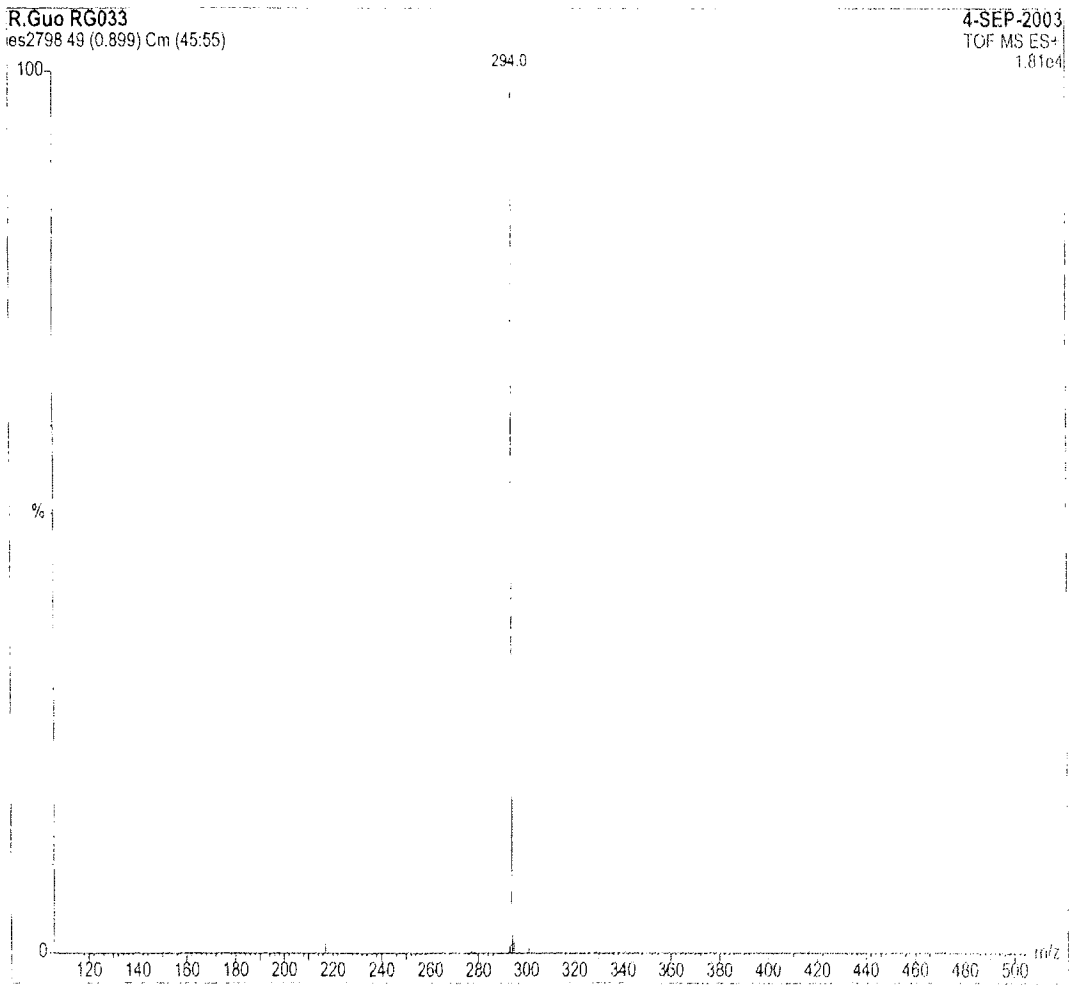
Compound 9: [2-amino,7,9-$^{15}N_3$,8-^{13}C]2'-deoxyguanosine

Elemental Composition Report **R.Guo RG 033**

Single Mass Analysis
Tolerance = 100.0 PPM / DBE: min = -0.5, max = 80.0

Monoisotopic Mass, Odd and Even Electron Ions
21 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

Minimum:				-0.5	
Maximum:		2000.0	100.0	80.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
294.0801	294.0810	-0.9	-3.0	6.5	12C9 13C H13 14N2 15N3 O4 Na



