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Post-synthetic Introduction of Labile Functionalities onto Purine Residues via 6-Methylthiopurines in Oligodeoxyribonucleotides§

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Abstract: Two methods are described for the preparation of oligodeoxynucleotides containing 6-methylthiopurine residues. 6-Methylthiopurine phosphoramidite (6) has been prepared and incorporated into oligomers. Methylation with methyl iodide of 6-thiopurine (or 6-thioguanine) in oligomers also exclusively produces oligomers containing 6-methylthiopurine (or 6-methylthioguanine). The methylthio group at defined purine residues in the deprotected oligomers can be oxidized selectively and converted at the final step into various functional groups including radioactive ^{35}S-thio group, a useful tag for cross-linking studies. Copyright © 1996 Elsevier Science Ltd

Introduction:

The availability of base-modified oligodeoxynucleotides has greatly stimulated research in many areas including carcinogenesis, DNA repair and DNA-protein interactions.[1] However difficulties are often encountered in the preparation of modified DNA, in particular DNA containing reactive or labile functional groups and DNA derived from hazardous reagents. The process of making DNA in general consists of the following stages: 1) preparation of required monomers; 2) incorporation of the monomers into DNA; 3) post-synthetic treatments including deprotection and purification. Conventionally, modified oligonucleotides have been synthesized by preparation and incorporation into DNA of the protected phosphoramidite of the modified base. Because of some limitations of this approach, we[2] and others[3] have been developing an alternative route - post-synthetic substitution in which the phosphoramidite of a versatile base is prepared and incorporated into DNA, then the versatile base is specifically converted into the desired modified base at the oligomer level. Such an approach makes it feasible to introduce functional groups which would have been unstable during preparation and/or incorporation. However the introduced groups are still subject to the deprotection step which is generally done with nucleophilic reagents, such as conc. ammonia, alkaline solution or alcohol/DBU (DBU: diazabicyclo[5,4,0]-undec-7-ene). Therefore the only groups which can be introduced are those which are sufficiently stable under nucleophilic conditions. One possible solution to overcome this limitation is to introduce the desired group *after* full deprotection or even *after* complete purification. This would allow one at the final step to introduce reactive functionalities (eg. cross-linkable groups) which might be unstable or at the final step to introduce functional groups from hazardous reagents (eg. radioactive or carcinogenic compounds), exposure of which could be minimized. However, by this approach the chosen leaving group on the base must not only remain stable throughout preparation, incorporation and nucleophilic deprotection, but also be replaceable by nucleophilic substitution under mild conditions. This seems to present a difficult dilemma. Very recently, we tried tackling such a problem by introducing labile functionalities on *pyrimidine* in fully deblocked oligodeoxynucleotides.[4]

It is equally difficult and challenging, if not more, to construct a ***purine*** building block bearing a satisfactory leaving group. Leaving groups previously used for the synthesis of oligomers carrying a modified purine by the post-synthetic substitution approach were either too reactive (such as 2-fluoropurine and 6-fluoropurine[5]) to survive during the deprotection procedures or too stable (such as 6-phenoxypurine[3b,c]) to be substituted by nucleophiles under mild conditions. Even in the case of 6-pentafluorophenoxypurine,[5c] an improved version of 6-phenoxypurine, its substitution at the nucleoside level still needs severe treatment (16 hr at 60°C for 3.3 N aqueous ammonia). Obviously, such procedure with a longer time and elevated temperature is not suitable for DNA containing a reactive or labile functionality.

In our previous work, we reported methods for the synthesis of versatile base monomers i.e. 2-amino-6-(2,4-dinitrophenyl)thiopurine and 6-(2,4-dinitrophenyl)thiopurine phosphoramidites[2] and their incorporation into oligomers. These 6-(2,4-dinitrophenyl)thiopurines in oligomers can be transformed at room temperature by treatment with mercaptoethanol to 6-thiopurines quantitatively or by treatment with other nucleophiles to their corresponding 6-substituted purines in good yields. However the formation of 6-substituted purine analogues by this approach is sometimes accompanied with a small amount of 6-thiopurine or 2-amino-6-thiopurine (i.e 6-thioguanine) analogues, although their separation can be easily achieved by chromatography under alkaline conditions.[6] The production of unwanted 6-thiopurine analogues could be attributed to the fact that 6-(2,4-dinitrophenyl)thiopurine has two nucleophilic sites (**Fig. 1**), since both dinitrophenylthio group (attacking route α) and dinitrophenyl group (attacking route β) are good leaving groups. In order to avoid the formation of unwanted thiopurines during nucleophilic substitutions, the strongly electron-withdrawing dinitrophenyl should be replaced by a less electron-withdrawing group, eg alkyl group, but such a replacement would inevitably inhibit the route α. A more attractive choice would be to employ oxidized forms of alkylthio group - sulphoxide or sulphone, since it is known that they are highly reactive.[7,8,9] In this report, we present in details effective approaches (chemical synthesis and selective methylation) to the preparation of DNA containing 6-methylthiopurine residues, report the results on the selective oxidation and conversion into a number of desired modified bases both prior to and after deprotection, and discuss their applications in DNA-related research fields.

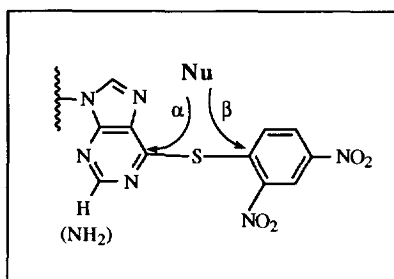
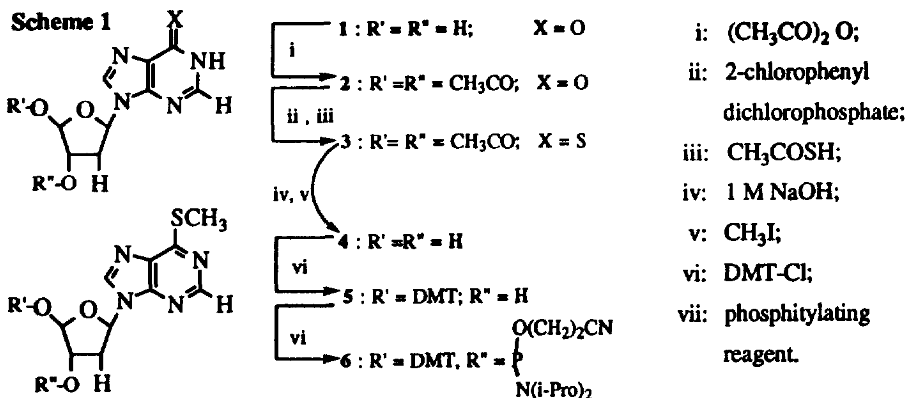


