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Incorporation Of 6-Thioguanosine Into Oligoribonucleotides

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Abstract: The preparation of N^2-benzoyl-S^6-cyanoethyl-6-thioguanosine and its incorporation into oligoribonucleotides, using standard phosphoramidite methods, is described. 6-Thioguanosine has been incorporated into the three guanosine sites of the central core of the hammerhead ribozyme resulting in 2- to 9-fold reductions in catalytic activity.

The chemical incorporation of sulphur into the phosphate backbone,[1,2] sugar[3] or bases[1] of oligonucleotides has largely been restricted to oligodeoxynucleotides. We are currently working on the incorporation of thiobases into oligoribonucleotides, recently reporting on 4-thiouridine[4] and 6-thioinosine.[5] Prior to this only the 2'-deoxy analogues, 4-thiothymidine,[6-8] 2'-deoxy-4-thiouridine,[6] 2'-deoxy-6-thioguanosine[9,10] and 2'-deoxy-6-thioinosine[11] had been incorporated into oligonucleotides. A number of modified nucleotides has been used to probe the interactions of the 6-carbonyl oxygen of guanosine, the most subtle being 2-aminopurine ribonucleoside (2-AP)[12,13]. In this case, the base is further altered by the removal of the N^1-imino hydrogen, which has the effect of reversing the hydrogen bonding properties at this position. An alternative approach would be to substitute oxygen for sulphur, forming 6-thioguanosine. The hydrogen bond strength of sulphur is approximately half that of oxygen, as suggested by spectroscopy in the gas phase.[14] The thiocarbonyl functional group also has different chemical and photo-chemical properties to the carbonyl group. These features can be used to probe protein-nucleic acid and nucleic acid-nucleic acid interactions. 4-Thiopyrimidines and 6-thiopurines have previously been used for post-synthetic modification[6] and photo-cross-linking studies.[15]

We report here the first synthesis of N^2-benzoyl-S^6-cyanoethyl-6-thioguanosine and its incorporation into chemically synthesised oligoribonucleotides. 6-Thioguanosine has been incorporated into three of the guanosine sites, G_5, G_8 and G_{12}, of the central core of a hammerhead ribozyme (Figure 1). Two modified 19-mer ribozymes GGCUCGACUGAU[^{6S}G]AGGCGC (ORN-5) and GGCUCGACUGAU[^{6S}G]AGGCGC (ORN-8) and a modified 24-mer substrate strand GCGCC[^{6S}G]AAACACCGUGUCUCGAGC (ORN-12) were prepared.

Synthesis of the fully protected phosphoramidite derivative of 6-thioguanosine (**6**) is shown in the Scheme. The exocyclic amine is protected with a benzoyl group. Connolly and Waters[10] found that ammonia deprotection of the benzoyl group occurred more rapidly for N^2-benzoyl-2'-deoxy-6-thioguanosine than for N^2-benzoyl-2'-deoxyguanosine. Protection of the thiocarbonyl group was achieved using the cyanoethyl group.[6,7,10] Clivio *et al.*[8] have used *S*-pivaloyloxymethylation to protect the thiocarbonyl group. The synthesis of **4** was similar to that of the 2'-deoxy analogue described by Waters and Connolly.[13] Standard methods for tritylation[16] and silylation[5,17] were used. The phosphitylation reaction was carried out over 18 h in the absence of a catalyst, usually DMAP or *N*-methylimidazole, affording **6** in

