Pergamon

SCIENCE DIRECT®

Tetrahedron 59 (2003) 1925–1932

TETRAHEDRON

Introduction of structural diversity into oligonucleotides containing 6-thioguanine via on-column conjugation

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Received 4 November 2002; revised 23 December 2002; accepted 23 January 2003

Abstract—A method is described for the introduction of structural diversity into the thiocarbonyl group of 6-thioguanine within support-bound, fully protected oligonucleotides via 'on-column' conjugation. 2′-Deoxy-6-thioguanosine with a chemically-labile trigger at its 6-thio function was incorporated at defined sites into chemically synthesized oligonucleotides. Following selective removal of the thio-protection group the support-immobilized oligonucleotides were conjugated with various groups on-column, and then deprotected and purified to produce a number of oligomers each containing a different modified base. Since the modification is accomplished on-column without affecting other functional and protecting groups in the oligomers this method is compatible with introducing structural diversity at multiple sites in DNA.

1. Introduction

Synthetic oligonucleotides containing modified nucleosides at specific positions provide increasingly useful tools for research in such areas as DNA–protein interactions, chemical carcinogenesis, therapeutic use of DNA, and molecular diagnostics (for latest reviews see[1–4]). One of the successful examples has been the utilization of oligonucleotides containing 6-methylthioguanine,[5] which is believed to play an important role in the delayed cytotoxicity of thioguanine that is used in the treatment of acute leukemia.

Oligonucleotides containing modified nucleosides can be prepared by both enzymatic and chemical approaches. The enzymatic method requires synthesis of a modified triphosphate, which is then incorporated into DNA by DNA polymerases.[6] Unlike the approach of chemical synthesis, incorporation of nucleoside analogues is normally carried out under mild conditions, and the resulting oligonucleotides are not subject to further harsh deprotection procedures. Hence this approach provides the possibility of synthesizing oligonucleotides containing alkaline-sensitive or thermally-labile nucleoside analogues. However DNA polymerases have certain requirements concerning substrate structure, and this can limit the types of modifications that can be introduced in nucleoside triphosphate analogues.

Using the traditional approach of chemical synthesis of site-specifically modified DNA, oligonucleotides are made by a route in which a modified phosphoramidite monomer is prepared (which can be a lengthy process), and then incorporated during the synthesis of oligomers. Each new modified nucleoside requires the synthesis of an appropriate monomer, which needs to be stable to the chemical treatments involved in DNA assembly, deprotection and purification. Since the conditions used in automated chemical DNA synthesis, including the post-synthetic deprotection procedures, are rather harsh, some modified nucleosides can be damaged by these conditions,[7] and therefore an alternative approach is required to overcome this obstacle. Over the past decade, we and others have been interested in developing a 'post-synthetic modification' strategy.[8–27] In this strategy, a convertible nucleoside is introduced into an oligonucleotide, which is stable towards the conditions of DNA synthesis and bears functionality capable of undergoing further transformations after synthesis. The transformations can be carried out while the oligonucleotide is still attached to the support,[24,25,27] or on a fully deprotected and purified oligomers in solution,[11,20]or concomitant with removal from the support and deprotection of other bases.[9,21] This strategy provides the possibility of making DNA containing a labile or chemically reactive nucleoside. In addition, a single synthesis of an oligonucleotide provides a source of DNA each containing a different modified nucleoside, which is potentially suitable for chemical synthesis of oligonucleotide libraries containing non-natural nucleosides. Here we wish to report the introduction of structural diversity into the thiocarbonyl group of 6-thioguanine from a convertible nucleoside within a support-bound, fully protected oligonucleotide via on-column conjugation. Convertible nucleoside **1** (Scheme 1) containing a chemically-labile trigger was incorporated at a defined site into an automated synthesized oligonucleotide

Keywords: post-synthetic modification; modified DNA; modified base; DNA synthesis; thioguanine.

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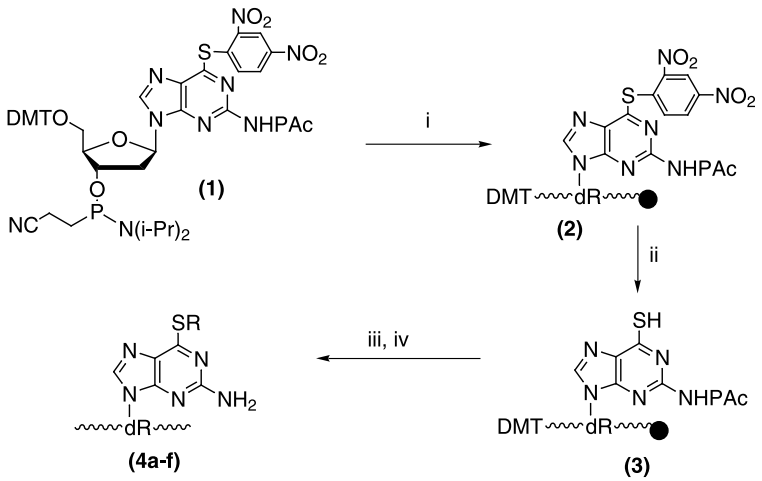
doi:10.1016/S0040-4020(03)00147-9



