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Synthesis by Post-Synthetic Substitution of Oligomers Containing Guanine Modified at the 6-position with S-, N-, O-derivatives

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Abstract: *6-(2,4-dinitrophenyl)thioguanine phosphoramidite monomers with the 2-amino group of the base protected by either the isobutyryl or phenylacetyl group were incorporated into oligodeoxynucleotides with an automatic DNA synthesizer. N^2- protection with the isobutyryl group was unsatisfactory because of the difficulty of removing it after synthesis of the oligomer. However, post-synthetic conversion of the N^2- phenylacetyl protected 6-(2,4-dinitrophenyl)thioguanine gives oligomers containing 6-thioguanine, 2,6-diaminopurine, 2-amino-6-methylaminopurine, O^6-methylguanine or guanine in high yield and purity. Potentially oligomers containing other labile functional groups at the 6-position could be produced by the procedure. DNA duplexes containing 6-thioguanine paired to cytosine had a markedly lower melting temperature (Tm) than their counterparts containing G:C. However a DNA duplex containing 4-thiothymine paired to A had a Tm similar to that of a DNA duplex containing T:A. The distortion in DNA structure caused by 6-thioguanine may play a role in the biological effect of this compound.*

INTRODUCTION

There is a great and increasing interest in the chemical synthesis of oligonucleotides containing modified bases for studies related to carcinogenesis, mutagenesis and DNA repair, and for investigation of protein-DNA interaction[1]. In particular there is an interest in base-modifications which lead directly to alteration of hydrogen bonding. Generally, modified bases have been introduced into the oligonucleotides at the polymerisation stage, but synthesis of oligomers containing a new modified base by that procedure always requires an experienced chemist to design and synthesize a modified monomer which is stable to the chemical treatments involved in assembly, in particular, the oxidation of the phosphite ester, the deprotection of the 5'-OH, and finally the deprotection of the oligomer after synthesis. Usually this cannot be achieved by design of the monomer alone and changes have to be made to the chemical treatments and or to the protecting groups. This is an insurmountable difficulty for all but the most experienced chemist. To overcome this problem Macmillan and Verdine[2] have recently suggested what they called a convertible nucleoside approach in which a versatile base was introduced into DNA during automatic synthesis, then converted into the modifed base after synthesis. They illustrated this by incorporating 4-O-(2,4,6-trimethylphenyl)-2'-deoxyuridine into DNA. After deprotection, the oligomer was treated with various aqueous amines to yield oligomers containing N^4-alkyl-deoxycytidine. Similarly, Webb and Matteucci[3] had reported conversion of oligomers containing 4-triazolothymine into oligomers containing 5-methyl-N^4,N^4-ethanocytosine, and we have used a post-synthetic

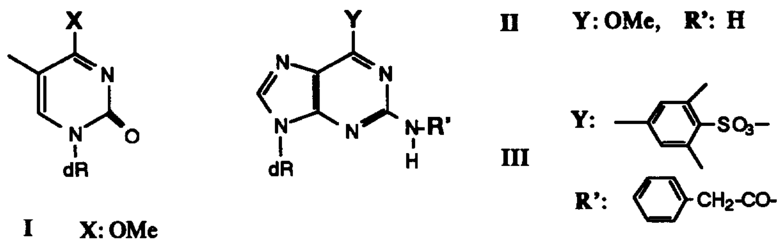
substitution strategy for making oligomers with a variety of modifications at the 4-position of thymine[4].

Previous papers[2,3,4] using the post-synthetic substitution strategy were all concerned with the synthesis of oligomers containing modified pyrimidines. Because of the importance of modified purines in carcinogenesis[1] and their use in therapy (eg. 6-thioguanine)[5], there is an equal, if not greater, need for a similar strategic approach to modified purines. Studies of the structural and biological properties of DNA containing modified purines have been limited by the fact that the modified oligomers were not available. Although syntheses of oligomers containing modified-purine such as 6-thioguanine (G^{S})[6,23], 2,6-diaminopurine (G^{NH2})[7,8], O^6-methylguanine (G^{OMe})[1,9,10] have been reported, some of the procedures are very difficult and tedious to perform[6,7,8], and, all of oligomers containing modified-guanine were made by a route in which a modified nucleoside was synthesized, converted to the corresponding phosphoramidite or phosphotriester monomer, and then incorporated during the synthesis of the oligomer. It is obvious that such a route is limited to the incorporation of relatively stable monomers which can withstand the conditions of synthesis.

It is more difficult to develop a post-synthetic substitution strategy for purines than for pyrimidines, but here we wish to report the development of the method for oligomers containing guanine modified at the 6-position, in which the versatile guanine monomer N^2-phenylacetyl-6-(2,4-dinitrophenyl)thiodeoxyguanosine-3'-phosphoramidite **XII** was prepared and incorporated into oligomers. The oligomer containing this versatile guanine ($G^{S\text{-}dn\varnothing}$,) was converted into oligomers containing 6-thioguanine (G^{S}), 2,6-diaminopurine (G^{NH2}), 2-amino-6-methylaminopurine (G^{NMe}), O^6-methylguanine (G^{OMe}) or guanine by treatment with appropriate reagents after synthesis. This method is more simple and effective than previously published methods[6,7,8], for oligomers containing 6-thioguanine and 2,6-diaminopurine and as simple and effective as the very recently published synthesis of an oligomer containing 6-thioguanine[23]. It is worth noting that as the amino, alkylamino, alkoxy and oxy functional groups at the 6-position are introduced after synthesis the method may be specially useful for the introduction of ^{15}N, ^{13}C, ^{17}O from [^{15}N]-NH_3, [^{13}C]-MeOH and [^{17}O]-H_2O for NMR study. Furthermore the method might be used for construction of oligomers containing other chemically reactive guanine derivatives such as 6-aziridino guanine[11] which might be used for DNA-crosslinking.