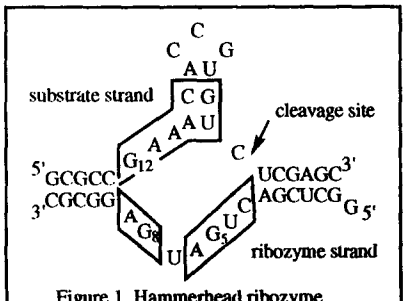
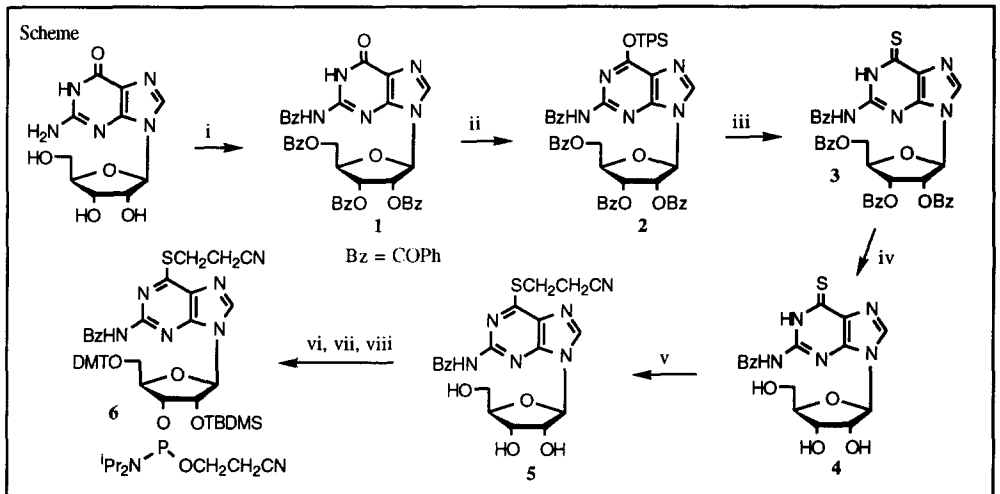


Figure 1 Hammerhead ribozyme
Nucleotides in boxes are essential for function

high yield (*ca.* 90%). We observed no 2'→3' isomerisation of the TBDMS group under these conditions, a result consistent with earlier observations.[5] NMR data are listed in the References and Notes.[18] Incorporation of **6** into the RNA oligomers was carried out on an ABI 391PCR-Mate DNA synthesiser (Applied Biosystems).[19] Deprotection of the *S*-cyanoethyl group was achieved with 1 M DBU in anhydrous acetonitrile for 5 h.[20] A previous report,[10] in which 2'-deoxy-6-thioguanosine was incorporated into oligodeoxynucleotides, used ammonia to cleave this group. In our hands, the use of methanolic ammonia resulted in lower yields of the final product (*ca.* 30% by HPLC of that obtained by initial treatment with DBU). Cleavage from the support and deprotection of the exocyclic amino and *O*-cyanoethyl groups were achieved in a sealed container with freshly prepared methanolic ammonia (methanol at 0°C purged with NH_3 for 30 min) at 30°C for 12-14 h. After evaporation, the residue was 2'-*O*-TBDMS deprotected with NEt_3.3HF/DMSO (1:1) for 24 h. Using TBAF in THF resulted in the complete degradation of the thiocarbonyl group. We have also found that diluting NEt_3.3HF in DMSO gave better yields than when neat NEt_3.3HF was used. Although the deprotection time is increased, from 8 to 24 h, a significant improvement in the stability of the 6-thiocarbonyl group was observed. When left to deprotect for 40 h no further degradation of the thiocarbonyl group was observed. In neat NEt_3.3HF, prolonged exposure resulted in further degradation. We have also found this method useful for preparing longer oligoribonucleotides which are often insoluble in neat NEt_3.3HF.[5] After *S*-cyanoethyl deprotection, thorough washing of the CPG to remove DBU is essential to prevent degradation of the thiocarbonyl group during desilylation. When N^2-benzoyl-6-thioguanosine was treated with 2 mM DBU in NEt_3.3HF, at 25°C, the thiocarbonyl group was degraded with a half-life of approximately 1 h.



i, Benzoic anhydride, pyridine, reflux; ii, 2,4,6-triisopropylbenzenesulphonyl chloride, DMAP, $^iPr_2NEt$, CH_2Cl_2; iii, Li_2S, THF; iv, 2.5 mM NaOH in MeOH; v, $BrCH_2CH_2CN$, K_2CO_3, DMF; vi, 4,4'-dimethoxytrityl chloride, pyridine; vii, TBDMSCl, imidazole, pyridine; viii, $^iPr_2NP(Cl)OCH_2CH_2CN$, $^iPr_2NEt$, THF, 20 h.