Fig. 1 the attacking routes α and β

Chemical Synthesis of 6-methylthiopurine deoxynucleoside phosphoramidite (6)

The synthetic route to 6-methylthiopurine phosphoramidite (**6**) is shown in **Scheme 1**. 2'-Deoxyinosine (**1**) was acetylated to give 3',5'-diacetyl-2'-deoxyinosine (**2**), which was in turn transformed into the 6-thio analogue (**3**).[2b,10] The product was then deacetylated and its thio group methylated in one pot to give 2'-deoxy-6-methylthiopurine (**4**). All these reactions produced desired compounds in excellent yields (70 % over-all yield from **1**). **4** was converted into its phosphoramidite monomer (**6**) with standard procedures. The stability of the methylthio functionality was a primary concern in making DNA containing 6-methylthiopurine, since this functionality could be rapidly oxidized.[7,9] Prior to the incorporation of **6** into DNA, the susceptibility was tested of the methylthio group towards the conditions for DNA synthesis and protection. It has been found that



6-methylthiopurine nucleoside (4) was completely intact in the oxidative solution (I_2 / H_2O / pyridine / THF) used in the synthesizer at 25°C, even after 24 hours. On the other hand, 4 was not very stable in conc. ammonia at 55°C used for standard deprotection, and about half of the nucleoside was damaged after overnight incubation. However, at reduced temperature, eg. 25°C, no damage to 4 was found. Therefore oligodeoxynucleotides containing 6-methylthiopurine were synthesized using the base-labile monomers and support.[11] As an example a 12 mer CGC XAG CTC GCG (where X is 6-methylthiopurine) has been successfully prepared. Its anion exchange chromatography[6] showed a single peak of the desired oligomer (**Fig. 2 inset**) and the presence of 6-methylthiopurine nucleoside was confirmed by nucleoside composition analysis (**Fig. 2**).

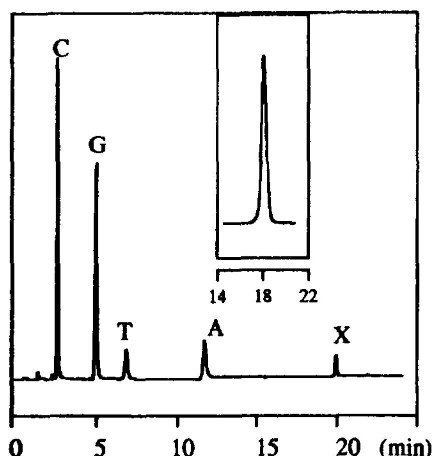


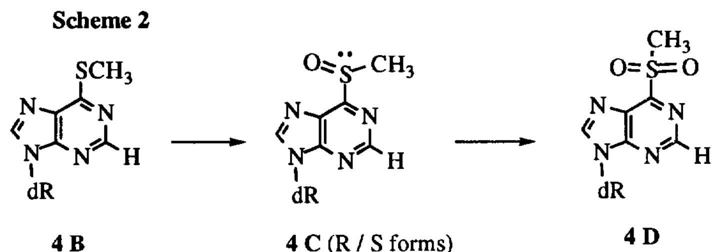
Fig. 2 Reversed phase HPLC profile of the nucleosides from enzymatic digestion of a synthetic 12 mer CGC XAG CTC GCG (X= 6-methylthiopurine). Conditions: Waters Nova-Pak C18 cartridge, 1.5 mL / min, 260 nm. The column was eluted for 8 min with 2.33% acetonitrile in 50 mM KH_2PO_4 (pH 6.2), then acetonitrile was increased to 16.7% over 12 min, then acetonitrile was kept at 16.7% for further 5 min. The figure inset is an anion exchange chromatographic profile of the modified 12 mer. Conditions: Pharmacia mono Q HR 5/5 column; 0.6 mL / min, 260 nm. The column was eluted with Eluant A (0.4 M NaCl, pH 12) for first 2 min, Eluant B (1.2 M NaCl, pH 12) was increased from 0% to 15% over 3 min, then to 35% for the following 20 min.

Oxidation and Conversion

The linkage between carbon and sulphur in sulphoxides and sulphones is generally stable. But when this bonding exists at the 6-position of a purine nucleoside (cf: Scheme 2) it becomes weak so that the related

sulphinyl and sulphonyl groups could be used as leaving groups.[7-9] However, there is a conflicting view on the reactivity of the sulphonyl functionality.[12] In order to have a better understanding and utilizing of the oxidation of 6-alkylthio functionality on purine and of the conversion of its oxidized derivatives, these reactions have been investigated at both the nucleoside level and the oligonucleotide level.

a) at the nucleoside level



3',5'-diacetyl-6-methylthiopurine 2'-deoxyribonucleoside **4B** (**Scheme 2**) was prepared by methylation of **3** (cf: **Scheme 1**) with methyl iodide under the basic condition. When treated with an equimolar amount of m-chloroperoxybenzoic acid (MCPBA) in CH_3CN, **4B** (Rf = 0.8, CH_3OH / $CHCl_3$ 10% v/v) was rapidly and almost quantitatively converted into the putative mono-oxidized derivatives **4C**. TLC showed only a single spot (Rf=0.4), but the presence and nearly equal amount of two stereoisomers (R / S forms resulting from the chiral centre of sulphur) was observed by NMR (cf: **Table 1**) after their isolation. Further addition of MCPBA immediately converted **4C** into **4D** (Rf = 0.55). **4D** was quite stable in absence of water and other nucleophiles and in fact it was isolated by fresh silica column. The structure of **4D** was confirmed by NMR and Mass spectroscopy. **Table 1** shows that the chemical shifts, notably for S-bonded methyl group, move downfield as the oxidative state for the sulphur increases from **4B** to **4C**, to **4D**.[13]

Table 1: Effects of Oxidative States of the Sulphur on the ^{1}H-NMR Chemical Shifts