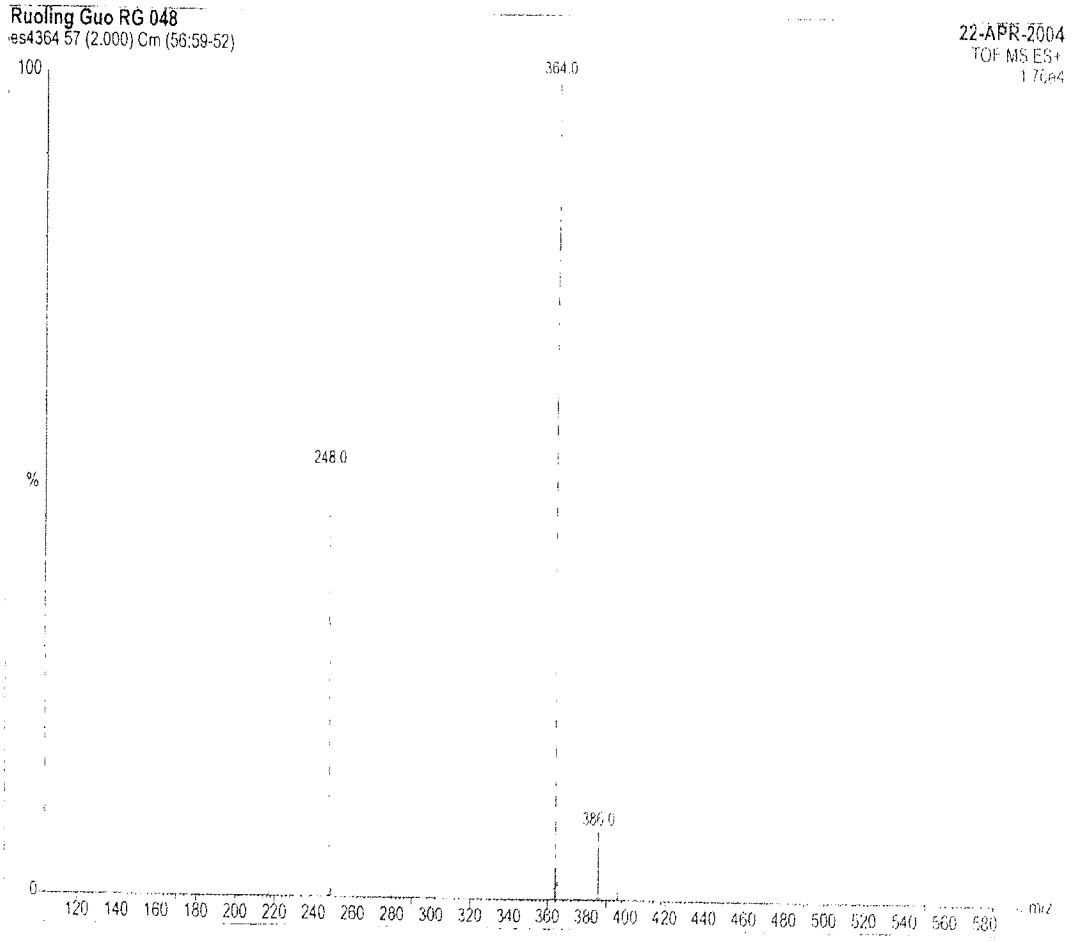
Compound 10: N^2-isobutyryl-[2-amino,7,9-$^{15}N_3$, 8-^{13}C] 2'-deoxyguanosine

Elemental Composition Report **Ruoling Guo RG 048**

Single Mass Analysis
Tolerance = 200.0 PPM / DBE: min = -0.5, max = 80.0

Monoisotopic Mass, Odd and Even Electron Ions
776 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

Minimum:				-0.5	
Maximum:		2000.0	200.0	80.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
364.1226	364.1228	-0.2	-0.7	7.5	12C13 13C H19 14N2 15N3 O5 Na