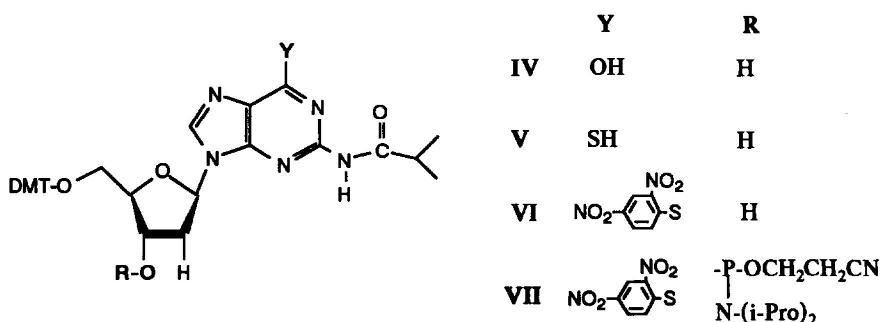
RESULTS AND DISCUSSION

The guanine analogue, which is to be converted to 6-modified guanines at the oligomer level, must contain at the 6-position a group which is stable during DNA synthesis and is also a good leaving group during the post-synthetic substitution. Comparing a similar functional group, for example, methoxy, at the 4-position of thymine **I** and at the 6-position of guanine **II** , one finds that the latter is more stable towards nucleophilic reagents (eg. MeOH/DBU or conc. ammonia), which makes substitution more difficult. Since the nucleoside, 6-mesitylenesulphonyldeoxyguanosine (6-Ms-deoxyguanosine) can be converted into O^6-methyldeoxy-guanosine[12], initially an attempt was made to use 6-Ms-guanine as a versatile guanine. 6-Ms-guanine monomer (**III**) was prepared in one step from commercial guanine phosphoramidite monomer and incorporated into oligomers with satisfactory coupling yield. However, the oligomers containing 6-Ms-guanine **III** did not give a satisfactory yield of the expected O^6-methylguanine when treated with N-methylpyrrolidine, then with CH_3OH/DBU[12]. A possible explanation is that N-methylpyrrolidine is less effective at the oligomer level than at the nucleoside level[12]. So our attention was turned towards the construction of another versatile guanine monomer.



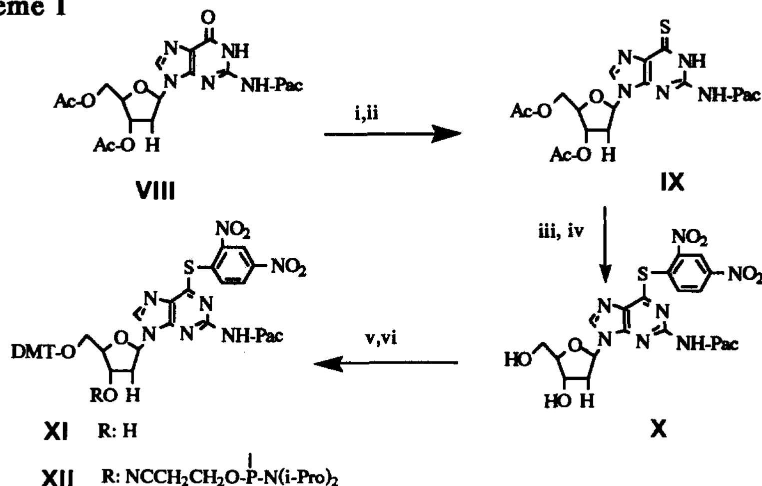
Concurrently with this work simple methods for direct thiation of the 4-position of pyrimidine nucleosides and the 6-position of deoxyguanosine were independently developed in our lab[13] and in other labs[14,15]. Because the C-S bond is more easily split than C-O, it seemed reasonable to base the versatile monomer on thioguanine rather than guanine itself. In order to protect this thioketo from oxidation by iodine, which is an essential reagent in phosphoramidite method, and to make the 6-substituent a better leaving group, 2,4-dinitrophenyl group was put on the 6-position of 6-thiodeoxyguanosine.

Another important point was the choice of protecting group on the 2-amino group of guanine. At first, the conventional protecting group, isobutyryl, was used, because this protected nucleoside, 5'-dimethoxytrityl-N^2-isobutyryl-2'-deoxyguanosine (IV), is commercially available. It was converted into its 6-thio-analogue (V) by one-pot reaction[13], in which the 6-position was first sulphonated with 2-mesitylenesulphonyl chloride (Ms-Cl), the Ms derivative was substituted with N-methylpyrrolidine, then the quaternary amino derivative was substituted with thiolacetic acid to form the 6-thio-analogue. Although the 6-keto and 3'-OH can both be sulphonated by Ms-Cl the former is more nucleophilic under these conditions and therefore the main product was sulphonated on the 6-position. Furthermore the Ms group on the 6-position can be substituted by N-methylpyrrolidine, then by thiolacetic acid[13] whereas that on the 3' position is unreactive with both reagents. The desired product (V) can be easily separated from impurities by a single silica gel column chromatography, converted into the 5' and N^2-protected 6-(2,4-dinitrophenyl)thiodeoxyguanosine (VI) with quantitative yield, then to the phosphoramidite monomer VII. The monomer was incorporated into a dodecamer AGCYAATTCGCT (Y: $G^{S\text{-}dn\phi}$) with satisfactory coupling yield (>98%) and then converted into the desired modified guanine (G^S, G^{NH2}, G^{NMe}, G^{OMe} or G) by treatment with the appropriate reagents.



However, removal of the isobutyryl group protecting the N^2-position of these modified guanines required much longer exposure to deblocking reagents (eg. 3 days, at room temperature in conc. ammonia) than was required for removal of the protecting groups on the other bases (8 h, at room temperature in conc

ammonia)[16]. This prolonged deprotection step increased the probability of destroying the modified guanine. To overcome this, the 6-(2,4-dinitrophenyl)thioguanine monomer ($G^{S\text{-}dn\emptyset}$) was prepared with a more base-labile group, the phenylacetyl group [9,10], protected on the N^2-position (Scheme 1).



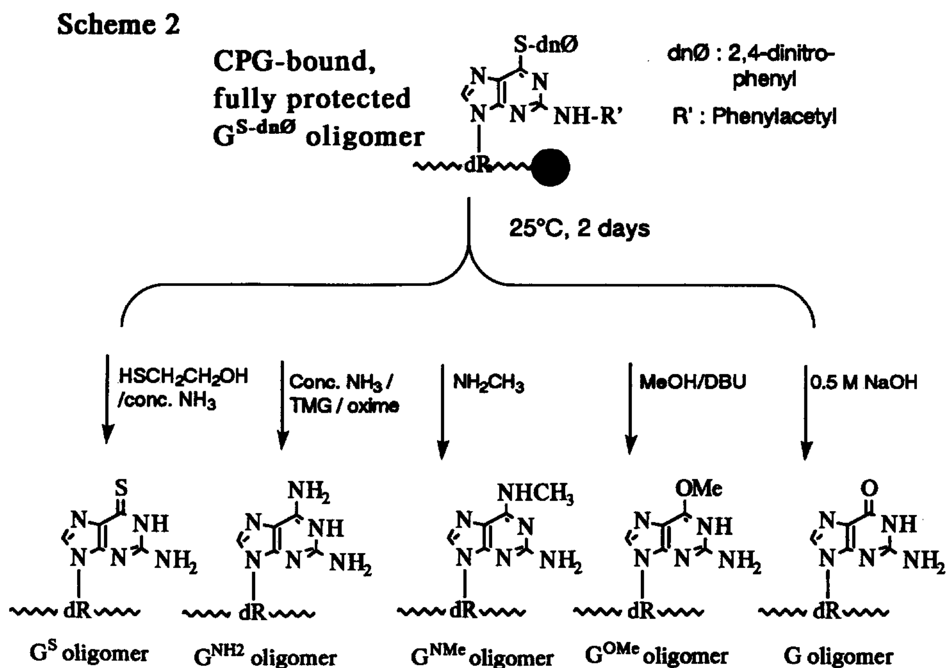
i: mesitylenesulphonyl chloride / N-methylpyrrolidine. ii: thiolacetic acid.
iii: 2 M NaOH. iv: 2,4-dinitrofluorobenzene.
v: 4,4'-dimethoxytriphenylmethyl chloride / pyridine.
vi: phosphitylating reagent. Ac: acetyl. Pac: phenylacetyl.