4a: R = CH_3; 4b: R = CH_3CH_2; 4c: R = $HOCH_2CH_2$; 4d: R = $(CH_3)_2CH$; 4e: R = $PhCH_2$; 4f: R = p-CH_3OPhCH_2.

i: automated DNA synthesis; ii: $HOCH_2CH_2SH/CH_3CN/(i\text{-}Pr)_2NCH_2CH_3$; iii: RX; iv: 0.5 M NaOH(aq).

Scheme 1. Post-synthetic modification of oligonucleotides on-column.

(**2**). Following the removal of the thio-protection group, the support-immobilized oligonucleotide **3** was conjugated with various groups, and then deprotected and purified using the usual procedures, producing a series of oligonucleotides (**4**) each containing a varied modified base. A number of workers have used thio-nucleosides as modifiable platforms within fully deprotected oligonucleotides for the post-synthetic modification of DNA in solution. Particularly related examples include those of Coleman and co-workers using 4-thio-2′-deoxyuridine[19,28] and 6-thio-2′-deoxyinosine,[20] and Xu[29] with 6-thio-2′-deoxyguanosine. However the advantage of carrying out the modification chemistry while the oligomer is attached to the support is the ease of separation of products from other reactants. Another advantage is that a CPG-immobilized, analogue-tagged oligonucleotide can be stored for a long period without removal of the protection group. When needed an aliquot can then be deprotected and modified as required. Finally, since the modification in this report is accomplished on-column without affecting other functional and protecting groups in the oligomer this method is compatible with the sequential introduction of diversity at multiple sites in oligonucleotides.

2. Results and discussion

2.1. Design and synthesis of convertible nucleoside phosphoramidite

For the strategy to be successful, the choice of protecting group at the 6-thio position of thioguanine is a crucial point. It needs to be stable during automated DNA synthesis, but should be removed selectively to unmask the thiol group without affecting other functional and protecting groups, and cleaving the DNA from the support. In our previous work,[9] we used the 2,4-dinitrophenyl group to protect the 6-thioguanine phosphoramidite monomer for the chemical synthesis of DNA containing 6-thioguanine. We observed that this protecting group could be quickly removed by treatment with conc. ammonia containing 10% of mercaptoethanol at room temperature. Further optimization carried out in this work revealed that removal of the 2,4-dinitrophenyl group can be accomplished within 30 min by 10% (v/v) mercaptoethanol in acetonitrile in the presence of 1% of *N*,*N*-diisopropylethylamine. We envisioned that such a mild treatment should not affect the other functional and protecting groups in the resin-bound DNA or break the resin–DNA linkage. To substantiate our anticipation, experiments were carried out to study the stability of four natural nucleosides phosphoramidites and DNA–resin linkage towards the above deprotecting mixture. Four commercially available UltraMild™ phosphoramidite monomers (from Glen Research) were treated individually with the mixture and monitored with TLC. No obvious damage to the monomers was observed for at least 3 h at room temperature. To test the stability of the DNA–resin linkage a 12 mer oligonucleotide (5′ AGG GAA TTC GCT 3′) was synthesised using UltraMild™ T-support. The final DMT group was left on the oligomer for easy identification of the oligomer peak by reverse phase HPLC. The support-bound oligomer was divided into aliquots and treated with the deprotection mixture. After 10 min, 30 min, 1 h, 2 h, and 3 h, the support was removed by filtration, and the filtrate was concentrated under reduced pressure and treated with conc. ammonia. HPLC analysis of the ammonia solutions showed no detectable oligomers cleaved from the support. These results demonstrated that phosphoramidite **1** would be an ideal convertible nucleoside for our purpose. We therefore prepared the phosphoramidite following a previously published method.[9]

2.2. Post-synthetic modification of oligonucleotides on-column

6-(2,4-Dinitrophenylthio)-2′-deoxyguanosine phosphoramidite (**1**) was incorporated into an oligonucleotide 5′-AGC XAA TTC GCT-3′ (X standing for 6-(2,4-dinitrophenyl)-thioguanine) using a DNA synthesiser (Scheme 1). The resulting oligomer (**2**) was then treated with 10%