	S-bonded CH_3	1'-H	8-H	2-H
4 B	2.661 (s)	6.453 (t)	8.653 (s)	8.753 (s)
4 C	3.063 (s)	6.542 (2 x t)	8.960(s) 8.949(s)	9.131 (s)
4 D	3.570 (s)	6.567 (t)	9.074 (s)	9.171 (s)

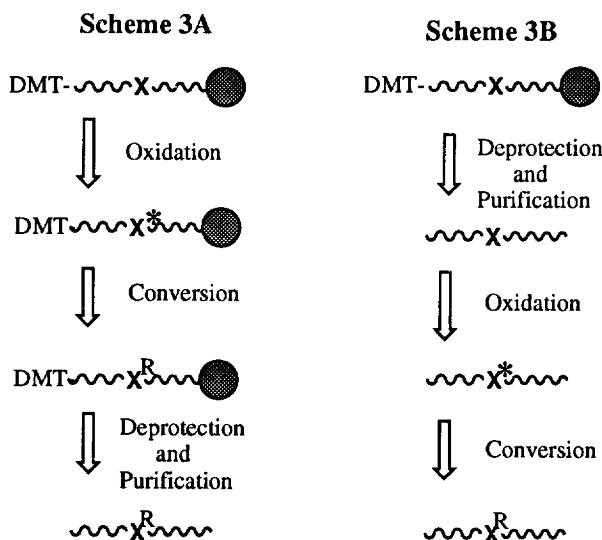
Compound **4C** and **4D** were further characterized and it has been found that both the 6-sulphinyl derivative (**4C**) and the 6-sulphonyl derivative (**4D**) could be rapidly converted into a 6-substituted compound by an appropriate reagent such as methylamine. This finding is consistent with one previous report,[7] but contrary to another.[10b] In an attempt to understand the underlying factors for this discrepancy,[12] aqueous magnesium monoperoxyphthalate (MMPP) solution[10b] instead of MCPBA in CH_3CN was also used. It was found that the oxidation of **4B** with MMPP to form **4C** was slower than with MCPBA, but **4C** could be further oxidized with excess of MMPP to form **4D**. However the newly formed **4D** was gradually converted into a new spot with an Rf of 0.25, which was the same as that of the hydrolysed product (compound **2** in **scheme 1**). Its formation was probably due to attack by water present in the reaction solution.

b) at the oligonucleotide level

From the above experiments it is clear that methylsulphinyl and methylsulphonyl groups at the 6-position of purine nucleosides, both of which can be made by oxidation of methylthio group on purine nucleosides, are very reactive towards nucleophiles and can be used as potential leaving groups for synthesis of 6-substituted purine nucleosides. Further investigations were carried out on the oxidation of the 6-methylthio functional group and conversion of the oxidized products at the oligomer level.

As a model, a pentamer CGXAT was synthesized (where X is 6-methylthiopurine). A sample of the pentamer was subject to mild deprotection (conc. ammonia, at RT overnight) and the standard purification (with Nensorb cartridge). Reversed phase HPLC analysis of the resultant oligomer showed a single peak (**Fig. 3a**) and the correct nucleoside composition was confirmed by HPLC analysis of the enzymatic digest. The remaining pentamer was treated as follows.

1) oxidation-conversion prior to deprotection



X: base bearing a readily oxidizable group; X*: base bearing an oxidized group;
X^R : base with an introduced functional group on it; ● : Support.

The starting pentamer, CGXAT (X: 6-methylthiopurine), still fully protected with the DMT group on 5'-terminal and attached to the CPG support at 3'-terminal (**Scheme 3A**), was treated with 1% MCPBA in CH_3CN for 10 min for oxidation of the methylthio group, followed by washing with 1% Et_3N in CH_3CN and with CH_3CN. The CPG support bearing the oxidized pentamer was then treated with a nucleophilic reagent (either 40% aqueous CH_3NH_2 solution or conc. NH_3 or 0.5 M aqueous NaOH solution) for 2 days at RT followed by standard purification.

Reversed phase HPLC (**Fig. 3**) showed that in each case this protocol produced one predominant peak, which was confirmed to be the desired oligomer by nucleoside composition analysis. These experiments also

demonstrated that the DMT group was stable[14] under the conditions used for oxidation with MCPBA therefore the DMT could be used to advantage for subsequent purification, and that the oxidation is highly selective for the methylthio group since in all cases only one major peak was produced. The above-described approach - oxidation-conversion prior to deprotection would be best suited to the preparation of DNA containing modified bases which are sufficiently stable towards the strong nucleophiles used for deprotection of the oligomer.

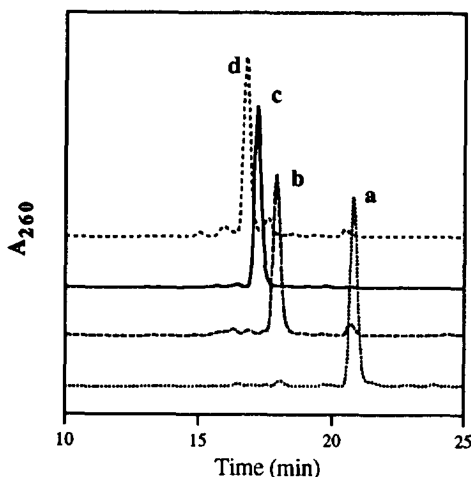


Fig. 3 HPLC profiles of the 6-methylthiopurine pentamer (CGXAT) and its transformed products. a) before the oxidation and substitution (X: 6-methylthiopurine); b) after the oxidation followed by substitution with 0.5 M aqueous NaOH (X: hypoxanthine); c) after the oxidation followed by substitution with aqueous methylamine (X: N^6-methyladenine); d) after the oxidation followed by substitution with concentrated aqueous ammonia (X: adenine). Conditions: Waters Nova-Pak C_{18} cartridge, flow rate 1.0 mL/min; Gradient: (0 to 30 min) 0-11% acetonitrile in 50 mM aqueous KH_2PO_4 (pH 6.2).

