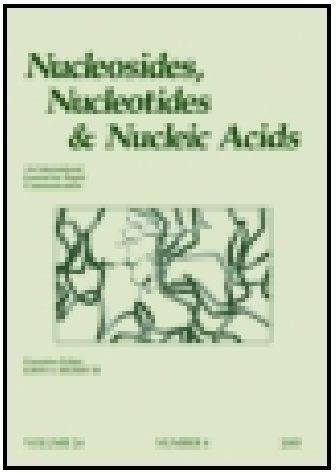

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Preparation of Oligodeoxynucleotides Containing 6-Methylthiopurine Residues by Chemical Synthesis or Specific Methylation

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PREPARATION OF OLIGODEOXYNUCLEOTIDES CONTAINING 6-METHYLTHIOPURINE RESIDUES BY CHEMICAL SYNTHESIS OR SPECIFIC METHYLATION

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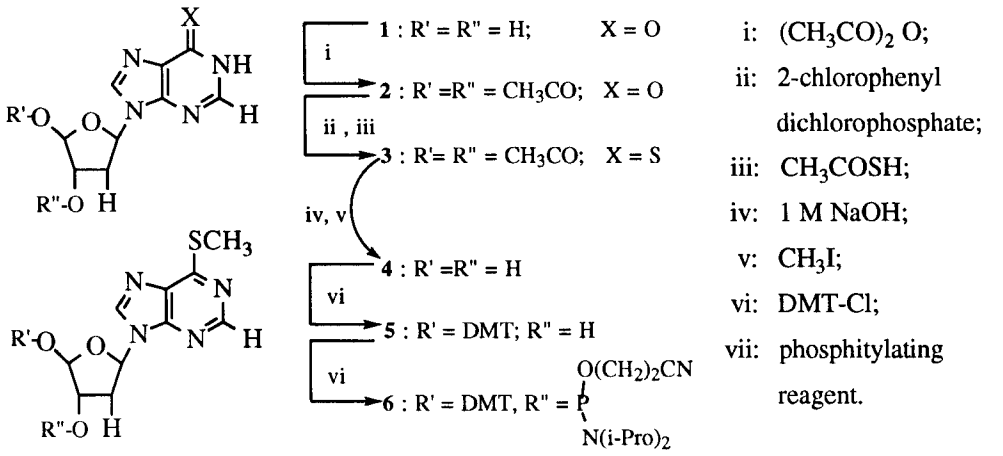
Abstract: Two methods (chemical synthesis and specific methylation) are described for the preparation of oligodeoxynucleotides containing 6-methylthiopurine residues. 6-Methylthiopurine phosphoramidite (**6**) is prepared and incorporated into oligomers. Methylation with methyl iodide of 6-thiopurine (or 6-thioguanine) in oligonucleotides also leads to exclusive production of 6-methylthiopurine (or 6-methylthioguanine) oligomers.

Incorporation into DNA of reactive nucleosides to which other groups can be attached after synthesis has considerable practical importance and several methods have been published. Previously, we reported methods for synthesis of versatile monomers— 6-(2,4-dinitrophenyl)thiopurine and 6-(2,4-dinitrophenyl)thioguanine phosphoramidites[1,2] and their incorporation into oligodeoxynucleotides. These modified purines in oligomers can be transformed by treatment with nucleophiles to the corresponding 6-substituted purines. However the formation of 6-substituted purines by this approach is sometimes accompanied by the formation of a small amount of 6-thiopurine resulting from the attack on the S—nitrophenyl bond rather than the S-C6 bond. One possible way to avoid this would be to oxidize the sulphur of a 6-alkylthio function to the sulphoxide or sulphone which are highly reactive leaving groups[3,4,5]. Nucleophiles would be expected to react with these oxidized forms exclusively at the S-C6 bond. Towards this end we wanted to prepare DNA containing 6-methylthiopurine and 6-methylthioguanine. Here we report the synthesis and incorporation into DNA of 6-methylthiopurine phosphoramidite and show that the same final product (and also 6-methylthioguanine) can be produced in DNA by the facile methylation of preexisting 6-thiopurine or 6-thioguanine residues incorporated into DNA by our previously reported methods[1,2]. The methylation of 4-thiouracil residue in the oligomer TTthiodUTT has been reported[6].



Scheme 1

Chemical Synthesis

Our synthetic route to 6-methylthiopurine phosphoramidite is shown in Scheme 1.

