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Synthesis of S^6-(2,4-Dinitrophenyl)-6-thioguanosine Phosphoramidite and Its Incorporation into Oligoribonucleotides

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Abstract—The preparation of N^2-phenoxylacetyl-S^6-(2,4-dinitrophenyl)-6-thioguanosine phosphoramidite and its subsequent incorporation into oligoribonucleotides is described. The identity of the oligonucleotides was confirmed by UV spectrophotometry and nucleoside composition analysis.

Oligonucleotides containing a thiol or a thione function on the base have been very useful tools in areas such as molecular biology and cancer research (for a latest review, see ref 1). They are also useful intermediates for the synthesis of other modified oligonucleotides by post-synthetic modification.[2] Photochemical cross-linking is a proven method for probing RNA-protein or RNA-RNA interaction at the atomic level as cross-linking can only take place between the molecules that are at the interface of these bio-molecules.[3] 4-Thiopyrimidine and 6-thiopurine nucleosides possess several desirable properties for such approach. The sulphur atom is only slightly larger than oxygen, but it otherwise chemically resembles oxygen, hence the introduction of these thionucleosides into oligonucleotides should not appreciably disturb the interaction between these molecules. Furthermore, these nucleosides are photoactive at long wavelength UV light (330–360 nm), which is well away from the usual absorption maxima of proteins (280 nm) and nucleic acids (260 nm), thus cross-linking can be carried out at a wavelength which there is no appreciable detrimental effect on proteins and nucleic acids. Finally, the cross-linking is site-specific as it can only occur between the photoactivable thiocarbonyl function and the target molecules. The photo-cross-linking approach using oligoribonucleotides bearing these thiobases has made it possible to probe the mechanism of hammerhead ribozymes[4] and to build the plausible three-dimensional models of biomolecules such as U small nuclear RNA (U snRNA),[5] rRNA[6] and hammerhead ribozymes.[7] In addition, it was reported that these photo-cross-linkable oligonucleotides were very useful probes for in situ hybridization assays[8] and for investigation of RNA-protein interactions.[9] The use of these oligomers for studying the mechanism of hammerhead ribozyme cleavage has also been documented.[10,11]

In our effort of developing methods for chemical synthesis of oligonucleotides containing thio-bases, we have reported the synthetic incorporation of 4-thiothymine,[12] 6-mercaptopurine[13] and 6-thioguanine[14] into DNA, and exploited the use of these oligonucleotides for studying DNA-protein interactions by photochemical cross-linking approach.[15] Recently, we reported a method for the introduction of structural diversity into the thiocarbonyl group of 6-thioguanine within support-bound, fully protected oligodeoxyribonucleotides via 'on-column' conjugation.[2a] As part of our continuing effort to develop methods for thio-modified oligonucleotide synthesis, we herein describe the synthesis of S^6-(2,4-dinitrophenyl)-6-thioguanosine phosphoramidite and its subsequent incorporation into oligoribonucleotides.[16] In comparison with oligodeoxyribonucleotides, less work has been reported with oligoribonucleotides containing thiobases, mainly because RNA synthesis is more difficult than DNA. To date there has been one report detailing the chemical incorporation of 6-thioguanosine into oligoribonucleotides, using a cyanoethyl group for the protection of the thiocarbonyl function.[11] The synthesis of the phosphoramidite involved eight synthetic procedures starting with guanosine. More recently, enzymatic ligation was applied to incorporate

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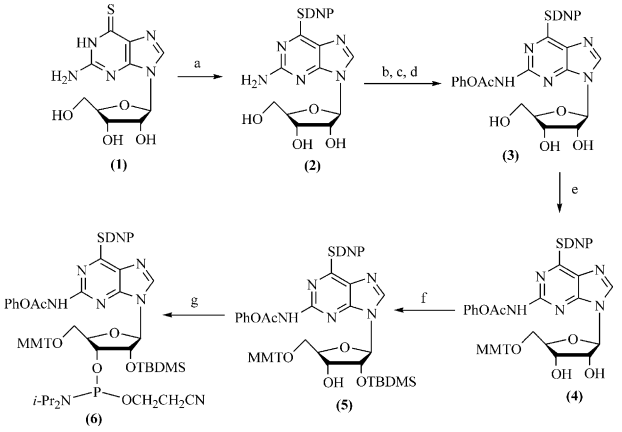
6-thioguanosine into oligoribonucleotides using its 3′,5′-bisphosphate derivative.[17]