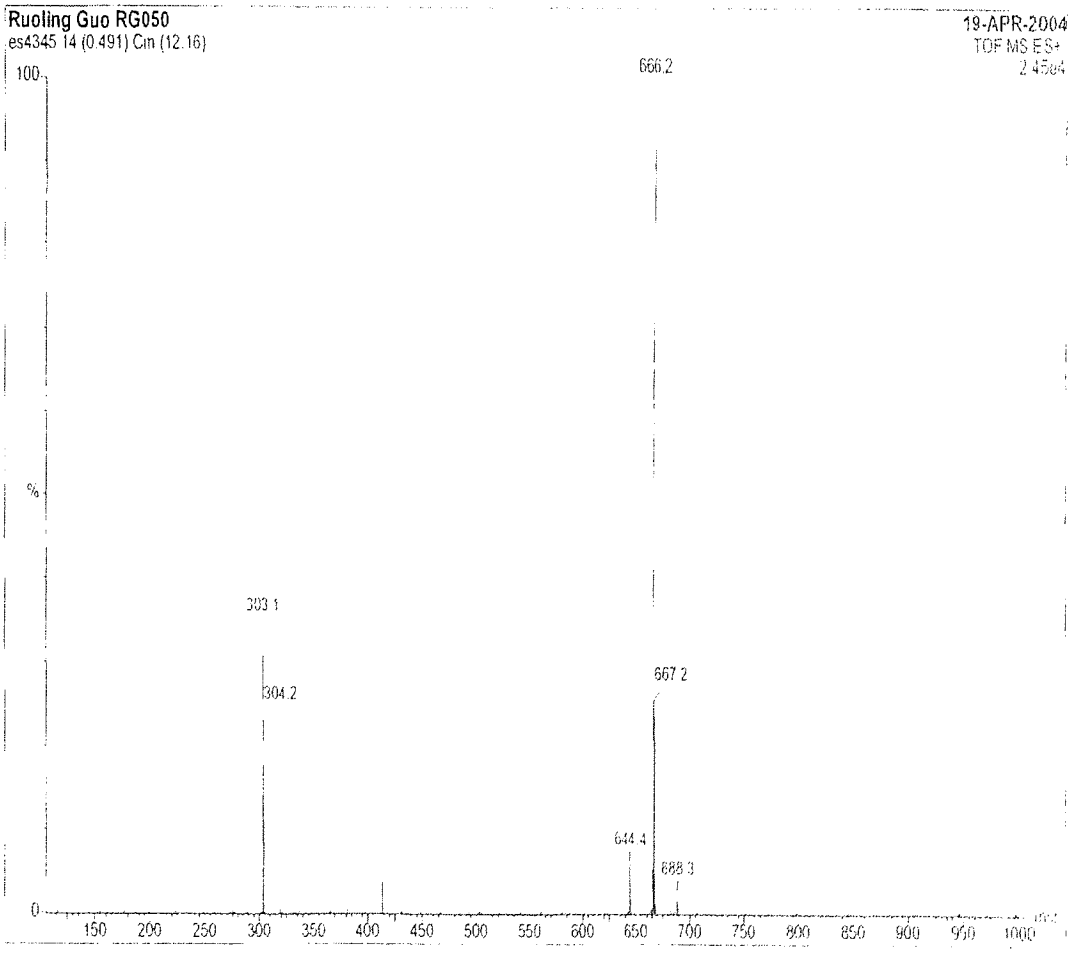
Compound 11: [2-Amino,7,9-$^{15}N_3$,8-^{13}C]5'-O-(4,4'-dimethoxytrityl)-N^2-isobutyryl-2'-deoxyguanosine

Elemental Composition Report **Ruoling Guo RG 050**

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -0.5, max = 80.0

Monoisotopic Mass, Odd and Even Electron Ions
322 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

Minimum:				-0.5	
Maximum:		2000.0	5.0	80.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
666.2529	666.2535	-0.6	-0.9	19.5	12C34 13C H37 14N2 15N3 O7 Na



Compound 12: [2-Amino,7,9-$^{15}N_3$,8-^{13}C]5'-O-(4,4'-dimethoxytrityl)-N^2-isobutyryl-2'-deoxyguanosine cyanoethyl-diisopropylamine phosphoramidite

Elemental Composition Report **Ruoling Guo RG 051**

Single Mass Analysis
Tolerance = 50.0 PPM / DBE: min = -0.5, max = 80.0

Monoisotopic Mass, Odd and Even Electron Ions
427 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

Minimum:				-0.5	
Maximum:		2000.0	50.0	80.0	
Mass	Calc. Mass	mDa	PPM	DBE	Formula
866.3626	866.3614	1.2	1.4	21.5	12C43 13C H54 14N4 15N3 O8 Na P