2. Oxidation-conversion after full deprotection

This approach is schematically shown in **Scheme 3B**. The starting material, CGXAT (X: 6-methylthiopurine), was fully deprotected (by conc. ammonia) and its chromatogram is shown in **Fig. 4** (the bottom line). A milder oxidizing reagent MMPP was used instead of MCPBA[15] to oxidize 6-methylthiopurine in oligomers, and the oxidized oligomers were then substituted with various nucleophiles. As a model study, methylamine was used as the substituting agent and the reaction course was monitored by HPLC (**Fig. 4**). The oxidation was almost completed within 1 hr (**Fig. 4**, the top line) and the substitution with methylamine was very rapid and completed within 10 min. This suggested that the oxidative form(s) of methylthio group even at the oligomer level was very reactive towards nucleophilic methylamine and that without nucleophilic agent, such as methylamine, the oxidized oligomer was stable and could be isolated by HPLC (cf. Peak b in **Fig. 5**). Also tested were its conversion with other nucleophiles, for instance azide and sulphide.[16] It was found that both pentamers containing 6-thiopurine and 6-azidopurine could be effectively produced and separated from the pentamer containing oxidized form of 6-methylthiopurine (**Fig. 5**). Therefore this method of oxidation-conversion after deprotection and purification would provide a useful approach to the preparation of oligomers containing labile or reactive bases since no further step is required before application.

Specific Methylation of thiopurine residues in nucleosides and in oligomers

During the preparation of **4**, it was noted that methyl iodide (CH_3I) reacted with thio group of **3** nearly instantly and quantitatively in alkaline aqueous solution,[17] therefore the selectivity of this reaction was further investigated under other conditions. It was found that both 2'-deoxyribo-6-thioinosine and 2'-deoxyribo-6-thioguanosine could be methylated selectively by CH_3I very rapidly in conc. ammonia and slowly in aqueous

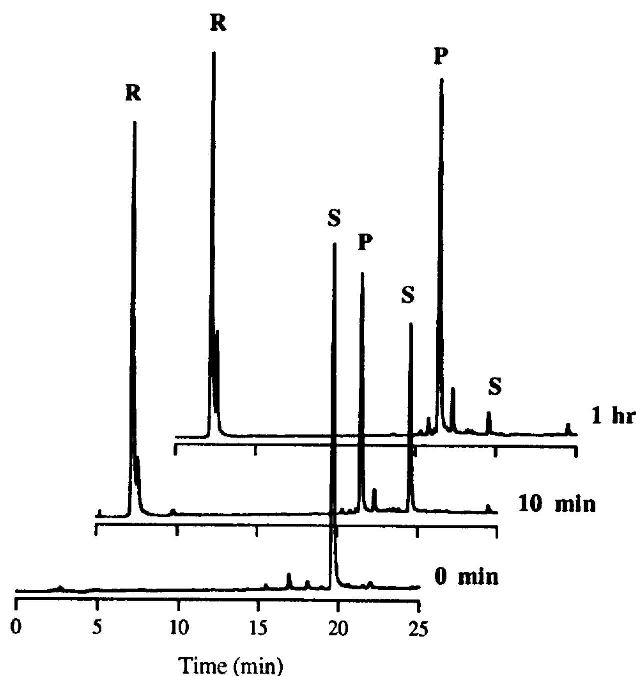


Fig.4 The reaction course of oxidation and conversion of 6-methylthiopurine pentamer (CGXAT) monitored by HPLC at 260 nm.
The modified pentamer (about 1 O.D) in 0. 1 M phosphate (pH 6.3) was treated with 100 μL of MMPP (3.56×10^{-3} M) for a given time (0 min, 10 min and 1 hr) at RT, then a small volume (20 μL) of the reaction solution was mixed with 5 μL of 40% aqueous methylamine for 10 min. Then the reaction solution was chromatographed under the conditions as described at the legend to Fig. 3.

Peak **S**: 6-methylthiopurine pentamer
Peak **P**: N6-methyladenine pentamer
Peak **R**: Reagent (MMPP)

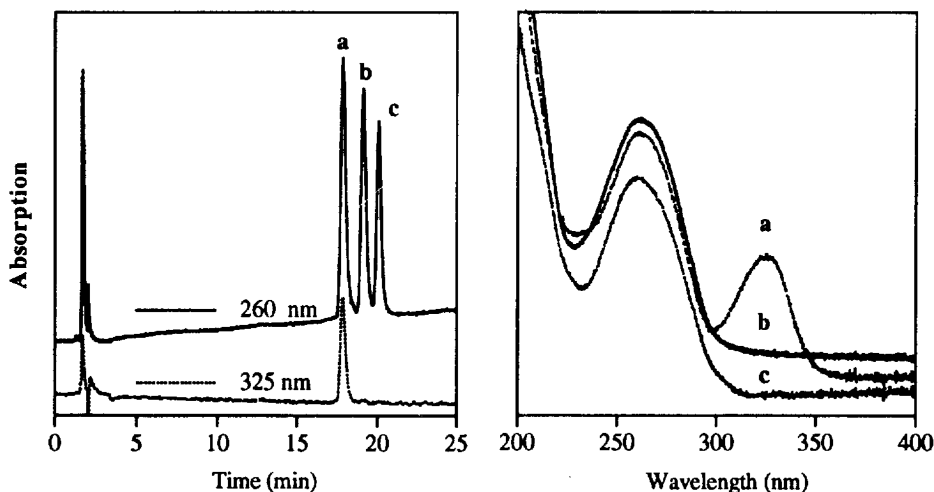


Fig. 5 Conversion into 6-substituted purines in the pentamer. A fully deprotected pentamer (CGXAT, X: 6-methylthiopurine) was oxidized with MMPP and its product then purified with HPLC. The isolated oligomer in 0.1 M KH_2PO_4 (pH 6.3) (Peak **b**, Rt=19 min) was reacted either with 0.1 M aqueous Na_2S (producing 6-thiopurine pentamer, Peak **a**, Rt=17.5) or with 0.1 M aqueous NaN_3 (producing putative 6-azidopurine oligomer, Peak **c**, Rt=20 min)[16]. The left part shows the chromatographic properties of the three pentamers (cf the Legend to Fig. 3 for HPLC conditions). The right part shows their UV spectra (Line **a** for 6-thiopurine pentamer, Line **b** for the oxidized pentamer and Line **c** for the putative 6-azidopurine pentamer).