3',5'-diacetyl-N^2-phenylacetyl-2'-deoxyguanosine (compound VIII), prepared as before[10], was converted into its 6-thio analogue (compound IX) using direct thiation[13] with isolated yields of 70-80%. After selective deacylation, the resulting 6-thiodeoxyguanosine was reacted with 2,4-dinitrofluorobenzene[17] in presence of triethylamine to give compound X with isolated yields of 80-90%. The reaction was highly regioselective because the 6-thioketo group of IX is much more nucleophilic than 3' and 5' hydroxyl groups. Using standard methods, compound X reacted with DMT-Cl / pyridine, then with phosphitylating reagent to give the designed phosphoramidite monomer (compound XII).

Since there is no previous report describing protection of 6-thioketo with the 2,4-dinitrophenyl group, the stability of 6-(2,4-dinitrophenyl)thioguanine monomer to the reagents used in oligonucleotide synthesis was studied before the monomer was used for DNA synthesis. The results showed that compound XI was stable towards the reagents used in oligomer assembly (N-methylimidazole in THF, acetic anhydride/lutidine/THF) for at least 24 h at room temperature, and compound X was stable towards 3% dichloroacetic acid in dichloroethane at room temperature for at least 3 hours. It was also found that the protecting group of the 6-thioketo function, 2,4-dinitrophenyl, can be removed completedly by 1M mercaptoethanol in ammonia within 30 mins. These results suggested that the monomer XII would be stable during the synthesis, and that the protecting groups easily removed after synthesis. Therefore the versatile monomer XII was incorporated into oligonucleotides by DNA synthesizer as before[18] without changing the normal program except that coupling time for XII was 3 mins. The coupling yield was monitored and estimated to be over 98%.

The two steps needed to get the desired modified oligomers: substitution and deprotection, were

investigated in detail. In all of these syntheses the normal bases were protected with labile groups[16] and the conditions under which they can be removed by conc. ammonia, alcohol/DBU and aqueous alkali at room temperature have been determined[4]. So the major question was the substitution and deprotection of the modified guanine. After synthesis the CPG-support bearing the synthetic, fully protected dodecamer (AGCG$^{S\text{-}dnØ}$AATTCGCT) with 5'-DMT still attached, was divided into parts, each of which was treated with appropriate chemical reagents (Scheme 2).



1) oligomer containing 6-thioguanine (G^S):

Treatment of the protected and CPG-bound oligomer containing N^2-phenylacetyl-6-(2,4-dinitrophenyl)thioguanine (CPG-G$^{S\text{-}dnØ}$ oligomer) with mercaptoethanol at room temperature for 3 h completely cleaved the 2,4-dinitrophenyl group from sulphur, then conc. ammonia was employed to cut all other protecting groups and produced an oligomer containing 6-thioguanine (G^S oligomer). It has been reported that 6-thiodeoxyguanosine is labile to aqueous alkali[6], but we found that 6-thiodeoxyguanosine both as the nucleoside and in the oligomer is stable to conc. ammonia for at least 3 days at room temperature, but not very stable at 55°C. Therefore we used conc. ammonia at 25°C to deprotect oligomers containing 6-thioguanine. Further optimization revealed that removal of 2,4-dinitrophenyl was very rapid so that conc. ammonia containing 10% of mercaptoethanol at room temperature was added for two days to directly give the desired product. The crude oligomer was purified in the same way as normal oligomer by Nen-sorb cartridge. More highly purified G^S oligomer can be obtained by FPLC (Fig. 1), because the desired G^S oligomer was well separated from impurities (mainly oligomers containing 2,6-diaminopurine and guanine). The total amount of impurity was usually less than 5% of the product. The base analysis (Fig.2) showed the presence of 6-thiodeoxyguanosine

(G^S) and no peak of 6-(2,4-dinitrophenyl)thiodeoxyguanosine ($G^{S\text{-}dn\emptyset}$) was observed at 260 nm and 330 nm. At the time this work was done the only published method[6] for the synthesis of oligomers containing G^S used the phosphotriester procedure with the conventional acyl groups protected on the bases. The greatest difficulty in that procedure was the deprotection step, where first 1 M benzenethiol in pyridine (8 h) was used for removal of the mesitylenesulphonyl group which the author assumed would have been produced on the 6-thio position during coupling. This was followed by tetramethylguanidine and E-2-nitrobenzaldoxime in pyridine (5 days) to cleave the oligomer from its support and remove the protecting groups on the phosphates, then NH_3 in anhydrous methanol (7 days) was used to remove protecting groups from bases. All these operations were carried out under nitrogen in anhydrous condition[6]. However very recently an oligomer containing G^S (AAACG^{S}TTT) has been synthesized using the 2-cyanoethyl group to protect the thio function of 6-thioguanine phosphoramidite[23], but there is no direct evidence that under their seperating system (reversed phase HPLC, pH 7.6) the G^S oligomer can be separated from the parent one. Therefore it is still uncertain whether the G^S oligomer is free from the parent one. We have employed anion exchange column at pH 12 to give a good separation of G^S oligomer from other oligomers of the same length (Fig. 1) and avoid the time-consuming purification and characterization steps previously used[6,23].

2) oligomer containing 2,6-diaminopurine (G^{NH2}):

Interest in synthetic oligomers containing 2,6-diaminopurine has been greatly stimulated by the finding that S-2L cyanophage DNA contains 2,6-diaminopurine instead of adenine[19]. Recently 2'-O-protected RNA oligomers containing 2,6-diaminopurine have been synthesized and shown to be useful antisense probes[20]. Preparation of short DNA oligomers containing G^{NH2}, in which an N^2,N^6-diacyl-protected monomer was used, has been reported [7,8], but the procedures are very difficult, not only in preparation of the modified monomer, but also in assembly and deprotection of the oligomer. Due to the extreme acid lability of the glycosidic linkage of the 2,6-di-N-acyl-derivative, the aprotic acid, $ZnBr_2$, was used for detritylation, which inevitably led to incomplete detritylation[7]. To circumvent this, Chollet et al[8] used 1-methyl-2,2-diethoxypyrrolidine for protection of the 6-amino group to reduce the depurination. However, deprotection in conc. ammonia at 65°C for 5-7 days was still necessary because of the slow hydrolysis of the two amino-protecting groups on the modified base[8]. It is clear these methods are far from satisfaction and needed to be considerably improved, and in a later paper Chollet et al[21] used a DNA polymerase to incorporate the triphosphate of 2,6-diaminopurine nucleoside to produce G^{NH2} oligomers.