Based on trityl assays, the overall yields of ORN-5, ORN-8 and ORN-12 were 53, 71 and 51%, respectively (average stepwise yields 96.5, 98.1 and 97.1%). Incorporation of ^{6}SG was similar to the standard phosphoramidites. All oligoribonucleotides were purified by HPLC as previously reported.[21] The final yields, after HPLC purification, for ORN-5 and ORN-8 were *ca.* 210 (9%), 300 (12%) and 270 μg (7%), respectively.

The UV spectrum of ORN-8 (Fig. 2) contains peaks at 260 and 348 nm, the latter being characteristic of the thiocarbonyl chromophore. Compositions of the modified oligoribonucleotides were confirmed by base composition analysis.[22] When recorded at 340 nm one peak was observed whose retention time[22] corresponded to an authentic sample of 6-thioguanosine.

A number of modified oligoribonucleotides have been prepared in order to study the mechanism of hammerhead ribozyme cleavage.[24] Replacement of the exocyclic oxygen with sulphur introduces a bulkier and weaker H-bonding atom but the same ring structure is retained. Single turnover reactions, carried out in duplicate, were performed at 37°C in a volume of 30 μL 50 mM Tris-HCl, pH 7.3, with 5'-^{32}P-labelled substrate at a concentration of 0.1 nM and a ribozyme concentration of 1 μM. Cleavage was initiated by the addition of $MgCl_2$ to a final concentration of 10 mM. Aliquots of 3 μL were removed at various time intervals and quenched with 5 μL of 8 M urea and 50 mM EDTA. The samples were analysed by PAGE on 20% (w/v) denaturing gels and the resulting bands were quantified by phosphorimaging. The half-life for cleavage of the modified strands ORN-5, ORN-8 and ORN-12 were 600 ± 50, 1900 ± 150 and 2700 ± 200 s, respectively, while the unmodified strand was 310 ± 20 s.

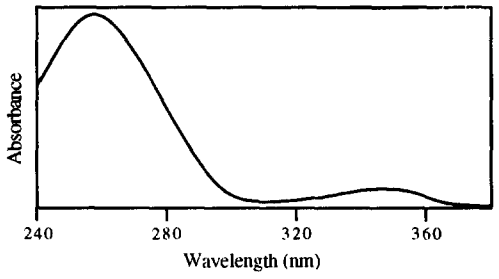


Figure 2. UV spectrum of GGCUCGACUGAU[^{6S}G]AGGCGC (ORN-8)

Incorporation of 2-AP at positions 5, 8 and 12 in the catalytic core of the hammerhead ribozyme resulted in substantial losses in activity.[13] Inosine substitution at positions 5 and 12 resulted in substantial reductions in activity, whereas, at position 8 only a small loss of activity was reported.[13] The small reduction in activity at position 5 (ORN-5) upon 6-thioguanosine substitution combined with the effects of inosine and 2-AP substitution is consistent with the exocyclic amino and N^1-imino, and not 6-carbonyl, groups being involved in hydrogen bonding. The 6- and 9-fold reductions in activity for ORN-8 and ORN-12 are consistent with the 6-carbonyl oxygen being involved in catalytically important interactions. The reduction in activity observed for ORN-8 is consistent with interactions observed in the recently reported crystal structure of a hammerhead ribozyme bound to a non-cleavable 2'-deoxy substrate strand.[25] However, no interactions by the 6-carbonyl oxygen atoms in G_5 and G_{12} were discussed.