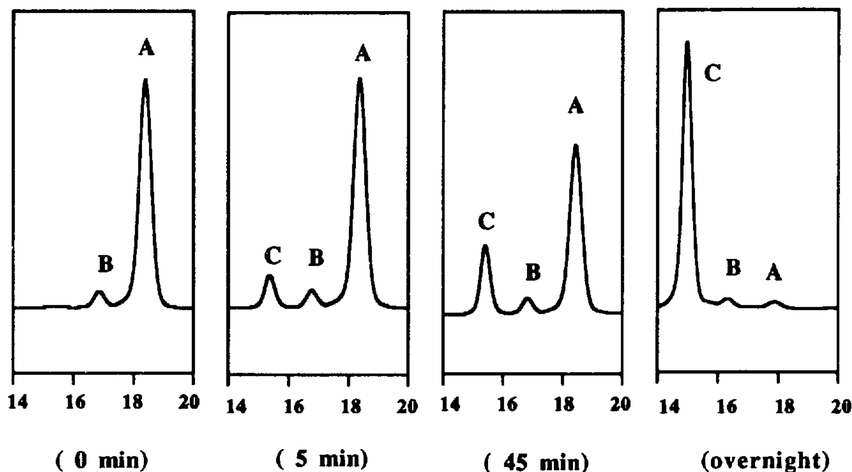
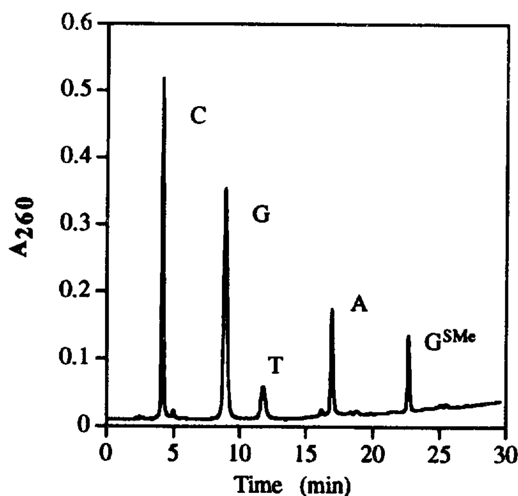


Fig. 6 The oligomers (1.5 OD) were dissolved in 0.05 M phosphate buffer (pH 8.5), to which 5 μL of 10% CH_3I / CH_3CN was added, then the methylation was followed by FPLC at the UV wavelength of 260 nm. The 6-thiopurine oligomer (peak **A**) was gradually converted to 6-methylthiopurine oligomer (peak **C**). Peak **A** : CGC XAG CTC GCG (X: 6-thiopurine), Peak **B** : CGC XAG CTC GCG (X: 6-hypoxanthine), Peak **C** : CGC XAG CTC GCG (X: 6-methylthiopurine). [For FPLC condition, see Fig 2 and Ref 6]



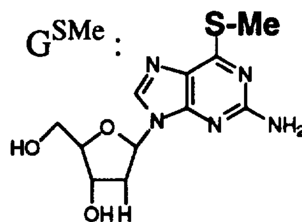


Fig. 7 The reversed phase HPLC profile of the nucleoside composition analysis of the 12 mer CGC XAG CTC GCG (X: 6-methylthioguanine). Conditions: Waters Nova-Pak C18 cartridge, 1 mL/min. The column was eluted for 10 min with 3.3% acetonitrile in 50 mM KH_2PO_4 (pH 6.3), then acetonitrile was increased to 20% over 10 min, then acetonitrile was increased at 26.6% over another 10 min.

phosphate solution (pH 8.5),[18] while under the same conditions their 6-oxy analogues remained unchanged. The selective reaction also took place with 6-thiopurine and 6-thioguanine residues in oligomers.[19] The reaction course of direct methylation of 6-thiopurine in a synthetic dodecamer was monitored by anion exchange chromatography and shown in Fig. 6. The modified oligomers prepared by this selective methylation were indistinguishable from the oligomer prepared by incorporation of 6-methylthiopurine monomer described above. The same results were also obtained for direct methylation of 6-thioguanine in oligomers. The formation of 6-methylthioguanine was confirmed by nucleoside composition analysis of the product (Fig. 7).

Site-specific ^{35}S-labelling of 6-thioguanine in oligodeoxynucleotide

As an example to demonstrate the advantages of the method described above, a 21 mer (see the experimental part and Note 20) containing 6-methylthioguanine was prepared from its 6-thioguanine oligomer by the direct methylation with methyl iodide. The resultant oligomer was oxidized and treated with Na_2S (from aldrich) and radioactive [^{35}S]-Na_2S (from Amersham). Both treatments resulted in the formation of the oligomer containing 6-thioguanine (Fig. 8 left part). The treatment with radioactive Na_2S produced the oligomer having ^{35}S-radioactivity (Fig. 8 right part) and nucleoside composition analysis of the radioactive oligomer showed that the 6-thio-2'-deoxyguanosine was ^{35}S-labelled.

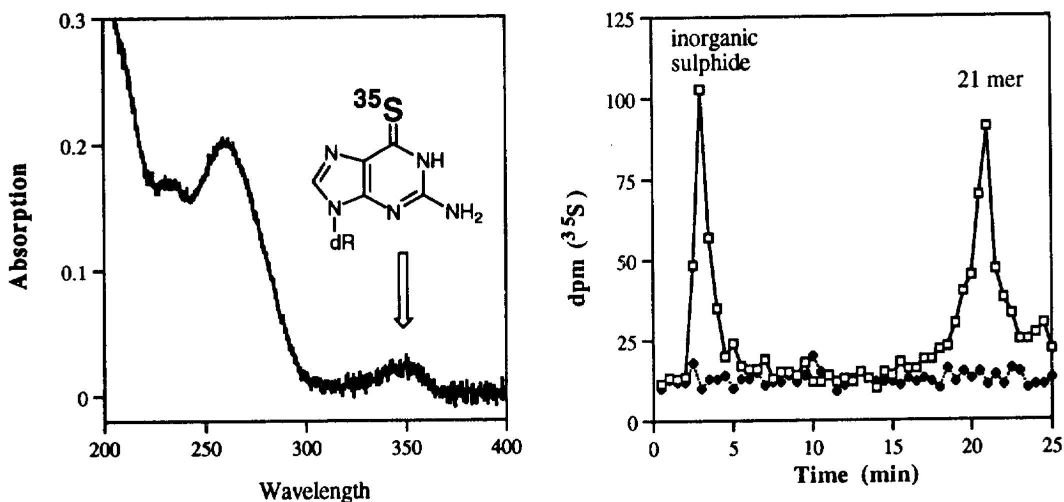


Fig. 8: Site-specific ^{35}S-labelling of an oligodeoxynucleotide. The left part is the UV spectrum of the transformed 21 mer obtained by the oxidation of the 6-methylthioguanine 21 mer followed by its conversion with [^{35}S]-Na_2S solution. The peak at the wavelength of 345 nm is characteristic of 6-thioguanine. The right part shows FPLC profiles of the transformed 21 mers from respective treatment with Na_2S (dark spot) and [^{35}S]-Na_2S (open square) detected by their radioactivity. For FPLC conditions, see Fig. 2 and Ref 6.