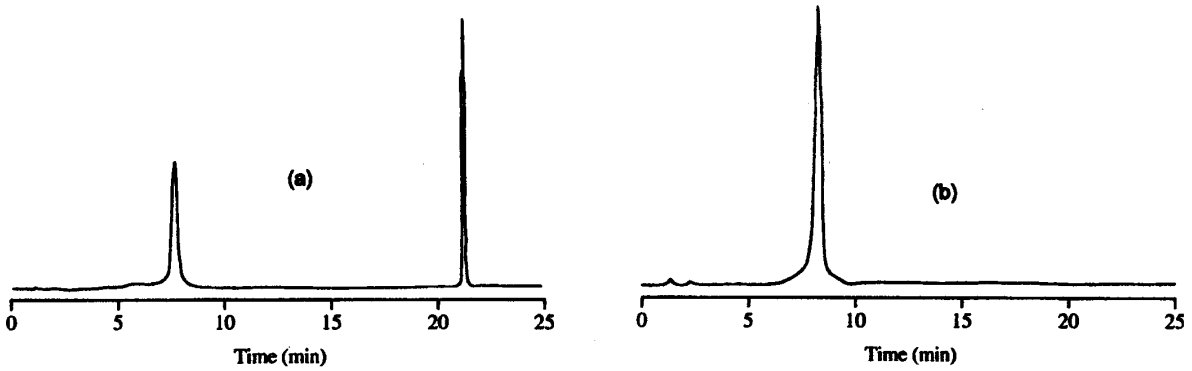


Figure 1. HPLC analysis of the removal of the phenylacetyl group with 0.5 M NaOH from N^2-phenylacetyl-6-methylthio-2′-deoxyguanosine. (a) after 2 h reaction; (b) after overnight reaction.

2-mercaptoethanol in CH_3CN in the presence of diisopropylethylamine. As the treatment only removed the 2,4-dinitrophenyl group, oligonucleotide **3** was fully protected and CPG-immobilized except with a naked thio-function. To convert the 6-thioguanine oligomer into a 6-conjugated thioguanine counterpart involves two major reactions: conjugation and deprotection. To test the feasibility of the strategy and to discover the optimum conditions for conjugation and deprotection, a detailed investigation was carried out on methylation of the oligomer **3** with methyl iodide.

It is well documented that alkoxy groups at the 6-position of guanine, such as the methoxy group, are labile to ammonia treatment at an elevated temperature,[30] which is the commonest procedure for the deprotection of chemically synthesized DNA. One can reasonably envision that alkylthio groups would be more susceptible to substitution by ammonia than alkoxy groups, as the former are better leaving groups. Therefore care must be taken in the deprotection of oligomers containing 6-alkylthioguanine to avoid the formation of 2,6-diaminopurine. The susceptibility of alkylthio groups was tested towards the conditions of deprotection using 2′-deoxy-6-methylthioguanosine as a model compound. The nucleoside was prepared[31] and treated with concentrated ammonia and monitored by TLC. It was found that the nucleoside was not completely stable in conc. ammonia at 55°C, in that about a quarter of the nucleoside was converted, after 6 h incubation, presumably into 2,6-diaminopurine deoxynucleoside. However, at room temperature the nucleoside remained unchanged after 3 days incubation. Experiments were also made with other deprotection reagents, namely, 0.5 M NaOH, and aqueous CH_3NH_2 (40%) solution.[9] The nucleoside was stable towards 0.5 M NaOH at 25°C for a prolonged period of time (3 days), but was unstable at 55°C, and was not stable toward aqueous CH_3NH_2 solution even at room temperature. Conc. ammonia and 0.5 M aqueous sodium hydroxide did not damage the molecule and were both being effective deprotecting agents,[9] however, 0.5 M sodium hydroxide was chosen as the deprotection agent since if there are any oligonucleotides produced by hydroxide substitution of the alkylthio group, a reaction which would transform an alkylthioguanine oligonucleotide into a guanine counterpart, they can be readily separated from alkylated oligomers and those containing the thio-base using anion-exchange HPLC.[32]