Our synthetic route for the preparation of 6-thioguanosine phosphoramidite (**6**) is shown in Scheme 1. To simplify the synthetic route commercially available 6-thioguanosine (**1**) was chosen as the starting material. To protect the thiocarbonyl group **1** was reacted with 2,4-dinitrofluorobenzene in the presence of triethylamine to give compound **2**. Although **1** was not protected, the reaction was highly chemoselective, most likely due to the fact that the thio-function is more nucleophilic than the 2′-, 3′- and 5′-hydroxyl group and the 2-amino function of 6-thioguanosine. We[2a,13,14] have previously used the 2,4-dinitrophenyl group for the protection of the thiocarbonyl function of deoxy-thionucleosides. Various other protective groups have also been used for the thio-function in the automated solid-phase synthesis of both DNA and RNA,[1] examples including the pivaloyloxymethyl group[18,19] and the 2-cyanoethyl group.[10,11] Adams et al.[11] used the latter for the protection of the thio-function of 6-thioguanosine. Removal of the *S*-cyanoethyl group needed 5 h DBU treatment in acetonitrile, and thorough washing resin after deprotection to remove any trace of DBU was essential to prevent degradation of the thiocarbonyl group during desilylation.[11] We chose the 2,4-dinitrophenyl (DNP) group since previous studies[2a,13,14] indicated that DNP was stable during automated oligonucleotide synthesis and easily removable with mercaptoethanol under very mild alkaline conditions. More significantly, its removal did not cleave the oligomers from the resin and effect other protecting groups within the oligomers, which provided a means of carrying out postsynthetic modification on the thio-function 'on-column'.[2a]

Adams et al.[11] used the benzoyl group for the protection of the 2-amino group of 6-thioguanosine, which was previously used for the protection of the 2-amino function of 2′-deoxy-6-thioguanosine.[20] We chose the phenoxylacetyl for the protection of the 2-amino group of 6-thioguanosine, as removal of N^2-phenoxylacetyl in 2′-deoxyguanosine can be achieved under mild conditions.[21] It was introduced into the exocyclic amino group of **2,** using a one-pot, three-step transient protection protocol[22] yielding N^2-phenoxylacetyl-S^6-(2,4-dinitrophenyl)-6-thioguanosine (**3**). After tritylation by the standard method,[23] compound **4** was reacted with *tert*-butyldimethylsilyl chloride (TBDMS-Cl), producing compound **5** and its 3′-TBDMS isomer and 2′,3′-diTBDMS 6-thioguanosine derivative, which on purification by flash chromatography yielded pure compound **5**. Our initial attempt of protecting the 2′-OH using the triisopropylsilyl group following a recommended procedure[24] resulted in no success. Treatment of compound **5** with 2-cyanoethyl-*N,N*-diisopropyl phosphonamidic chloride for 12 h at room temperature[24] produced the desired phosphoramidite **6**. The structures of these compounds were confirmed by NMR and high resolution mass spectrometry.[25]



Scheme 1. The synthetic route for the thioguanosine phosphoramidite (**6**). Reaction conditions were as follows: (a) dinitrofluorobenzene/Et_3N; (b) $(CH_3)_3SiCl$/pyridine; (c) phenoxyacetyl chloride/pyridine; (d) NH_3H_2O; (e) MMTCl/pyridine; (f) *t*-butyldimethylsilyl chloride/$AgNO_3$/pyridine; (g) *i*-$Pr_2NP(Cl)OCH_2CH_2CN$/*i*-Pr_2Ne_t.

The stability of S^6-(2,4-dinitrophenyl)-thioguanosine phosphoramidite (**6**) to the reagents used in phosphoramidite chemistry had been tested before the monomer was used for RNA synthesis. Compound **3** was dissolved in dichloroacetic acid/dichloroethane (deblocking reagent); and compound **5** in acetic anhydride/lutidine/THF (capping reagent A), *N*-methylimidazole in THF (capping reagent B), and iodine in THF/pyridine/water (oxidation reagent). By monitoring the changes in these solutions with TLC, it was found that compound **5** was stable towards the reagents for at least 24 h at room temperature, and compound **3** was stable towards the deblocking solution for at least 3 h. It was also observed that, in agreement with our previous observations,[2a] the S^6-(2,4-dinitrophenyl) group could be readily removed within 30 min by 10% mercaptoethanol in CH_3CN containing 1% of Et_3N. These results indicated that monomer **6** would be stable during the synthesis, and the protecting group (2,4-dinitrophenyl) easily removable after synthesis.