Discussion

In this report, we describe two methods for the preparation of oligodeoxynucleotides containing 6-methylthio purine residues. The first method is to synthesize the phosphoramidite of 6-methylthiopurine nucleoside, then incorporate it into oligomers with an automated synthesizer. The second one is to prepare

oligomers containing 6-thiopurine residues with our previously reported protocols,[2] and then directly methylate the thio-keto group of the modified purines in the synthetic oligomer with methyl iodide. The latter method has an additional advantage: the 6-thioguanine phosphoramidite[2a] is commercially available (Glen Research, USA). In addition, this S-alkylation could introduce other functional groups and this chemical property would be of great potential for selective functionization of oligonucleotides. In a previous report[21] 4-thiouracil in a pentamer of homopyrimidine was directly methylated with CH_3I, however it was not tested whether other bases (G, A, C) in DNA would be methylated in addition to the 4-thio-keto functionality. The results in this report demonstrate the selective methylation of the thio-keto group in synthetic oligomers containing 6-thiopurine (or 6-thioguanine) without methylation of the four normal bases.

6-Methylthiopurine residues are stable under normal conditions (such as in presence of weak acids or bases or nucleophiles), but the methylthio group can be oxidized selectively to form methylsulphinyl and methylsulphonyl groups. These oxidized forms of the methylthio group on purines are reactive and replaceable by various nucleophiles at the nucleoside level[7] as well as at the oligonucleotide level.[10] In the work presented here the emphasis was placed on detailed studies of the oxidation of the methylthio purine in synthetic oligomers and of conversion of the oxidized products prior to and after deprotection and purification.

In the case of fully protected oligomers still attached to CPG support, the methylthio group was oxidized with MCPBA for a short time (5-10 min) at RT, and then replaced with strong and basic nucleophiles, which also acted as the agent to cleave the oligomers from CPG support and to remove all protecting groups. This protocol efficiently produced desired DNAs containing modified bases in good yield and satisfactory quality and since it avoided forming unwanted thiopurine analogue by the attacking route β (**Fig. 1**). By this new approach (with ^{15}N-ammonium hydroxide) we have successfully prepared a 20 mer containing site-specifically ^{15}N-labelled adenine, confirmed by high resolution NMR spectroscopy.[24]

Although the chemicals (0.5 M aqueous NaOH, or 40% aqueous methylamine or conc. aqueous ammonia) used in the demonstrations (**Fig. 3**) are of high nucleophilicity and strong basicity and served as the agents for the substitution, cleavage and deprotection in one pot, it is also possible to separately carry out these three reactions by use of one reagent for the substitution and use of others for the cleavage and deprotection, as the substitution of the leaving group (the oxidized form(s) of methylthio group) on purine residues is quicker than the deprotection. By such an approach, it could allow one to introduce functional groups which derive from chemicals of less nucleophilicity and basicity (eg. alcohol), followed by use of a deprotecting agent such as conc. ammonia or diluted NaOH to get desired DNA provided that the formed substituted purine in oligomers is stable under the conditions where the deprotecting agent is used.

For the introduction of labile groups onto purine residues in oligomers, an alternative method was developed. Fully deprotected and purified oligodeoxynucleotides containing 6-methylthiopurine residues were first selectively oxidized, then substituted with a suitable chemical as the donor of chosen functionality. This approach was exemplified by the preparation of pentamers containing 6-azido-adenine and 6-thiopurine. Since the conversion is done at the last step, it is also feasible to introduce other reactive or labile functionalities. Furthermore it would be ideal for the conversion to be done just prior to biological applications. This approach has been applied to site-specific introduction of radioactive ^{35}S onto the 6-position of predetermined purine residues in oligomers. It has been reported that thiopurines in oligonucleotides can be photo-crosslinked with proteins[22] or RNA.[23] The specific introduction of radioactive ^{35}S atom in DNA can provide a useful tag for

monitoring at the molecular level the course of the interaction of DNA with other macromolecules such as proteins. If upon photo irradiation the ^{35}S atom becomes attached to an amino acid(s) of the protein, then the radioactivity from ^{35}S could help to locate the interaction or recognition sites between DNA and the protein. This could be of great potential in studies of DNA-protein interactions.

One of remaining concerns is the stability of the oxidized form(s) of the methylthio group on purine in a ready-to-use DNA. It has be found that they remained active in reaction with methylamine (CH_3NH_2) even after the intermediate (presumed to be the mono-oxidized form) had been kept in an aqueous buffer (pH 6.3) at minus 20°C over 2 months. This sufficient stability of the highly reactive groups provides a useful chemical property which allows one to conveniently carry out biological applications. Detailed investigation of the interaction of ^{35}S-labelled DNA with proteins is underway.

Conclusions

This article illustrates a simple and generalizable method for making oligodeoxynucleotides containing modified bases by using oxidation-conversion approach, in which the purine bearing a unique functionality (methylthio group) was incorporated into oligomers followed by normal deprotection and purification. After specific activation (oxidation), the newly formed functionality [the oxidized form(s) of the methylthio group] could be readily converted into desired functionalities under mild conditions. This approach has allowed one to introduce isotopic atoms (^{15}N and ^{35}S) and chemically reactive agents (azido group) onto the 6-position of the pre-determined purine residue, and the resultant base-modified DNA could be of many uses in DNA-related studies. It could be envisaged that similar approaches could be developed for the introduction of designed functionalities (such as carcinogens to form DNA-adducts) onto the 2-position and the 8-position of guanine in DNA.