Another important point was the removal of protecting group (phenylacetyl) on the 2-amino position of 6-alkylthioguanine. It has been observed that alkylation of the O^6-position of guanine retards the removal of protecting group from the 2-amino position of the nucleoside compared with unmodified 2-deoxyguanosine.[30,34] To investigate the removal of the phenylacetyl group from the 6-alkylthioguanine counterpart, N^2-phenylacetyl-6-methylthio-2′-deoxyguanosine was treated with 0.5 M NaOH (aq) in an Eppendorf tube at room temperature and the deprotection was followed by RP-HPLC. It was found that complete removal of the group was accomplished after overnight reaction (Fig. 1). Although removal of the phenylacetyl group would be slower at the oligomer level, it is reasonable to expect that its complete removal at the oligomer level could be accomplished by 2 days deprotection with the aqueous alkali, a protocol that was used successfully by Xu[33] in the deprotection of the N^2-phenylacetyl group from O^6-carboxymethylguanine at the oligomer level. For natural nucleoside building blocks, base-labile monomers and supports (UltraMild™ phosphoramidites and supports, Glen Research) were used, with the phenoxyacetyl group for the protection of the exocyclic amino group of adenine, 4-isopropylphenoxyacetyl on guanine and the acetyl group on cytosine. Removal of these protecting groups and the cleavage of the oligomers from the supports can be achieved under mild conditions, such as 0.5 M aqueous sodium hydroxide (2 days at rt) without the risk of transforming N^6-acyladenine or N^4-acylcytosine into hypoxanthine or uracil, respectively.[33]

Having established the stability of 2′-deoxy-6-alkylthioguanosine towards 0.5 M NaOH and the rate of removal of the phenylacetyl group from its N^2-phenylacetyl derivative, CPG-bound oligomer **3** with an unmasked thiol group was then reacted with methyl iodide in acetonitrile in the presence of 5% $EtN(i\text{-}Pr)_2$ for 30 min. After 2 days deprotection with 0.5 M NaOH (aq) and then Nen-sorb column purification, the oligomer was assessed by anion-exchange HPLC (FPLC), showing one major peak (Fig. 2a). Pure oligomer was obtained by further purification with FPLC (Fig. 2b). A UV spectrum of the purified oligomer

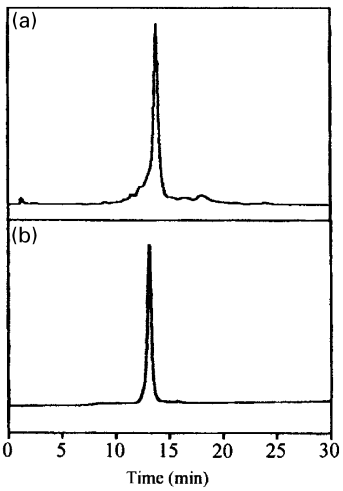


Figure 2. HPLC profiles of the methylated oligonucleotide 5′ AGC X1AA TTC GCT 3′ (X1 standing for 6-methylthio-2′-deoxyguanosine). (a): after Nensorb column purification; (b): after further purification with FPLC).

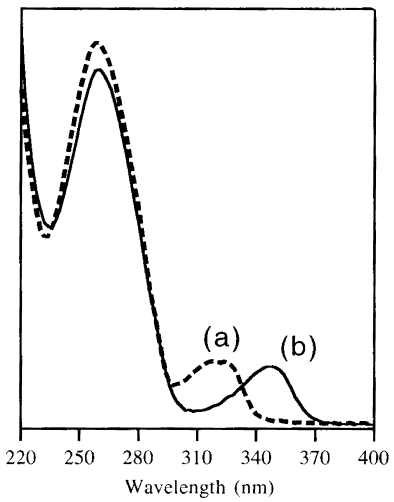


Figure 3. UV spectra of the methylated oligonucleotide (a) and of the oligonucleotide containing thioguanine (b).

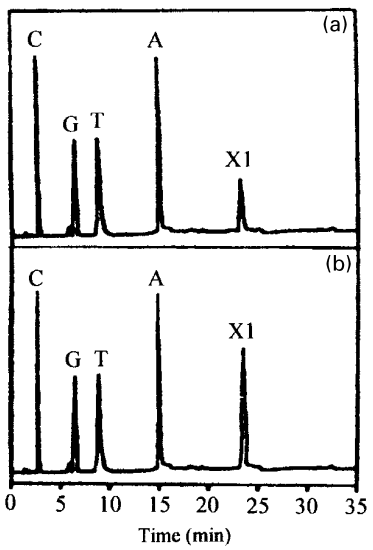
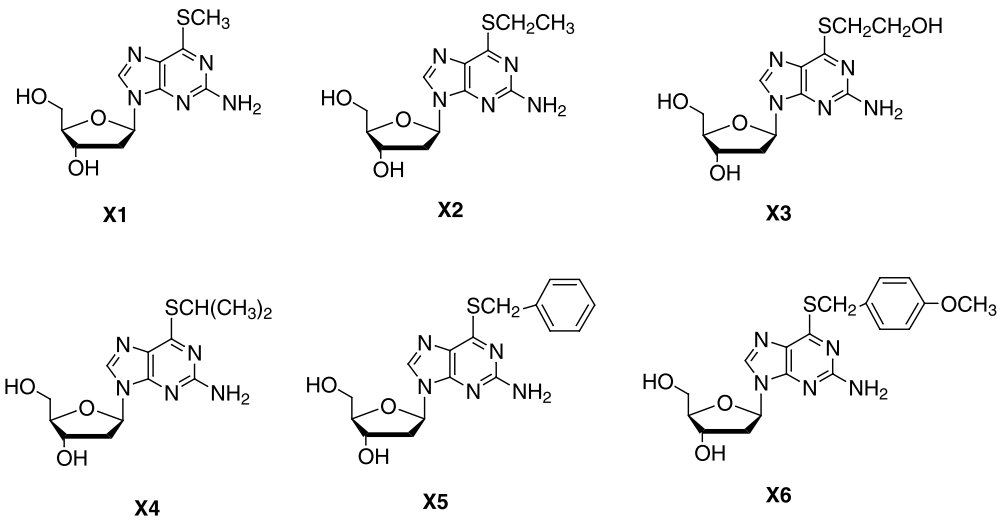