In first attempt to synthesize oligomers containing 2,6-diaminopurine, the CPG-$G^{S\text{-}dn\emptyset}$ oligomers were treated with conc. ammonia at 55°C overnight to cleave the oligomer from the CPG, remove the protecting groups and substitute the 1-thio-2,4-dinitrophenyl group. However as well as the desired oligomer containing G^{NH2} a substantial amount of oligomers containing G^S and guanine itself was obtained. Presumably the G^S oligomer was formed because the nucleophile ammonia attacked the 1-position of the dinitrophenyl group leaving sulphur in the 6-position. The amount of G^S oligomer was reduced dramatically by lowering the temperature, and treatment with conc ammonia at 25°C (2 days) gave the desired G^{NH2} oligomer with negligible amount of G^S oligomer. However there was still an unacceptable amount of oligomer containing guanine, resulting from attack by hydroxide ion. We had previously observed[9] that conc. ammonia containing tetramethylguanidine (TMG) and E-2-nitrobenzaldoxime removed the protecting groups from oligonucleotides faster than ammonia alone, suggesting that TMG and the oxime must favour the role of ammonia as a

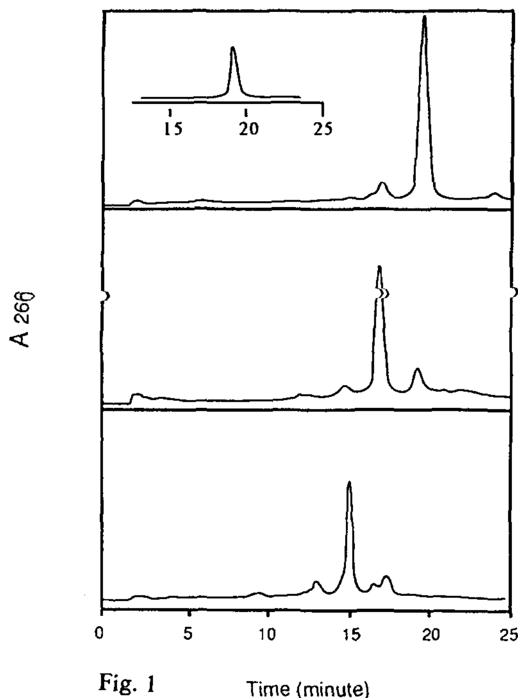


Fig. 1

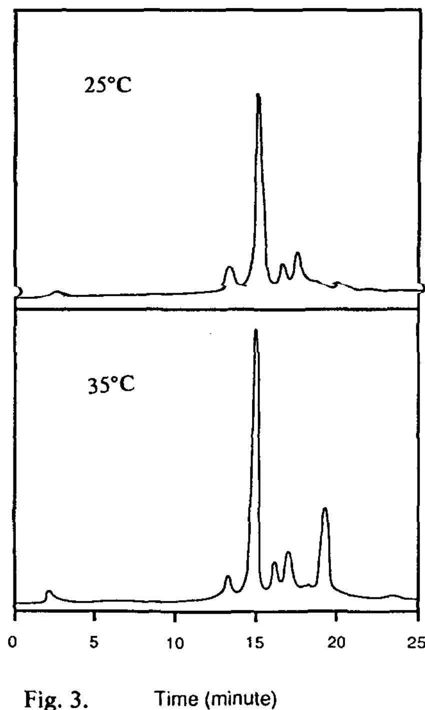


Fig. 3.

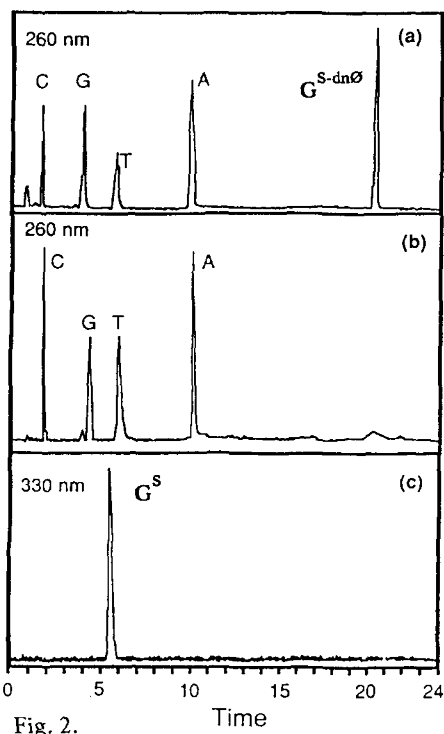


Fig. 2.

Fig. 1 FPLC profiles of unpurified 12 mer AGCYAATTCGCT prepared by post-synthetic substitution
top figure: Y= G^S; (Inserted fig. for the purified G^S 12 mer)
middle figure: Y= G;
bottom figure: Y= G^{NH2}.

Fig. 2. Base analysis of purified AGC G^SAA TTC GCT
(a): HPLC profile of deoxyribonucleosides: C, G, T and A (equal amount), plus $G^{S\text{-}dnØ}$ (excess amount).
(b): HPLC profile of an enzymic digest of the 12 mer measured at 260 nm;
(c): the same as (b) but measured at 330 nm)

Fig. 3. FPLC profiles of unpurified 12 mer AGC YAA TTC GCT prepared by post-synthetic substitution with MeOH/DBU at two different temperatures
top figure: at 25°C; bottom figure: at 35°C
The bottom figure shows that if the substitution is carried out at slightly higher temperature (35°C), then a greater amount of oligomer comtaining 6-thioguanine (the peak eluting at 19 min) will be produced.

nucleophile. So the CPG-$G^{S\text{-}dn\emptyset}$ oligomer was treated at 25°C with conc. ammonia containing TMG/oxime for 2 days. This produced G^{NH2} oligomer with negligible amount of oligomer containing G^S and G (Fig.1). This method provides a more simple and practical way to prepare oligomers containing 2,6-diaminopurine (G^{NH2}) than previous methods[7,8] and furthermore allows one to introduce ^{15}N using easily available ^{15}N ammonia.

3) oligomers containing 2-amino-6-methylaminopurine (G^{NMe})

Treatment of the CPC-$G^{S\text{-}dn\emptyset}$ oligomers with methylamine (25°C, 2 days) effected both substitution and deprotection and gave an oligomer containing 2-amino-6-methylaminopurine with negligible amount of oligomers containing guanine. The oligomer containing G^{NMe} was easily purified by FPLC at pH 12. This is a good example of the use of post-synthetic substitution to make oligomers containing different alkylamines. Because alkylamines are generally good nucleophiles they can be easily introduced into the 6-position of guanine at the oligomer level by the present method. It is noteworthy that oligomers containing bifunctional group, eg. ethylenediamine group could be useful for DNA crosslinking or attachment of non-radiactive labelling reagents.