The phosphoramidite **(6)** was incorporated into a 5-mer (5′-GCG^{SH} AU-3′) and a 12-mer (5′-UAC CAG^{SH} UGA GCU-3′) oligoribonucleotides, together with four natural nucleoside phosphoramidites (from Glen Research) protected with base-labile groups on the exocyclic amino functions (phenoxylacetyl for adenosine, *i*-propylphenoxyacetyl for guanosine and acetyl for cytidine). Standard automated procedures were used except the coupling time for **6** was extended to 30 min. Based on trityl assay, the overall yields of the 5-mer and the 12-mer were 92.7 and 71%, respectively (average stepwise yields 98.5 and 97.2%). Coupling of **6** was similar to the standard phosphoramidites. Deprotection of the 2,4-dinitrophenyl was achieved with 10% of mercaptoethanol in acetonitrile containing 1% of triethylamine, and the excessive deprotection reagent was removed and the resin-attached oligomers washed with acetonitrile by filtration. Cleavage from the support and removal of the protecting groups on the exocyclic amino functions and *O*-cyanoethyl groups in phosphate esters were accomplished with saturated ammonia solution in ethanol (freshly prepared) at 25 °C for 8 h. After

removal of the ammonia solution, the oligomers were treated with $Et_3N{\cdot}3HF$/DMSO (1:1)[11] to remove the 2′-*O*-TBDMS groups. Using tetrabutylammonium fluoride (TBAF) in THF, a standard procedure for removing the silyl group,[24] resulted in a product which showed multiple small peaks in HPLC analysis and loss of the identity peak with the thiol function (340–350 nm) in the UV spectrum. The 2′-*O*-TBDMS deprotected oligomers were then purified with Nen-sorb column (Fig. 1a). Further purification with HPLC resulted in pure oligoribonucleotides as a single peak (Fig. 1b). The UV spectrum of the HPLC purified 5-mer (5′-GCG^{SH} AU-3′) showed peaks at 260 and 346 nm (Fig. 2a). The peak at 346 nm is characteristic of the thiocarbonyl chromophore (Fig. 2b). Nucleoside composition analysis[26] of the purified oligoribonucleotides showed a nucleoside ratio consistent with that expected, as shown in Figure 3a for (5′-GCG^{SH} AU-3′). Peaks on the HPLC trace were identified by retention time comparison with the authentic samples. No adenosine was observed due to the deamination of adenosine by adenosine deaminase. This has also been previously observed by others.[24] The adenosine deaminase is a common contaminant in the enzyme preparations. When monitored at 345 nm only one peak was observed with the retention time corresponding to an authentic sample of 6-thioguanosine (Fig. 3b). The identity of the oligomers was also determined by ESI mass spectrometry.[27]

In conclusion, S^6-(2,4-dinitrophenyl)-6-thioguanosine phosphoramidite was prepared by a reliable and short synthetic procedure. Its incorporation into the oligonucleotides proceeded with good yield, and the integrity of these oligomers was fully confirmed by UV spectrometry, nucleoside composition analysis, and mass spectrometry. Use of easily removable protection groups for the thiol and 2-amino functions minimised the possible damage to the alkaline-labile thiol group during the postsynthetic procedures. It is worth pointing out that as the S^6-(2,4-dinitrophenyl) group in 6-thiodeoxyguanosine can be removed under mild conditions with-

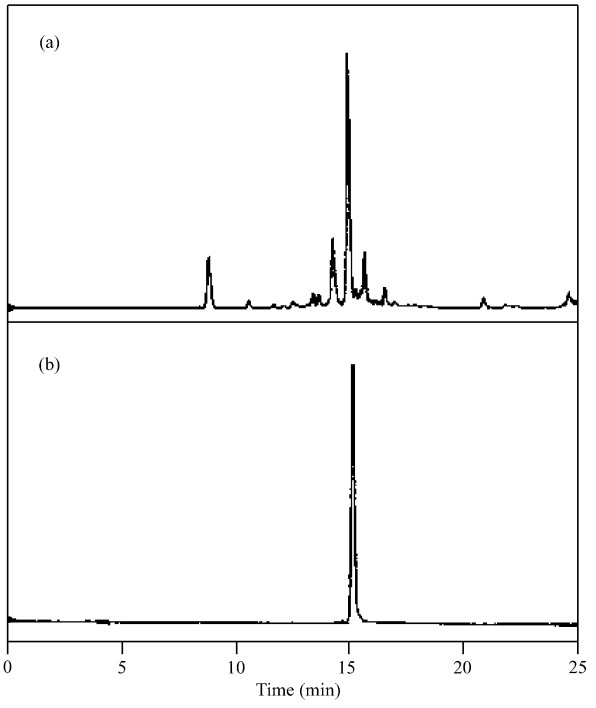


Figure 1. The reverse phase HPLC chromatograms of the oligoribonucleotide 5′-GCG^{SH} AU-3′. (a) After Nen-sorb column purification; (b) after further purification with HPLC. C18 columns were used with 0.1 M triethylammonia acetate, pH 6.5, and acetonitrile gradients of 2–20% over 25 min at a flow rate of 1 mL/min, recorded at 260 nm.