ACKNOWLEDGEMENT

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EXPERIMENTAL

Chemicals and General Methods

Syntheses of oligomers were carried out by ABI 391 DNA synthesizer (Applied Biosystems), using Millipore's Expedite monomers and supports in which amino groups of the bases are protected with t-butylphenoxyacetyl group. All other chemicals were from either Aldrich or Sigma and used directly without further purification unless stated otherwise. General methods such as purification with Nensorb Prep cartridges (Du Pont) or Fast protein liquid chromatography (FPLC) on a Mono Q HR 5/5 column (Pharmacia) and nucleoside composition analysis by reversed phase HPLC were carried out as described before.[2]

Synthesis of 5'-O-(4,4'-dimethoxytriphenylmethyl)-2'-deoxy-6-methylthio-purine-3'-O-(2-cyanoethyl-N,N-diisopropylamino)-phosphoramidite (6)

Compound 3 (2.82 g, 8 mmoles), prepared by acylation of 2'-deoxyinosine with acetic anhydride followed by transformation into 6-thio analogue as described before,[2b] was dissolved in 25 mL of 1 N NaOH under stirring at RT. After 30 min, TLC (CH_3OH / $CHCl_3$, 10/90, v/v) showed that the starting material (Rf: 0.65) was converted into a new spot with very low Rf. Then 800 μL (12.8 mmoles) of methyl iodide was added to the

reaction solution. After vigorous stirring for 30 min, TLC showed that the intermediate was changed to a new spot (Rf: 0.5). The solution was extracted with 8 x 40 mL of ethyl acetate and the combined organic layers dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a colourless powder, which was crystallized from acetone to give pure compound **4** in 85% yield. ^{1}H NMR data (in DMSO-d_6): 2.34-2.75 (2H, m, 2'-H and 2"-H), 2.66 (3H, s, SCH_3), 3.52-3.59 (2H, m, 5'-H), 3.88 (1H, m, 4'-H), 4.42 (1H, m, 3'-H), 4.99 (1H, t, 5'-OH, ex), 5.36 (1H,d, 3'-OH, ex), 6.42 (1H, t, 1'-H), 8.65 (1H, s, 8-H) and 8.73 (1H, s, 2-H). UV λmax: 284 nm. Elementary analysis: $C_{11}H_{14}N_4O_3S$, Calc. C: 46.80, H: 4.96, N: 19.86. Found C: 47.05, H: 5.01, N: 19.86. Then compound **4** was tritylated at the 5'-OH position to give compound **5** in yield of 63%. ^{1}H NMR data (in DMSO-d_6): 2.46-2.95 (2H, m, 2'-H and 2"-H), 2.66 (3H, S,-SCH_3), 3.16 (2H, m, 5'-H), 3.71 and 3.72 (6H, s, CH_3O of DMT), 4.01 (1H, m, 4'-H), 4.51 (1H, m, 3'-H), 5.37 (1H, d, 3'-OH, ex), 6.45 (1H, t, 1'-H), 6.75-7.30 (13H, m, aromatic-H of DMT), 8.55 (1H, s, 8-H), and 8.65 (1H, s, 2-H). The resulting **5** was then converted into compound **6** in yield of 60%. ^{31}P NMR data (in $CDCl_3$): 145.50 and 145.33.

DNA Synthesis, Conversion and Purification

General methods for the automated synthesis of modified oligomers were employed as before,[2] except that the monomer of 6-methylthiopurine deoxyribonucleoside (**6**) was added manually and its coupling time was 3 min.

Oxidation and conversion prior to deprotection-- A typical protocol is as follows:

After DNA synthesis, the cartridge (on the synthesizer) containing a synthesized and fully protected oligomer (CGXAT, X: 6-methylthiopurine) with the DMT group on 5'-terminal and the CPG support at 3'-terminal was treated with 10 mL 1% MCPBA in CH_3CN by manual injection for 5 min, then washed with 10 mL of 1% Et_3N in CH_3CN and with 2 x 10 mL of CH_3CN. After the cartridge had been disconnected from the synthesizer and dried to remove remaining CH_3CN, the CPG support was divided into four parts. Each of them was placed into an Eppendorf tube and treated with 1 mL of one of three nucleophilic reagents (40% aqueous CH_3NH_2; conc. NH_3; 0.5 M aqueous NaOH) for 2 days at RT. These nucleophiles replaced the oxidized form(s) of methylthio group on the 6-position of the purine as well as deprotected the oligomer. The resultant oligomers were purified by Nensorb Prep cartridge.

Oxidation and conversion after full deprotection-- A typical protocol is as follows:

A synthetic oligomer containing 6-methylthiopurine (CGXAT, X: 6-methylthiopurine) was deprotected with conc. ammonia for 2 days at RT and purified with Nensorb Prep cartridge. The deprotected and purified oligomer was completely dried, then treated with 100 μL of freshly prepared aqueous magnesium monoperoxyphthalate (MMPP) solution (3.56×10^{-3} M) for 2.5 hr. The solution was desalted with Sep-Pak cartridge or NAP-10 cartridge. Fractions containing oligomers were located with UV measurements, freeze-dried and stored at -20°C until use. For conversion, the oxidized and desalted oligomer was reacted with appropriate reagents (cf: Note 16).

Site-specific ^{35}S labelling of oligodeoxyribonucleotide :

1.4 Unit (A_{260}) of 21-mer containing 6-methylthioguanine (TCT ATC ACC GCA AGX GAT AAA, X: 6-methylthioguanine)[20] in 100 μL of water was treated with 200 μL of the MMPP solution for 2.5 hr at RT as described above. The oxidized oligomer was purified by using NAP-10 and 1.2 Unit of the oligomer recovered and freeze-dried. 0.2 Unit of the oxidized oligomer was redissolved in 200 μL of 0.3 M phosphate buffer (pH 7.7) and incubated with 10 μL of aqueous [^{35}S]-Na_2S store solution [made from 100 μCi of [^{35}S]-Na_2S (from Amersham) in 1 ml of water] for 20 hr at RT. The mixture was applied onto NAP-10 cartridge, eluted with water and collected in 0.5 mL / tube. The oligomer was found in fractions 2 and 3.