4) oligomers containing O^6-methylguanine (G^{OMe}):

Several methods for the synthesis of oligomers containing O^6-methylguanine have been described[1,9,10], but post-synthetic substitution provides an alternative with the advantage that NMR sensitive group such as ^{13}C from [^{13}C]-CH_3OH could be introduced at the last step. Treatment of CPG-$G^{S\text{-}dn\emptyset}$ oligomers with MeOH/DBU (25°C, 2 days) effected both sustitution and deprotection to give G^{OMe} oligomer (Fig. 1). The temperature of the substitution was also crucial. When a slightly higher temperature (35°C) was employed overnight, a greater percentage of G^S oligomer was produced (Fig. 3). Both 2,4-dinitrophenyl and 1-thio-2,4-dinitrophenyl groups are good leaving groups and presumeably higher temperature favours the substitution of the former group to give G^S oligomer. Oligomers containing O^6-methylguanine prepared by this method were identical to ones prepared previously[10].

5) oligomers containing guanine:

Treatment of the CPG-$G^{S\text{-}dn\emptyset}$ oligomers with 0.5 M NaOH at 25°C for 2 days gave an oligomer containing guanine. This parent oligomer was purified by the usual method (Fig. 1). This present method may prove useful to synthesize the parent oligomer, as a reference sample, during the same synthesis as the modified oligomer and to introduce NMR sensitive ^{17}O at the last step from ^{17}O-water.

Stability of DNA duplexes containing 6-thioguanine and 4-thiothymine: The thermal melting profiles of DNA duplexes containing 6-thioguanine were measured and compared with control DNA, and with DNA containing 4-thiothymine. Unexpectedly the presence of 6-thioguanine produced a much greater depression of the melting temperature (Tm) than the presence of 4-thiothymine. The Tm of a self-complementary duplex (AGCG^{S}AATTCGCT) containing two 6-thioguanine: C base pairs was 12.8 °C lower than that of the control DNA (table 1). The Tm of a non self-complementary duplex (CAGG^{S}AATTCGC) containing one 6-thioguanine: C pair was 6.9 °C lower than that of the analogous control. The presence of 4-thiothymine produced less instability. The Tm of a self-complementary duplex (AGCGAAT^{S}TCGCT) containing two 4-thiothymine: A pairs was only 1 °C lower than the control. This result is consistent with previous results[22]. However a non self-complementary duplex (CAGGAATT^{S}CGC) containing only one 4-thiothymine: A pair had a Tm 3 °C lower than the control. Although 6-thioguanine and 6-mercaptopurine,

Table 1. The Melting Temperature (Tm) Values of DNA Duplexes Containing 6-Thioguanine and 4-Thiothymine

Self-complementary Duplexes	Tm	ΔTm	Non-self-complementary Duplexes	Tm	ΔTm
5' AGC GAA TTC GCT 3' TCG CTT AAC CGA	64.5	0	5' CAG GAA TTC GC 3' GTC CTT AAG CG	58.7	0
5' AGC G^SAA TTC GCT 3' TCG C TT AAG^S CGT	51.2	12.8	5' CAG G^SAA TTC GC 3' GTC C TT AAG CG	51.8	6.9
5' AGC GAA T^STC GCT 3' TCG CTT^S A AG CGT	63.5	1	5' CAG GAA TT^SC GC 3' GTC CTT AA G CG	55.7	3

which is converted to 6-thioguanine in vivo, are widely used in cancer therapy, and the 6-thioguanine analogue, azathioprine, is used in transplant surgery, very little is known about their mode of action at the molecular level. The cytotoxicity occurs at cell division and it seems possible that the distortion of the DNA structure, which can be seen here as a reduction in Tm, plays a role in the cytotoxicity of these drugs. The availability of synthetic DNA containing 6-thioguanine should be of significant value in advancing an understanding of this very important question.

EXPERIMENTAL

Abbreviation: DBU, 1,8-diazabicyclo-[5.4.0]-undec-7-ene; DMT, 4,4'-dimethoxytriphenylmethyl; TMG, tetramethylguanidine; CPG, controlled-pore-glass; G^S, 6-thioguanine; G^{NH_2}, 2,6-diaminopurine; G^{NMe}, 2-amino-6-methylaminopurine; $G^{S\text{-}dn\varnothing}$, 6-(2,4-dinitrophenyl)thioguanine; G^{OMe}, O^6-methylguanine.

Chemicals, enzymes, and general methods

The CPG-linked monomers and the chemicals used on the synthesizer were obtained from Cruachem (Glasgow, Scotland) and the 2-cyanoethylphosphoramidites protected with phenoxyacetyl on the amino functions of adenine and guanine and with isobutyryl on the amino function of cytosine (PAC amidites) were from Pharmacia. 5'-DMT-N^2-isobutyryl-2'-deoxyguanosine was from Cruachem, conc. ammonia (d=0.88 Aristar) from BDH and 40% aqueous methylamine from Aldrich. Anhydrous acetonitrile was obtained by drying acetonitrile (HPLC grade, Rathburn) with molecular sieve 4A at least overnight. All other chemicals were from either Aldrich or Sigma. All chemicals and solvents, unless stated otherwise, were used directly without further purification. The water content of anhydrous solvents was checked by Karl Fischer titration. Reverse phase HPLC for base analysis was carried out on a Gilson 320, with a 620 Datamaster for integration and Shimadzu SPD6A UV spectrophotometric detector, using a Waters 8MBC18 10μ column. Gradients were formed from 0.05 M aqueous KH_2PO_4 (pH 4.5) (buffer A) and 0.05 M aqueous KH_2PO_4 (pH 4.5) containing 33% CH_3CN (buffer B) at a flow rate of 3 ml/min. Fast protein liquid chromatography (FPLC) was carried out on a Dionex BIOLC system with a Dionex variable wavelength detector using a Pharmacia monoQ HR5-5 column. Gradients were formed from 0.4 M NaCl, 0.01 M NaOH aqueous solution (pH 12) (buffer C) and 1.2 M NaCl, 0.01 M NaOH aqueous solution (pH 12) (buffer D) at a flow rate of 0.7 ml/min. Thin layer chromatograghy (TLC) was carried out on Merck Kieselgel 60 F_{254} aluminium backed TLC sheets developed with 5% $CH_3OH/CHCl_3$ (solvent A) or CH_2Cl_2/ethylacetate/diisopropylamine (75:25:2, v/v/v, solvent B). Nensorb Cartridges (NEN Research Products, Du Pont Co., Boston, MA 02118, USA) were used to remove failure sequences from the oligomers according to the makers' instruction.