Figure 4. HPLC traces of the enzymatic digest of the methylated oligonucleotide (a) and of the digest with added authentic 6-methylthio-2′-deoxyguanosine (b).

(Fig. 3), together with that of oligonucleotide containing thioguanine, shows the presence of 6-methylthioguanine ($\lambda_{max} = 313$ nm) and the disappearance of thioguanine ($\lambda_{max} = 350$ nm). To further confirm the identity of the methylated oligomer, the purified oligomer was desalted and digested first with snake venom phosphodiesterase followed by spleen alkaline phosphate to their constituent 2-deoxynucleosides in a standard assay buffer. The fact that 6-thio-2-deoxyguanosine was no longer present in the digest of the oligomer (Fig. 4a), in conjunction with the appearance of a retained residue (**X1** Scheme 2) is consistent with full alkylation of the thiocarbonyl group in the oligomer. No covalent modification of other bases was evident by RH-HPLC. To verify the identity of the retained residue, HPLC analysis was carried out of a pre-mixed sample of the oligomer digest with the synthetic 6-methythio-2′-deoxyguanosine,[31] which confirmed the identity of the retained residue (Fig. 4b). Having confirmed the feasibility of the approach for preparation of oligomers containing 6-methylthioguanine we extended the method for the conjugation of other groups at the thiocarbonyl function (Scheme 1).

Although methylation of the thiocarbonyl group on DNA was complete within a short period of time (30 min), as one would expect, the reaction would be slower when less electrophilic alkylating reagents are used. For example our preliminary experiments indicated that when isopropyl iodide was used the reaction did not take place after 2 h in acetonitrile, and only 50% of alkylation was observed after 5 h reaction in DMF (a more pro-nucleophilic substitution solvent than CH_3CN). Therefore further experiments were done to follow the progress of these reactions in order to find the optimum reaction time for conjugation. CPG-immobilized oligomer **3** was treated with various alkylating agents at room temperature in DMF (or CH_3CN for methyl iodide) in the presence of 5% ethyl diisopropylamine for a different period of time. Following deprotection the extent of alkylation of the oligomers was assessed by FPLC (pH 12) after being subject to Nen-sorb column purification. The unalkylated oligomer (containing thioguanine) can be easily separated from the alkylated oligomer (containing 6-alkylthioguanine) as the former carries one more negative charge under alkali conditions.[32] Among the alkylating agents tested, methyl iodide was the most effective alkylating agent, and methylation was finished within 20 minutes in acetonitrile (Fig. 5). For other alkylating reagents, reactions needed to be carried out in a more pro-nucleophilic-substitution solvent (DMF) and longer reaction times were needed.

Using the above protocol, five more alkylated oligomers were prepared (Scheme 1). FPLC profiles of those oligomers (after Nensorb column purification) showed only one major peak, similar to that of the methylated oligomer (Fig. 2a). Enzymatic digestion of each of the FPLC purified oligonucleotides produced four natural nucleosides and a modified nucleoside as expected (Fig. 6). The identity of the modified nucleoside was confirmed by co-injection of the DNA digest with the authentic modified nucleosides prepared in a similar method as 2′-deoxy-6-methylthioguanosine.[31] In addition to five primary alkyl groups a secondary alkyl group (isopropyl) was also



Scheme 2. Structures of alkylated thioguanine nucleotides.

introduced into the oligomer, albeit at a much slower rate. It is worth pointing out that in the work of utilizing thio-nucleosides as modifiable platforms for post-synthetic modification of oligonucleotides in solution by Coleman and co-workers[19,20,28] and by Xu,[29] only primary alkyl halides were used. Our efforts to append an isopropyl group, using isopropyl iodide, to the thio-function of 6-thioguanine in a deprotected oligomer in solution produced no satisfactory results.