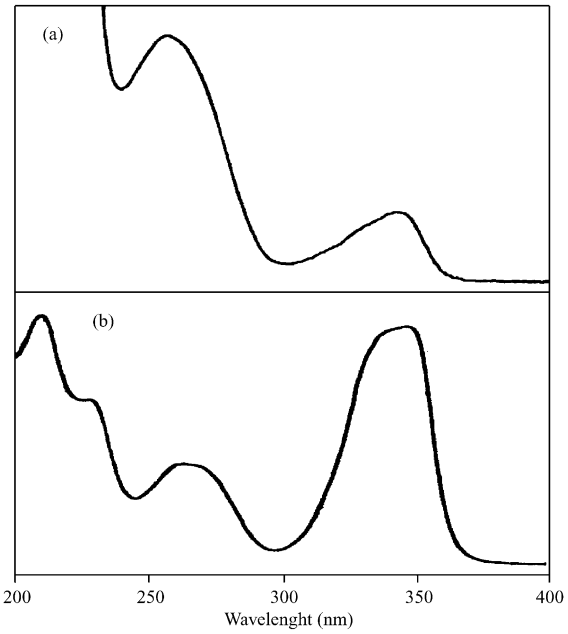


Figure 2. UV spectrum of the oligoribonucleotide 5′-GCG^{SH} AU-3′ showing peaks at (a) 260 and 346 nm; and (b) that of 6-thioguanosine showing a major peak at 343 nm.

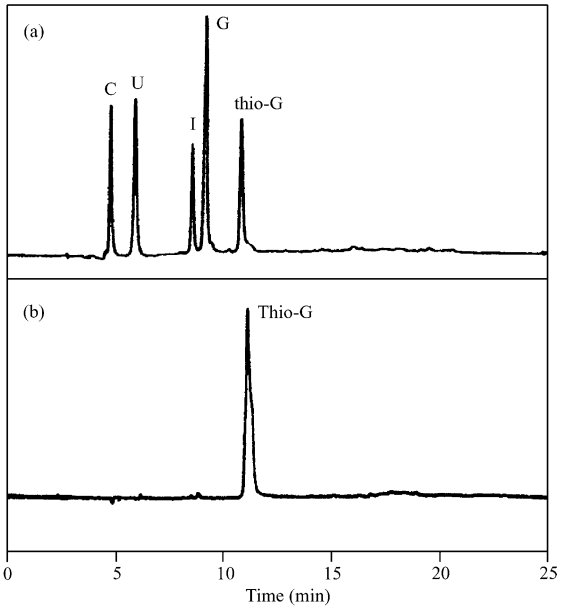


Figure 3. Nucleoside composition analysis[26] of 5′-GCG^{SH} AU-3′. The nucleosides generated from digestion with snake venom phosphordiesterase and alkaline phosphatase were separated by HPLC with monitoring at (a) 260 nm; and at (b) 345 nm. The identity of the peaks was confirmed by retention time comparison with the authentic samples. Inosine was resulted from the deamination of adenosine by adenosine deaminase, which is a contaminant in the enzyme preparations.

out affecting other functional and protecting groups and removing the oligomers from the resin,[2a] phosphoramidite **6** could be compatible with introducing structural diversity at multiple sites in RNA via 'on-column' conjugation. This is currently being investigated.