Base analysis

The purity of the oligomers was assessed by analysis of the nucleosides obtained from enzymic digestion. In general 0.5 A_{260} units of an oligomer was dissolved in 160 μl H_2O and 20 μl 600 mM Tris·HCl, 60 mM $MgCl_2$, pH 8.5. Snake venom phosphodiesterase I (10 μl, 10 μg protein) was added and the mixture incubated

(37°C, 30 min), then alkaline phosphatase (10 µl, 5 µg protein) was added and incubated at 37°C for another 30 min. The deoxyribonucleosides were analyzed by HPLC using 93% buffer A and 7% buffer B for first 7 min, then with a linear gradient from 7% to 80% of buffer B over the following 10 min, then remaining at this concentration for another 5 min in order to detect any remaining of un-substituted $G^{S\text{-dn}Ø}$ nucleoside. The eluate was generally monitored at 260 nm, but for the G^S oligomer an extra run was monitored at 330 nm for the detection of 6-thiodeoxyguanosine. The amount of each nucleoside was measured by integration of the absorbance of each peak[4,10]. Retention times were: dC: 1.5 min; dI (from enzymatic hydrolysis of dA): 3.5 min; dG: 3.9 min; T: 5.5 min; G^S: 5.7 min; G^{NH2}: 7.0 min; dA: 9.5 min; G^{NMe}: 11.2 min; G^{OMe}: 11.7 min; $G^{S\text{-dn}Ø}$: 20.2 min.

Melting Curve Measurement

Oligomers containing 4-thiothymine were prepared as the previous paper[4]. The DNA strands were annealed in 0.1 M Hepes pH 7.5, 0.2 M NaCl and 0.02 M $MgCl_2$. The temperature dependent change in absorbance at 260 nm, of the solution in this buffer, was followed using a CARY3 spectrophotometer connected to a Cary temperature controller (Varian Techtron Pty Ltd, Australia). The rate of temperature increase was 1°C/min. The Tm values were determined as the maximum values of the first derivative graph of the absorbance vs temperature.

Synthesis of 5'-O-(4,4'-dimethoxytriphenylmethyl)-N^2-isobutyryl-2'-deoxy-6-(2,4-dinitrophenyl)thioguanosine-3'-O-(2-cyanoethyl-N,N-diisopropylamino)-phosphoramidite (VII)

5'-Dimethoxytriphenylmethyl-N^2-isobutyryl-2'-deoxy-6-thioguanosine (**V**): 5'-Dimethoxytriphenylmethyl-N^2-isobutyryl-2'-deoxyguanosine **IV** (1.28 g, 2 mmol) was dissolved in anhydrous CH_2Cl_2 (25 ml) and triethylamine (1.2 ml), 2-mesitylenesulphonyl chloride (0.88 g, 4 mmol), and 4-(dimethylamino)pyridine (10 mg) added sequentially. After 1 h stirring at room temperature, the solution was cooled with an ice-bath and N-methylpyrrolidine (1 ml) slowly added. The solution was left stirring for 20 min in the ice-bath and then for another 30 min at room temperature. Thiolacetic acid (1 ml) in CH_2Cl_2 (5 ml) was added dropwise and stirred for another 1 h. The reaction mixture was washed with saturated aqueous $NaHCO_3$ (2 x 25 ml) and then with saturated aqueous NaCl (2 x 25 ml). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to solid, and purified by column chromatography on Kieselgel 60 H using CH_2Cl_2 followed by a gradient of 0.5% to 1.5% $CH_3OH/CHCl_3$ (v/v) with a few drops of pyridine added. The fractions containing the desired product were pooled and evaporated under reduced pressure to give a slightly yellow solid (0.53g, 40.0%). ^1H NMR data (in DMSO-d_6): 1.13 (6H, d, -C$(CH_3)_2$- of isobutyl), 2.37-2.78 (3H, m, 2'-H, 2"-H and -CH- of isobutyl), 3.09-3.19 (2H, m, 5'-H), 3.71 (6H,s, OCH_3 of DMT), 3.95 (1H, m, 4'-H), 4.40 (1H, m, 3'-H), 5.35 (1H, d, 3'-OH, ex), 6.25 (1H, t, 1'-H), 6.78-7.40 (13H, m, aromatic-H), 8.30 (1H, s, 8-H), 11.93 (1H, s, 2-NH, ex) and 13.41 (1H, s, 6-SH). UV: λmax=332.0 nm (in MeOH)

5'-Dimethoxytriphenylmethyl-N^2-isobutyryl-2'-deoxy-6-(2,4-dinitrophenyl)thioguanosine (**VI**): To a solution of compound **V** (0.33 g, 0.5 mmol) in anhydrous CH_3CN (25 ml) was added triethylamine (0.5 ml) and 2,4-dinitrofluorobenzene (0.11g, 0.6 mmol). After 40 min, TLC showed that the starting material was entirely converted to a new compound with higher Rf (solvent A), which was yellow under visible light. The reaction mixture was concentrated under reduced pressure into a small volume and diluted with $CHCl_3$ (25 ml). The solution was washed with saturated aqueous $NaHCO_3$ (25 ml), then with saturated aqueous NaCl (25 ml). The organic layer was dried over anhydrous Na_2SO_4, evaporated to give a yellow solid, and purified by silica gel column chromatography using CH_2Cl_2 followed by 1% CH_3OH/CH_2Cl_2 (v/v) with a few drops of pyridine added. The fractions containing the desired product were combined and evaporated to a yellow solid (0.38 g, 92%). ^1H NMR data (in DMSO-d_6): 1.03 (6H, d, -C$(CH_3)_2$- of isobutyl), 2.37-2.90 (3H, m, 2'-H, 2"-H and -CH- of isobutyl), 3.12 (2H, m, 5'-H), 3.70 (6H, s, OCH_3 of DMT), 3.98 (1H, m, 4'-H), 4.55 (1H, m, 3'-H), 5.31 (1H, d, 3'-OH, ex), 6.40 (1H, t, 1'-H), 6.73-7.36 (13H, m, aromatic-H), 8.09 (1H, d, 6-H of 2,4-dinitrophenyl), 8.29 (1H, m, 5-H of 2,4-dinitrophenyl), 8.54 (1H, s, 8-H), 8.90 (1H, d, 3-H of 2,4-dinitrophenyl) and 10.52 (1H, s, 2-NH, ex) UV: λmax=233.3 nm and 348.0 nm (in MeOH)

5'-Dimethoxytriphenylmethyl-N^2-isobutyryl-2'-deoxy-6-(2,4-dinitrophenyl)thioguanosine-3'-O-(2-cyanoethyl-N,N-diisopropylamino)-phosphite (**VII**): Compound VI (250 mg, 0.3 mmol) was dissolved in 2 ml dry THF and 0.25 ml of N,N-diisopropylethylamine added. The solution was cooled in an ice-bath and 150 µl of 2-cyanoethyl-N, N-di-isopropylchlorophosphoramidite in 1 ml of dry THF was added dropwise under stirring. After 10 min, the ice-bath was removed and the reaction left stirring at room temperature until TLC showed the complete conversion of starting material into two compounds with higher Rfs in solvent B. The mixture was diluted with ethyl acetate (25 ml) and washed with saturated aqueous NaCl (2 x 25ml). The organic layer was dried over Na_2SO_4 and evaporated to give a yellow solid, which was purified by column chromatography eluted with CH_2Cl_2/ethyl acetate/diisopropylamine (85:15:1, v/v/v). The fractions containing

the desired product were combined and evaporated to a yellow solid, redissolved in benzene and lyophilized to give a yellow powder (190 mg, 62.1%). ^{31}P NMR data (in $CDCl_3$): 149.41 and 149.16.