2.3. Introduction of multiple modified nucleosides into oligonucleotides

The ease and effectiveness of the unmasking/alkylation process presented above suggested that this approach will be useful for preparation of libraries of chemically synthesized modified oligonucleotides. Since alkylation is accomplished on-column without affecting other functional and protecting groups in the oligomer, this method is compatible with introducing modifications at multiple sites in the oligo-nucleotide sequentially (Scheme 3). It is worth to mention that convertible nucleosides involving the introduction of diversity via nucleophiles are not amenable to this,[9,21,23] as one can expect that these nucleophiles can at least partially deprotect the oligomer, making subsequent elongation of the oligonucleotide impossible.

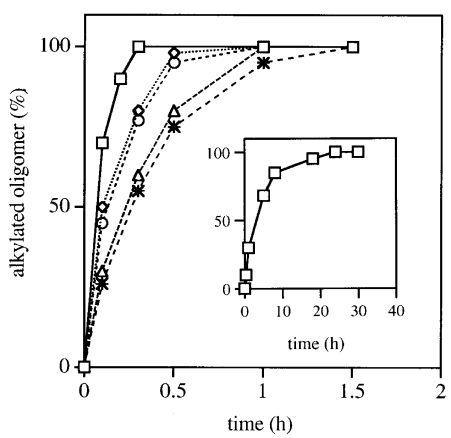


Figure 5. Reaction time course of oligonucleotide 5′6AGC XAA TTC GCT 3′ (X standing for 6-thio-2′-deoxyguanosine) with various alkylating agents. □: CH_3I; ◇:CH_3CH_2I; ●: $HOCH_2CH_2I$; △: $PhCH_2Br$; ∗: *p*-CH_3OPhCH_2Br; inserted figure: $(CH_3)_2CHI$.

To demonstrate the principle a two-site, four-component library was prepared. A pentamer oligonucleotide (**5**, Scheme 3) was synthesised using standard procedures. Following removal of the masking group at the 6-position of thioguanine, resin-bound pentamer was treated with an equal molar mixture of iodoethane and iodoethanol. After removing the excess reagents and washing the resin **6** with acetonitrile, an aliquot of the resin was taken, deprotected and the resulting oligonucleotide purified and enzymatically digested. HPLC analysis of the digest (Fig. 7) revealed the presence of the expected alkylated nucleosides **X2** and **X3** (Scheme 2) alongside four natural DNA components. The remainder of the resin was placed back in an empty column, and the elongation was continued on the DNA synthesiser until the preparation of a 10 mer oligonucleotide (**7**) was complete. The second thio-carbonyl function was alkylated with a mixture of benzyl bromide and 4-methoxybenzyl bromide. The purified oligomer was enzymatically digested and analysed by reverse-phase HPLC. In addition to **X2** and **X3**, another two thio-deoxyguanosine derivatives (**X5** and **X6**) were also present (Fig. 8). It is worth pointing out that although the same convertible nucleoside was introduced at the second site, any other suitable convertible bases can be introduced at this stage.

3. Conclusions

The work presented here demonstrates that introducing structural diversity into 6-thioguanine oligonucleotides can be achieved via on-column conjugation of protected oligonucleotides containing a single reactive thio function. The function is chemically revealed in a site-specific manner under mild conditions that do not affect other parts of the oligonucleotides. The use of such a convertible nucleoside has the advantage that only a single phosphor-amidite needs to be prepared, making the method suitable for constructing libraries of modified oligonucleotides. As the conjugation chemistry is carried out while the oligomers are still on the solid support it is easy to separate the