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25. ^{1}H NMR and HRMS data: **2**: ^{1}H NMR spectrum (DMSO-d_6): 3.47–3.64 (m, 2H, 5′-H, 5″-H), 3.89 (m, 1H, 4′-H), 4.10 (m, 1H, 3′-H), 4.47 (m, 1H, 2′-H), 5.07 (t, 1H, 5′-OH), 5.20 (d, 1H, 3′-OH), 5.50 (t, 1H, 2′-OH), 5.80 (d, 1H, 1′-H), 6.77 (s, 2H, 2-NH_2, ex), 7.96 (d, 1H, 6-H of 2,4-dinitrophenyl), 8.32 (s, 1H, 8-H), 8.40 (d, 1H, 5-H of 2,4-dinitrophenyl), 8.88 (s, 1H, 3-H of 2,4-dinitrophenyl). HRMS: calcd for $C_{16}H_{15}N_7O_8S$ $[M+Na]^+$ 488.0601, found 488.0592. **3**: ^{1}H NMR spectrum (DMSO-d_6): 3.34–3.58 (m, 2H, 5′-H, 5″-H), 3.94 (m, 1H, 4′-H), 4.18 (m, 1H, 3′-H), 4.59 (m, 1H, 2′-H), 4.79 (s, 2H, –CH_2– of $PhOCH_2CO$), 5.94 (d, 1H, 1′-H), 6.80–6.92 (m, 3H, Ar), 7.25 (m, 2H, Ar), 8.21 (d, 1H, 6-H of 2,4-dinitrophenyl), 8.37 (d, 1H, 5-H of 2,4-dinitrophenyl), 8.72 (s, 1H, 8-H), 8.87 (s, 1H, 3-H of 2,4-dinitrophenyl), 10.86 (s, 1H, 2-NH, ex). HRMS: calcd for $C_{24}H_{21}N_7O_{10}S$ $[M+Na]^+$ 622.0968, found 622.0975. **4**: ^{1}H NMR spectrum (DMSO-d_6): 3.13–3.16 (m, 2H, 5′-H, 5″-H), 3.35 (m, 1H, 4′-H), 3.69 (s, 3H, CH_3O–), 4.05 (m, 1H, 3′-H), 4.29 (m, 1H, 2′-H), 4.73 (s, 2H, –CH_2– of $PhOCH_2CO$), 6.01 (d, 1H, 1′-H), 6.75–6.96 (m, 4H, Ar), 7.15–7.29 (m, 15H, Ar), 8.15 (d, 1H, 6-H of 2,4-dinitrophenyl), 8.46 (d, 1H, 5-H of 2,4-dinitrophenyl), 8.61 (s, 1H, 8-H), 8.89 (s, 1H, 3-H of 2,4-dinitrophenyl), 10.84 (s, 1H, 2-NH, ex). HRMS: calcd for $C_{44}H_{37}N_7O_{11}S$ $[M+Na]^+$ 894.2169, found 894.2150. **5**: ^{1}H NMR spectrum ($CDCl_3$): −0.11 (s, 3H, CH_3–Si), 0.02 (s, 3H, CH_3–Si), 0.84 (s, 9H, *t*-Bu-Si), 3.45 (m, 2H, 5′-H, 5″-H), 3.76 (m, 3H, CH_3O–), 4.25 (m, 1H, 4′-H), 4.43 (m, 1H, 3′-H), 4.53 (s, 2H, –CH_2– of $PhOCH_2CO$), 4.90 (m, 1H, 2′-H), 6.04 (d, 1H, 1′-H), 6.77–6.87 (m, 4H, Ar), 7.18–7.38 (m, 15H, Ar), 8.21 (s, 1H, 2-NH, ex), 8.37 (m, 2H, 5-H and 6-H of 2,4-dinitrophenyl), 8.75 (s, 1H, 8-H), 8.96 (s, 1H, 3-H of 2,4-dinitrophenyl). HRMS: calcd for $C_{50}H_{51}N_7O_{11}SSi$ $[M+Na]^+$ 1008.3034, found 1008.3063. **6**: ^{31}P NMR spectrum ($CDCl_3$): 153.79, 152.82. HRMS: calcd for $C_{59}H_{69}N_7O_{12}SSiP$ $[M+Na]^+$ 1186.1707, found 1186.1723.
26. In a typical nucleoside composition analysis, 1 O.D. of oligoribonucleotide was dissolved in 1.0 mL of a solution containing 0.2 mM $ZnCl_2$, 16 mM $MgCl_2$, 250 mM Tris–HCl pH 6.0, 0.2 unit of snake venom phosphodiesterase (Sigma), and 4 units of calf-intestinal alkaline phosphatase (Sigma), and the mixture was incubated at 37 °C for 8 h. The digested sample was then analysed with reverse-phase HPLC (C18 column), which was eluted with buffer A (0.1 M TEAA/acetonitrile, 98:2) and buffer B (0.1 M TEAA/acetonitrile, 20:80). A linear gradient was formed from 0 to 20% of the buffer B over 25 min. Peaks on the HPLC trace were identified by retention time comparison with the authentic samples. Retention times were: C: 4.50 min, U: 5.63 min, I: 8.26 min, G: 8.85 min, G^{SH}: 10.50 min, and A: 13.12 min.
27. ESI-MS: calcd for (5′-GCG^{SH} AU-3′) $[M-H]^-$ 1583.23, found 1583.02; calcd for (5′-UAC CAG^{SH} UGA GCU-3′) $[M-H]^-$ 3808.51, found 3808.16.