Synthesis of 5'-O-(4,4'-dimethoxytriphenylmethyl)-N2-phenylacetyl-2'-deoxy-6-(2,4-dinitrophenyl)thioguanosine-3'-O-(2-cyanoethyl-N,N-diisopropylamino)phosphoramidite (XII)

3',5,-diacetyl-N2-phenylacetyl-2'-deoxy-6-thioguanosine (**IX**): 3',5,-diacetyl-N2-phenylacetyl-2'-deoxyguanosine **VIII** (2.8 g, 6 mmol), prepared as before[10], was dissolved in anhydrous CH_2Cl_2 (50 ml), and triethylamine (3 ml), 2-mesitylenesulphonyl chloride (2.0 gm, 9 mmol) and 4-(dimethylamino)pyridine (20 mg) sequentially added. After 1 h stirring at room temperature, the solution was cooled in an ice-bath and N-methylpyrrolidine (5 ml) in CH_2CH_2 (5 ml) was added slowly. The solution was left stirring for 30 min in the ice-bath and then for another 30 min at room temperature. Thiolacetic acid (5 ml) in CH_2Cl_2 (10 ml) was added dropwise and stirred for another 1 h. The reaction mixture was washed with saturated aqueous $NaHCO_3$ (2 x 75 ml) and then with saturated aqueous NaCl (2 x 75 ml). The organic layer was dried (Na_2SO_4) and evaporated to solid, and purified by column chromatography using CH_2Cl_2 followed by a gradient from 0.5% to 2% $CH_3OH/CHCl_3$ (v/v). The fractions containing the desired product were pooled and evaporated under reduced pressure to give a slightly yellow solid. ^1H NMR data (in DMSO-d_6): 2.02-2.08 (6H, 2 s, 3' and 5' -$COCH_3$), 2.56-2.98 (2H, m, 2'-H and 2"-H), 3.84 (2H, s, -CH_2- of phenylacetyl),4.19-4.25(3H, m, 4'-H and 5'-H), 5.32 (1H, m, 3'-H), 6.23 (1H, t, 1'-H), 7.27-7.34 (5H, m, aromatic -H of phenylacetyl),8.43 (1H, s, 8-H), 12.23 (1H, s, 2-NH, ex) and 13.27 (1H, s, 6-SH).

N2-phenylacetyl-2'-deoxy-6-(2,4-dinitrophenyl)thioguanosine (**X**): Compound **IX** was selectively deprotected using a published procedure[10] to give N2-phenylacetyl-2'-deoxy-6-thioguanosine, which was confirmed by NMR spectroscopy; ^1H NMR data (in DMSO-d_6): 2.57-2.99 (2H, m, 2'-H and 2"-H), 3.83 (2H, s, -CH_2- of phenylacetyl), 3.54 (2H, m, 5'-H), 3.83 (1H, m, 4'-H), 4.37 (1H, m, 3'-H), 4.98 (1H, t. 5'-OH, ex), 5.33 (1H, d, 3'-OH, ex), 6.20 (1H, t, 1'-H), 7.27-7.35 (5H, m, aromatic -H of phenylacetyl),8.41 (1H, s, 8-H), 12.23 (1H, s, 2-NH, ex) and 13.26 (1H, s, 6-SH); UV: λmax=331.0 nm (in MeOH). The purified N2-phenylacetyl-2'-deoxy-6-thioguanosine was quantitatively converted to compound X using a similar procedure as for compound VI. The identity was confirmed by NMR spectroscopy; ^1H NMR data (in DMSO-d_6): 2.30-2.72 (2H, m, 2'-H and 2"-H), 3.55 (2H, m, 5'-H), 3.71 (2H, s, -CH_2- of phenylacetyl), 3.85 (1H, m, 4'-H), 4.41 (1H, m, 3'-H), 4.90 (1H, t. 5'-OH, ex), 5.34 (1H, d, 3'-OH, ex), 6.34 (1H, t, 1'-H), 7.27 (5H, m, aromatic -H of phenylacetyl), 8.12 (1H, d, 6-H of 2,4-dinitrophenyl), 8.36 (1H, m, 5-H of 2,4-dinitrophenyl), 8.68 (1H, s, 8-H), 8.88 (1H, d, 3-H of 2,4-dinitrophenyl) and 10.88 (1H, s, 2-NH, ex).

5'-O-(4,4'-dimethoxytriphenylmethyl)-N2-phenylacetyl-2'-deoxy-6-(2,4-dinitrophenyl)thioguanosine-3'-O-(2-cyanoethyl-N,N-diisopropylamino)-phosphoramidite (**XII**): Compound **X** was tritylated with dimethoxyltriphenylmethyl chloride in presence of pyridine by standard procedure and the resulting compound (XI) was confirmed by NMR spectroscopy; ^1H NMR data (in DMSO-d_6): 2.35-2.89 (2H, m, 2'-H and 2"-H), 3.08-3.24 (2H, m, 5'-H), 3.71 (8H, s, -CH_2- of phenylacetyl and CH_3O of DMT), 3.96 (1H, m, 4'-H), 4.51 (1H, m, 3'-H), 5.38 (1H, d, 3'-OH, ex), 6.38 (1H, t, 1'-H), 6.66-7.35 (18H, m, aromatic-H of phenylacetyl and of DMT), 8.04 (1H, d, 6-H of 2,4-dinitrophenyl), 8.25 (1H, m, 5-H of 2,4-dinitrophenyl), 8.55 (1H, s, 8-H), 8.87 (1H, d, 3-H of 2,4-dinitrophenyl) and 10.82 (1H, s, 2-NH, ex). **XI** was then converted into compound **XII** using the same procedure as for compound **VII**. ^{31}P NMR data (in $CDCl_3$): 149.41 and 149.16.

Stability Tests

Stability of compound **X** *and* **XI** *towards the conditions used in oligonucleotide assembly* Compound X (20 mg) was dissolved in 3% dichloroacetic acid/dichloroethane and changes in the solution monitored by TLC ($CH_3OH/CHCl_3$, 10: 90, v/v) over 24 h at room temperature; Compound **XI** (20 mg) was dissolved respectively in a) 4.4% N-methylimidazole in THF; b) acetic anhydride/lutidine/THF 1:1:8; and c) 0.1 M iodine in THF/pyridine/water 40:9:1. Changes in the solution were monitored by TLC ($CH_3OH/CHCl_3$, 5: 95, v/v) over 24 h at room temperature.