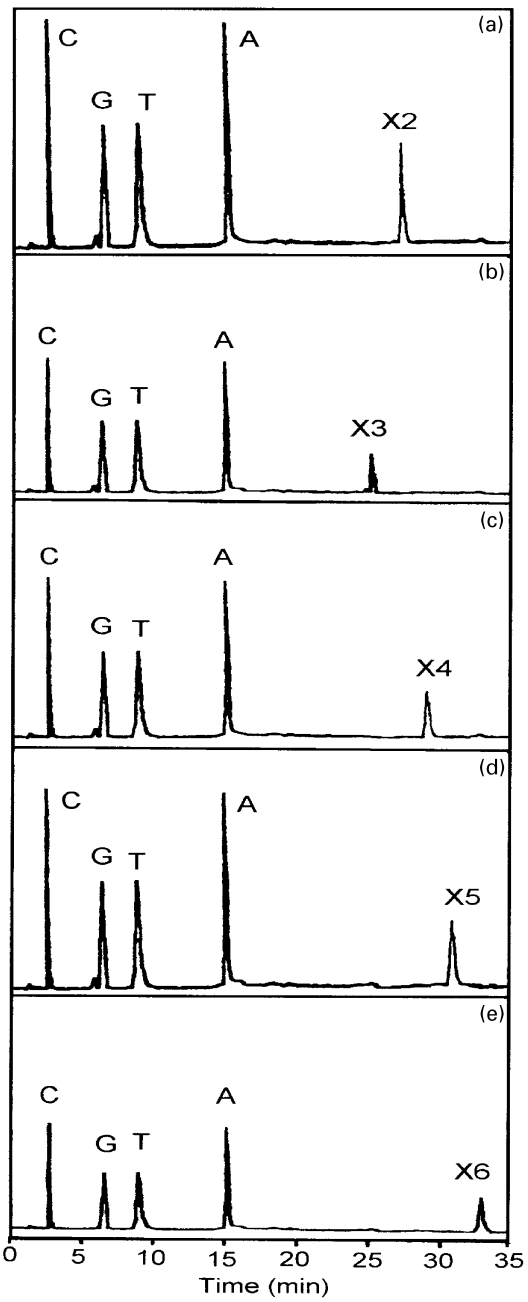


Figure 6. HPLC traces of the enzymatic digests of the alkylated oligonucleotides. X2: 6-ethylthio-2′-deoxyguanosine (a); X3: 6-hydroxyethylthio-2′-deoxyguanosine (b); X4: 6-isopropylthio-2′-deoxyguanosine (c); X5: 6-benzylthio-2′-deoxyguanosine (d); X6: 6-(*p*-methoxybenzylthio)-2′-deoxyguanosine (e).

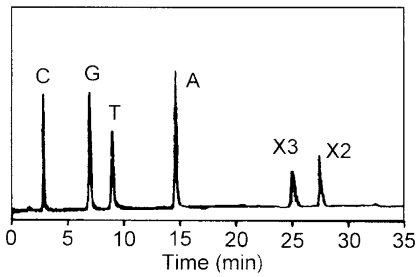


Figure 7. HPLC profile of the enzymatic digest of oligomers CAX2GT and CAX3GT.

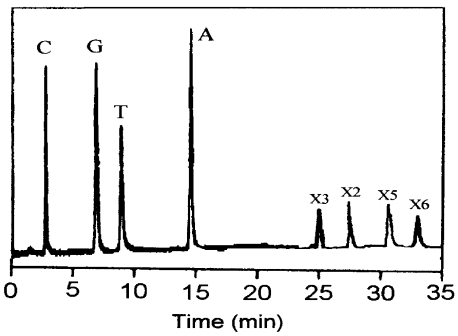


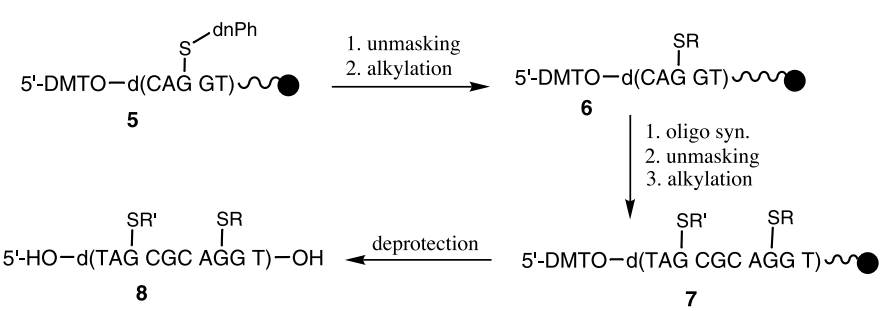
Figure 8. HPLC analysis of the enzymatic digest of an oligonucleotide containing two 2′-deoxy-6-thioguanosine sites which have each been modified in two different ways.

products from other reactants. In addition since coupling is achieved on-column without affecting other functional and protecting groups the method is compatible with introducing diversity at multiple sites of the oligonucleotides sequentially.

4. Experimental

4.1. General methods

Oligonucleotides were synthesised using a Beckman Oligomer 1000 DNA Synthesizer using standard synthetic cycles except the modified monomer was injected into the column manually, and its coupling time extended to 3 minutes. The CPG-linked monomers and phosphoramidites (UltraMILD™ phosphoramidites) and other chemicals for DNA synthesis were from Glen Research. Thioguanine phosphoramidite was prepared using a published method.[9] All other chemicals and solvents were from either Aldrich



Scheme 3. Introduction of multiple modified nucleosides into oligonucleotides.

or Sigma, and used directly without further treatment unless otherwise stated. Reverse phase HPLC was carried out on Hewlett Packard series 1000 using C-18 column, and ion exchange HPLC (FPLC) was carried out as previously described.[9,32]