2'-Deoxyinosine (**1**) was acetylated to give 3',5'-diacetyl-2'-deoxyinosine (**2**), which was in turn transformed into its 6-thio analogue (**3**)[2,7]. The product was then deacetylated and its thio group methylated in one pot to give 2'-deoxy-6-methylthioinosine (**4**)[8]. All these reactions produced the desired compounds in excellent yields (70 % over-all yield from **1**). **4** was tritylated, then converted into its phosphoramidite monomer (**6**)[9] with standard procedures[10] without any problem. Since the sulphur can be rapidly oxidized[3-5], we tested the susceptibility of the methylthio group towards the conditions for DNA assembly and deprotection before the incorporation of the monomer (**6**) into DNA. It was found that 6-methylthiopurine nucleoside (**4**) was completely intact in the oxidative solution (I_2 / H_2O / pyridine / THF) at 25°C, even after 24 hours. On the other hand, **4** was not very stable in conc. ammonia at 55°C and about half of the nucleoside was damaged after overnight incubation, however, at reduced temperature of 25°C, no damage to **4** was found. Therefore DNA containing 6-methylthiopurine was synthesized by automated synthesizer without altering normal procedures except using phosphoramidites of the unmodified bases with labile protecting groups (Expedites, Millipore). As an example, a 12 mer CGC XAG CTC GCG (where X is 6-methylthiopurine) was synthesized. Ion exchange chromatography[11] showed a single peak of the desired oligomer (Fig. 1). The presence of 6-methylthiopurine in the modified oligomer was confirmed by nucleoside composition analysis.

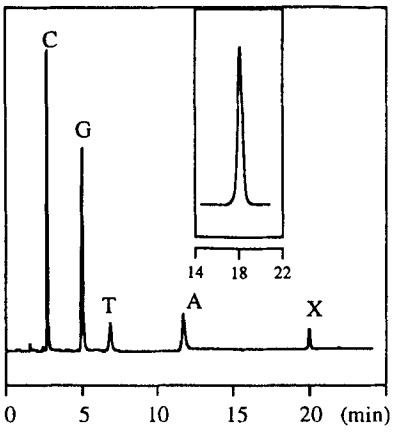
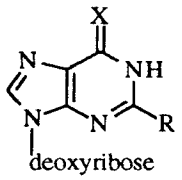


Fig. 1 The reversed phase HPLC profile of the nucleosides from enzymatic digestion of a synthetic 12 mer CGC XAG CTC GCG (X= 6-methylthiopurine). Conditions: Waters Nova-Pak C18 cartridge, 1.5 mL/min, 260 nm. The column was eluted for 8 min with 2.33% acetonitrile in 50 mM KH_2PO_4 (pH 6.2), then acetonitrile was increased to 16.7% over 12 min, then acetonitrile was kept at 16.7% for further 5 min. The figure inset is the ion exchange chromatographic profile of the modified 12 mer. Conditions: Pharmacia mono Q HR 5/5 column; 0.6m L/min, 260 nm.The column was eluted with A (0.4 M NaCl, pH 12) for first 2 min, the B (1.2 M NaCl, pH 12) was increased to 15% over 3 min, then to 35% for the following 20 min.

Specific Methylation

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[12]. This observation has prompted us to further investigate the selectivity of this reaction under other conditions. It has been found that both 2'-deoxyribo-6-thioinosine (**7**) and 2'-deoxyribo-6-thioguanosine (**8**) could be regio-specifically methylated by CH_3I very rapidly in conc. ammonia and slowly in phosphate aqueous solution (pH 8.5), while under the same conditions their 6-oxy analogues (**9** and



7: X = S, R = H
8: X = S, R = NH_2
9: X = O, R = H
10: X = O, R = NH_2

10) remained unchanged. The same specific reaction also took place with 6-thiopurine and 6-thioguanine residues in DNA[13]. The modified oligomers prepared by this specific methylation were indistinguishable from the oligomer prepared by the incorporation of 6-methylthiopurine monomer described above. Since the phosphoramidite used for the preparation of DNA containing 6-thiogunine is commercially available (Glen Research, USA), we believe that this S-alkylation will be of great potential for specific functionization of oligonucleotides. Furthermore, the easy oxidation of the alkylthio group may be suitable for introducing a variety of functionals into the 6-position of purines in DNA. Previous work[3-5] has shown that certain nucleophiles could replace the oxidized forms of the alkylthio groups. Such a replacement, combined with easy preparation of oligomer containing 6-methylthiopurines could allow one to prepare DNA containing purines onto which very reactive groups are mildly added just before use. This work is in progress.

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8. Compound **3** (2.82 g, 8 mmole) was dissolved in 25 ml of 1 N NaOH aqueous solution at RT. After 30 min, TLC (15% CH_3OH / $CHCl_3$) showed the deacylation was completed. Methyl iodide (800 µl,12.8 mmole) was slowly added and the solution was stirred vigorously for another 30 min. TLC showed that the intermediate had been converted into a new spot with a higher Rf. After work-up, a colourless powder was obtained, which was crystallized to give pure compound **4**. Data from NMR, UV and elementary analysis confirmed that **4** was a mono-methylated thiopurine nucleoside. The view that **4** is an S-methylated derivative rather than an N1-methylated was further supported by the fact that oxidation with 3-chloroperoxybenzoic acid and subsequent substitution with methylamine, aziridine or thiolacetic acid give corresponding 6-substituted purine deoxynucleosides.
9. Compounds **5** and **6** were confirmed by ^1H NMR and ^{31}P NMR.

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13. 1 OD of a fully deprotected and purified oligomer containing 6-thioinosine (or 6-thioguanine) was dissolved in 100 µl of 0.05 M phosphate buffer (pH 8.5). 20 µl of 10% 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).