Stability of 2'-deoxy-6-thioguanosine towards conc. ammonia at 25°C and 55°C 2'-deoxy-6-thioguanosine (10 mg) was dissolved in 1 ml of conc. ammonia and the solution was kept at 25°C for 3 days and at 55°C for a day respectively. The reaction course was monitored by TLC ($CH_3OH/CHCl_3$,15: 85, v/v).

Synthesis, Conversion and Purification of Oligonucleotides

Oligonucleotides were synthesized on a Cruachem PS200 automatic DNA synthesizer (Cruachem Ltd., Glasgow, Scotland) using PAC amidites of the normal bases (see above). The general procedure was carried out as before[4,18]. The portion of the oligonucleotide 3' to the modified guanine was synthesized on the machine, then the versatile guanine monomer (compound **VII** or **XII**) was added manually. 10 mg of the

monomer was dissolved in 0.1 ml of anhydrous CH_3CN and 0.1 ml of 0.5 M tetrazole in anhydrous CH_3CN added. The bottom end of the cartridge containing the solid and partially synthesized oligomer was disconnected from the machine and the mixture of monomer and tetrazole injected from a gas tight syringe. The syringe was used to draw the solution in and out of the cartridge several times over a period of 3 min, then the cartridge was immediately reconnected to the synthesizer to complete the synthesis.

Preparation of oligomers containing O⁶-methylguanine (G^{OMe}): CPG-support bearing the 12 mer $G^{S\text{-}dnØ}$ oligomer, with the 5'-DMT still on (CPG-$G^{S\text{-}dnØ}$ oligomer) was put into Eppendorf tubes and MeOH/DBU (1ml, 9:1, v/v) was added and left at 25°C for 2 days. The solution was neutralized (90 ml 50% aqueous acetic acid,1.5 equivalent to DBU) and immediately passed through a Dowex 50 x 8, Na^+ form, 400 mesh ion exchange column (10 ml wet volume) eluted with water and collected in 1 ml fractions. The oligomers were usually found in fractions 4 to 6 by measuring A_{260}. The oligonucleotides were separated from failure sequences and the DMT group finally removed using a Nensorb Prep cartridge (Du Pont company). Further purification of the modified oligomer by FPLC and its base composition analysis were carried out as before[10].

Preparation of oligomers containing guanine from the $G^{S\text{-}dnØ}$ oligomer: The CPG-$G^{S\text{-}dnØ}$ oligomer was treated with 0.5 M aqueous NaOH (25°C, 2 day). The deprotected and substituted oligomer was purified with a Nensorb Prep cartridge and FPLC as above.

Preparation of oligomers containing 6-thioguanine: The CPG-$G^{S\text{-}dnØ}$ oligomer was treated with 10% mercaptoethanol in conc. ammonia for 2 day at 25°C, then the product was purified by Nensorb cartridge and FPLC as described above. The correct base analysis of the modified oligomer was presented in Fig. 2.

Preparation of oligomers containing 2,6-diaminopurine (G^{NH2}) : The CPG-$G^{S\text{-}dnØ}$ oligomer was treated with conc. aqueous ammonia (d=0.880) containing 65 mM tetramethylgunidine and 75 mM 2-nitrobenzaldoxime for 2 days at 25°C. The resulting oligomer was purified with a Nensorb Prep cartridge and further with FPLC. The correct base composition was confirmed.

Preparation of oligomers containing 2-amino-6-methylaminopurine (G^{NMe}): The CPG-$G^{S\text{-}dnØ}$ oligomer was treated with 40% aqueous methylamine for 2 days at 25°C. The resulting oligomer was purified with a Nensorb Prep cartridge and further with FPLC. The correct base composition was confirmed.

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REFERENCES

1. Singer, B. *Cancer Research* 1986 *46* 4879-4885; Basu, A. K.; Essigmann, J. M. *Chem. Res. in Toxicology* 1988 *1* 1-18; Swann, P.F. *Mutation Research* 1990 *233* 81-94; Hayashibara, K.C.; Verdine, G. L. *J. Am. Chem. Soc.* 1991 *113* 5104-5105; Englisch, U.; Gauss, D. H. *Angew Chemie Int. Edi. Eng.* 1991 *30* 613-629.
2. Macmillan, A.M.; Verdine, G.L. *Tetrahedron* 1991 *47* 2603-2616
3. Webb, T.R.; Matteucci, M.K. *Nucleic Acids Research* 1986 *14* 7661-7674
4. Xu, Y.-Z.; Zheng, Q.; Swann, P.F. (1991) Submitted to J. Org. Chem.
5. Elion, G.B. *Science* 1989 *244* 41-47
6. Rappaport, H.P. *Nucleic Acids Research* 1988 *16* 7253-7267
7. Gaffney, B.L.; Marky, L.A.; Jones, R.A. *Tetrahedron* 1984 *40* 3-13
8. Chollet, A., Chottet-Damerius, A and Kawashima E.H. *Chemica Scripta* 1986 *26* 37-40
9. Li, B.; Swann, P.F. *Biochemistry* 1989 *28* 5779-5786
10. Smith, C.A.; Xu, Y.-Z.; Swann, P.F. *Carcinogenesis* 1990 *11* 811-816
11. Cowart, M.; Benkovic, S.J. *Biochemistry* 1991 *30* 788-796
12. Reese, C.B.; Skone, P.A. *J .Chem. Soc. Perkin Trans.* 1984 *1* 1263-1271
13. Xu, Y.-Z.; Zheng, Q.; Swann P.F. *Tetrahedron Letters* 1991 *32* 2817-2820
14. Nikiforov, T.T.; Connolly, B.A. *Tetrahedron Letters* 1991 *32* 3851-3854
15. Kung, P.-P.; Jones, R. A. *Tetrahedron Letters* 1991 *32* 3919-3922
16. Schulhof, J .C.; Molko, D.; Teoule, R. *Tetrahedron Letters* 1987 *28* 51-54
17. Shaltiel, S. *Biochemical and Biophysical Research Communications* 1967 *29* 178-183
18. Xu, Y.-Z.; Swann, P.F. *Nucleic Acids Research* 1990 *18* 4061-4065
19. Kirnos, M.D.; Khudyakov, I.Y.; Alexandrushkina, N.I.; Vanyyushin, B. F. *Nature* 1977 *270* 369-370.
20. Sproat, B.S.; Iribarren, A.M.; Garcia, R.G.; Beijer, B. *Nucleic Acids Research* 1991 *19* 733-738. Lamm, G.M.; Blencowe, B.J.; Sproat, B.S.; Iribarren, A.M.; Ryder, U.; Lamond, A.I. *Nucleic Acids Research* 1991 *19* 3193-3198
21. Chollet, A and Kawashima, E. *Nucleic Acids Research* 1988 *18* 305-317
22. Connolly, B.A. and Newman, P.C. *Nucleic Acids Research* 1989 *17* 4957-4974
23. Christopherson, M.S.; Broom, A.D. *Nucleic Acids Research* 1991 *19* 5719-5724