4.2. General procedure for alkylation of protected resin-bound oligonucleotides

Oligonucleotide **2** was synthesised on a 1 μmol scale with standard DNA synthesis cycles. The oligonucleotide was transferred into an Eppendorf tube and treated with 0.5 ml of unmasking reagent (10% of 2-mercaptoethanol in acetonitrile containing 1% of diisopropylethylamine) for 1 h. The reagent was removed and the resin washed with acetonitrile (5×1 ml) and dried under vacuum. Resin-bound oligonucleotide **3** equivalent to 0.2 μmol of the original loading was treated with one of the following halide reagents (5%, 0.5 ml) containing 5% of $(i\text{-Pr})_2$NEt at room temperature with shaking, respectively, CH_3I/CH_3CN (30 min); CH_3CH_2I/DMF (1 h); $HOCH_2CH_2I$/DMF (1 h); $C_6H_5CH_2Br$/DMF (1 h); *p*-$CH_3OC_6H_4CH_2Br$/DMF (1.5 h); and $(CH_3)_2CHI$/DMF (24 h). The halide reagents were removed and the CPG-support was washed with acetonitrile (3 x 1 ml) and dried, and then deprotected with 0.5 M NaOH (aq) for two days at room temperature. The deprotected oligomer was separated from the failure sequences and the DMT group removed using a Nensorb Prep cartridge (Du Pont Company). Further purification of the oligomer using ion exchange HPLC (FPLC) was carried out as previously described.[9,32]

4.3. Synthesis of oligonucleotides containing multiple modified nucleosides

Pentamer **5** was assembled on a 1 μmol scale with standard DNA synthesis cycles. Resin was put into an Eppendorf tube and treated with the unmasking reagent and conjugated as described above except with a mixture of CH_3CH_2-$I/HOCH_2CH_2I/(i\text{-Pr})_2$NEt/DMF (5/5/5/85, v/v/v) for 1 h. The resin was divided into two equal portions, one was deprotected and purified as above, and the other was placed back on the DNA synthesizer and second convertible monomer (**1**) was incorporated and the oligomer extended to decamer **7**. Unmasking and conjugation of the second modified base was carried out as the first except with a mixture of $C_6H_5CH_2Br$/p-$CH_3OC_6H_4CH_2Br/(i\text{-Pr})_2$NEt/DMF (5/5/5/85, v/v/v) for 1.5 h.

4.4. General method for enzymatic digestion of modified oligonucleotides

The oligonucleotide substrate, purified with ion exchange HPLC, was desalted with a Water Sep-pak C_{18} cartridge. The oligomer (≈1 O.D. unit) was then evaporated to dryness in an Eppendorf tube. The residue was redissolved 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 incubation continued for 60 min. The nucleosides were separated by HPLC using 97% buffer A (0.05 M aqueous KH_2PO_4, pH 4.5) and 3% buffer B (0.05 M aqueous KH_2PO_4, 35% CH_3CN, pH 4.5) for the first 8 min, then with a linear gradient from 3 to 35 % of buffer B over the following 17 min followed by a linear gradient from 35 to 70% of buffer B over the remaining 10 min. The eluate was monitored at 260 nm for the first 20 min for detection of d*C*, d*G*, d*T* and d*A* and the remainder at 310 nm for detection of the modified nucleosides. Peaks on the HPLC traces were identified by retention time comparison with authentic samples. Retention times were: d*C*, 2.7 min; d*G*, 6.8 min; d*T*, 8.9 min; d*A*, 14.5 min; X1, 20.7 min; X2, 27.3 min; X3, 25.1 min; X4, 29.6 min; X5, 30.6 min; and X6, 33.1 min (see Scheme 2 for the structures of X1–X6).

Acknowledgements

We wish to thank The Royal Society for their financial support.

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31. 2′-Deoxy-6-methylthioguanosine[12] was prepared as follows. 2-Deoxy-6-thioguanosine (100 mg, 0.35 mmol) was dissolved in 5 ml of conc. ammonia and methyl iodide (31 μl, 50 mmol) was added to the above under stirring. After 1 h additional methyl iodide (10 μl) was added, and the reaction continued for 30 min. The mixture was then evaporated to a small volume, cooled on ice and 10 ml of water added. The precipitate was filtered and re-crystallized with acetone. 58 mg of the product was obtained. ^{1}H NMR (in DMSO-d_6): 2.26–2.66 (2H, m, 2′-H and 2″-H), 2.63 (3H, s, SCH_3), 3.42–3.55 (2H, m, 5′-H), 3.91 (1H, m, 4′-H), 4.43 (1H, m, 3′-H), 6.22 (1H, t, 1′H), 6.52 (2H, br, 2-NH_2, ex) and 8.20 (1H, s, 8H). UV λ_{max}=313 nm. HRMS: calcd for $C_{10}H_{13}N_5O_3S$ $[M+Na]^+$ 306.0637, found 306.0646.
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