REFERENCES AND NOTES

§ A preliminary account of part of this work was presented as a poster at 11th International round table

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12. 6-Methylsulphonyl purine ribonucleosides have been prepared using chlorine as an oxidizing agent and the sulphonyl group was found to be very reactive and displaceable by various nucleophiles.[7] On the other hand, 6-methylthiopurine-2'-methyl-ribonucleoside could be oxidized with an equimolar quantity of magnesium monoperoxyphthalate (MMPP) to form 6-methylsulphinyl derivative, which was reactive towards pyridine, but when it was further oxidized with excess of MMPP to form a compound which, the authors[10b] assumed to be 6-methylsulphonyl derivative, was inactive towards pyridine. The marked difference in these reports[7,10b] could not be explained just by their chemical structural difference - the only difference being the presence of 2'-methyl group in the latter case.[10b] In the case of deoxynucleosides, 6-ethylsulphinyl purine derivative, but not its sulphonyl derivative, has been prepared and found to be replaceable.[8] In another paper[9] the methylthio group at the 6-position of purine in a synthetic oligothymidylate was oxidized and *in situ* converted into N^6, N^6-ethanoadenine. Although the methylthio group was assumed to be converted into its sulphonyl group, however no direct evidence was provided.
13. It has been noted that 8-H of **4C** gave two well-split peaks while 2-H of **4C** gave only a single peak. This difference may be related to the orientation of the chiral methylsulphinyl group. Further investigation is underway. The chemical shift for the S-linked methyl was changed from 2.66 ppm ($-SCH_3$ of **4B**) to 3.06 ppm ($O{=}S{-}CH_3$ of **4C**), and the NMR peak at 3.37 ppm was due to trace water present in the DMSO-d_6. However it has also been noted that a paper[8] reported that the NMR signal at 3.37 ppm assigned for the S-linked methylene in the ethylthio derivative ($-SCH_2CH_3$) was also assigned for the methylene (3.37 and 3.38 ppm) in the ethylsulphinyl derivative ($O{=}S{-}CH_2CH_3$).
14. Other lines of evidence are: (i) the solution of 5'-DMT-thymidine in 1% MCPBA in CH_3CN did not show any detectable amount of detritylated thymidine within 30 min at RT; (ii) the isolated yield of the substituted pentamer (e.g. CGXAT, X: N^6-methyladenine) obtained with this protocol of the oxidation (1% MCPBA in CH_3CN) and conversion (40% aqueous CH_3NH_2) is virtually the same as that of the non-substituted pentamer (i.e. CGXAT, X: 6-methylthiopurine) obtained by direct

purification without the oxidation and conversion.

15. At an earlier stage, the pentamer containing 6-methylthiopurine was dissolved in an aqueous buffer (1 M phosphate, pH 6.3) and treated with 1% MCPBA in CH_3CN for 5 min at room temperature. The unreacted MCPBA was removed by extraction with diethyl ether. Methylamine was then added to the aqueous solution for conversion. HPLC analysis showed that only one third of the pentamer was converted into the desired pentamer (containing N^6-methyladenine). A prolonged substitution with methylamine did not increase the yield of the product. When the oxidizing time was increased from 5 min to 1 hr, most of the starting pentamer was transformed into the desired pentamer. It was unexpected that the oxidation of the fully deprotected pentamer was slower than that of the pentamer undeprotected and attached to the CPG support. However, it was reasoned that the slower oxidation might be due to the poor solubility of MCPBA in the aqueous buffer. As MCPBA is a rather strong oxidizing agent and could cause adverse effects on the oligomer in a prolonged treatment, therefore a mild oxidizing reagent MMPP was used instead of MCPBA for oxidation of oligomers in the aqueous solution.

16. A fully deprotected pentamer containing 6-methylthiopurine, after oxidized with MMPP, desalted and dried, was dissolved in 0.1 M potassium dihydrogen phosphate (pH 6.3). The MMPP-treated oligomer solution was mixed with 0.1 M aqueous sodium sulphide (pH 12) at RT. After 1 hr, HPLC showed that the retention time of the oligomer has changed from 19 min (Peak **b** in **Fig. 5**) to 17.5 min (Peak **a**) and it could also be detected at the wavelength of 325 nm. When the MMPP-treated oligomer solution was mixed with 0.1 M aqueous sodium azide, HPLC showed that the new peak (Rt = 20 min, Peak **c** in **Fig. 5**) eluted later than the starting oligomer (Rt=19 min) and the hydrolysed product (i.e. the pentamer containing hypoxanthine, Rt=18 min). These observations suggest that the later-eluting (Peak **c**) is very likely to be the pentamer containing 6-azido-purine.

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18. The preparation of 2'-deoxy-6-methylthioguanosine was carried our as described below. 180 mg of 2'-deoxy-6-thioguanosine was dissolved in 2 mL of conc. ammonia at RT, to which 47.5 μL of methyl iodide was added. The solution was stirred vigorously for 30 min, by which time most of the starting material (Rf: 0.3 in 20% CH_3OH / $CHCl_3$) was converted into a new spot (Rf: 0.7). Then additional 10 μL of methyl iodide was added. After 10 min, the solution was evaporated to a small volume, co-evaporated with ethanol. The residue was dissolved in 5 mL of hot acetone. Crystalline product was obtained (90 mg). ^{1}H NMR (in DMSO-d_6): 2.22-2.59 (2H, m, 2'-H and 2"-H), 2.56 (3H, s, SCH_3), 3.48-3.58 (2H, m, 5'-H), 3.81 (1H, m, 4'-H), 4.34 (1H, m, 3'-H), 6.20 (1H, t, 1'-H), 6.50 (2H, br, 2-NH_2, ex) and 8.14 (1H, s, 8-H). UV λmax=315 nm, λmin=274 nm. The preparation of 2'-deoxy-6-methylthiopurine nucleoside (**4**) has been described in the experimental section.

19. 1 OD of a fully deprotected and purified oligomer containing 6-thiopurine (or 6-thioguanine) was dissolved in 100 μL of 0.05 M phosphate buffer (pH 8.5). 20 μL of 5% CH_3I / CH_3CN (v/v) was added and vigorously mixed for 5 min. The solution was left overnight and the desired oligomer was isolated using a Sep-Pac cartridge (Waters).

20. The DNA duplex consisting of this sequence has been shown to have a higher yield of crosslinkage with Cro-protein [Zheng, Q. Ph.D thesis (University of London) **1995**].

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