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18. NMR data were recorded on either General Electric QE 300, Brucker AM 400 or Varian Unity 500 spectrometers. Selected data: **2**: ^{13}C NMR: δ ($CDCl_3$) 23.50, 24.62, 24.64 (3 × q, $CH(\underline{C}H_3)_2$), 29.87, 34.31 (2 × d, $\underline{C}H(CH_3)_2$), 64.21 (t, C-5'), 71.83 (d, C-2'), 75.00 (d, C-3'), 80.92 (d, C-4'), 88.30 (d, C-1'), 119.90 (s, C-5), 123.89 (d, Ph(TPS)), 127.39-129.80 (7 × d, Ph), 128.83, 129.45, 130.96 (3 × s, ipso Ph), 132.28, 133.15, 133.44, 133.68 (4 × d, Ph), 134.05 (s, ipso Ph(TPS)), 142.82 (d, C-8), 150.85 (s, ipso Ph(TPS)), 151.46 (s, C-4), 154.24 (s, C-6), 154.62 (s, C-2), 154.93 (s, ipso Ph(TPS)), 163.83, 165.17, 165.32, 166.12 (4 × s, COPh). **4**: ^{1}H NMR: δ (d^6-DMSO) 3.57, 3.67 (2 × 1H, 2 × dd, *J* 12, 4 Hz, H-5',5"), 3.94 (1H, q, *J* 3.5 Hz, H-3'), 4.17 (1H, t, *J* 4 Hz, H-4'), 4.49 (1H, t, *J* 5 Hz, H-2'), 5.91 (1H, d, *J* 6 Hz, H-1'), 7.58 (2H, t, *J* 7.5 Hz, Ph(*m*-H)), 7.69 (1H, t, *J* 7.8 Hz, Ph(*p*-H)), 8.08 (2H, d, *J* 7 Hz, Ph(*o*-H)), 8.50 (1H, s, H-8), 12.24 (1H, s, N<u>H</u>COPh). **5**: ^{1}H NMR: δ (d^6-DMSO) 3.19 (2H, t, *J* 6.5 Hz, CH_2CN), 3.54 (2H, m, SCH_2), 3.57 (partial overlap with CH_2CN peak) (dd *J* 4 Hz (other *J* obscured), H-5'), 3.68 (1H, dd, *J* 12, 4 Hz, H-5"), 3.95 (1H, d(br), *J* 3 Hz, H-3'), 4.20 (1H, t(br), *J* 4 Hz, H-4'), 4.63 (1H, t, *J* 5.3 Hz, H-2'), 5.97 (1H, d, *J* 6 Hz, H-1'), 7.54 (2H, t, *J* 7.3 Hz, Ph(*m*-H)), 7.62 (1H, t, *J* 7 Hz, Ph(*p*-H)), 7.97 (2H, d, *J* 7 Hz, Ph(*o*-H)), 8.64 (1H, s, 8-H), 11.06 (1H, s, N<u>H</u>COPh). ^{13}C NMR: δ (d^6-DMSO) 18.23 (t, $\underline{C}H_2CN$), 24.67 (t, SCH_2), 61.80 (t, C-5'), 70.89 (d, C-2'), 74.12 (d, C-3'), 86.21 (d, C-4'), 87.68 (d, C-1'), 119.97 (s, C≡N), 128.54 (s, C-5), 128.87, 128.71, 132.70 (3 × d(br), Ph), 134.94 (s, ipso Ph), 142.24 (d(br), C-8), 150.25 (s, C-2), 152.74 (s, C-4), 159.00 (s, C-6), 166.28 (s, COPh). N^2-Benzoyl-S^6-cyanoethyl-5'-*O*-dimethoxytrityl-6-thioguanosine: ^{1}H NMR: δ ($CDCl_3$) 2.92 (2H, t, *J* 11.8 Hz, CH_2CN), 3.20, 3.39 (2 × 1H, 2 × dd, *J* 9, 2.4 & 9, 3 Hz, H-5',5"), 3.63 (2H, q, *J* 6.3 Hz, SCH_2), 3.75 (6H, s, OCH_3), 4.43 (1H, d, *J* 4.8 Hz, H-3'), 4.50 (1H, s, H-4'), 5.04 (1H, dd(br), *J* 6.3, 4.8 Hz, H-2'), 5.99 (1H, d, *J* 6.3 Hz, H-1'), 6.69 (4H, d, *J* 8.4 Hz, Ph), 7.08-7.60 (12H, m, Ph), 7.98 (2H, d, *J* 7.2 Hz, Ph), 8.19 (1H, s, 8-H), 9.02 (1H, s, N<u>H</u>COPh). δ ($CDCl_3$) ^{13}C NMR: δ ($CDCl_3$) 19.16 (t, $\underline{C}H_2CN$), 24.46 (t, SCH_2), 55.15 (q, OCH_3), 63.79 (t, C-5'), 73.93 (d, C-2'), 76.65 (d, C-3'), 86.55 (s, $O\underline{C}Ar_3$), 87.09 (d, C-4'), 92.22 (d, C-1'), 112.98, 113.00 (2 × d, DMT arom. C), 118.55 (s, C≡N), 126.77-129.83 (7 × d, Ph), 128.82 (s, C-5), 132.81 (d, COPh arom. C), 133.46 (s, COPh ipso arom. C), 135.21, 135.29 (s, DMT ipso arom. C), 141.34 (d, C-8), 144.09 (s, DMT ipso arom. C), 148.20 (s, C-4), 151.34 (s, C-2), 158.37 (s, DMT ipso arom. C), 160.33 (s, C-6), 165.25 (s, COPh). N^2-Benzoyl-2'-*O*-TBDMS-S^6-cyanoethyl-5'-*O*-dimethoxytrityl-6-thioguanosine: ^{1}H NMR: δ ($CDCl_3$) -0.232, -0.016 (2 × 3H, 2 × s, $SiCH_3$), 0.83 (9H, s, $SiC(CH_3)_3$), 3.11 (1H, dd, *J* 11, 3.0, H-5'), 3.26 (2H, t, *J* 2.8 Hz, CH_2CN), 3.55-3.78 (3H, m, H-5", SCH_2), 3.66, 3.69 (2 × 3H, 2 × s, OCH_3), 4.26, 4.35 (2 × 1H, s, d, *J* 5 Hz, H-3',4'), 5.37 (1H, dd, *J* 6, 5 Hz, H-2'), 5.84 (1H, d, *J* 7 Hz, H-1'), 6.62-7.68 (18H, m, Ph), 7.88 (1H, s, N<u>H</u>COPh), 8.01 (1H, s, H-8). ^{13}C NMR: δ ($CDCl_3$) -5.08, -4.98 (2 × q, $SiCH_3$), 17.80 (s, $Si\underline{C}(CH_3)_3$), 18.54 (t, $\underline{C}H_2CN$), 25.38 (t, SCH_2), 25.51 (q, $SiC(\underline{C}H_3)_3$), 55.12, 55.17 (2 × q, OCH_3), 63.59 (t, 5'-C), 71.08 (d, C-2'), 73.62 (d, C-3'), 84.51 (d, C-4'), 86.31 (s, $O\underline{C}Ar_3$), 88.64 (d, C-1'), 113.23, 113.28 (2 × d, DMT arom. C), 118.77 (s, C≡N), 126.82-129.92 (7 × d, Ph), 129.46 (s, C-5), 131.53 (d, COPh arom. C), 133.61 (s, COPh ipso arom. C), 135.75, 136.54 (2 × s, DMT ipso arom. C), 142.89 (d, C-8), 145.13 (DMT ipso arom. C), 149. 54 (s, C-4), 151.91 (s, C-2), 158.50, 158.59 (s, DMT ipso arom. C), 160.68 (s, C-6), 164.59 (s, CO). **6**: ^{1}H NMR: δ ($CDCl_3$) -0.28*, -0.25, -0.04, -0.03* (4 × 3H, 4 × s, $SiCH_3$), 0.75*, 0.76 (2 × 9H, 2 × s, $SiC(CH_3)_3$), 0.92, 1.15*, 1.21* (6H, 12H (overlapping doublets), 6H, 3 × d, *J* 6.5, 6.5, 7.0 Hz, $N(CH(C\underline{H}_3)_2)_2$), 2.60*, 2.68 (2 × 2H, 2 × t, *J* 6.5 Hz, $OCH_2C\underline{H}_2CN$), 3.00*, 3.05 (2 × dd, *J* 11, 2.5 & 11, 2 Hz, H-5'), 3.27 (m, $SCH_2C\underline{H}_2CN$), 3.46-3.78, 3.89, 4.00 (m, H-5", $POCH_2$, SCH_2 and $N(C\underline{H}(CH_3)_2)_2$), 3.64*, 3.667, 3.671*, 3.69 (4 × 3H, 4 × s, OCH_3), 4.24 (1H, s(br), H-3'), 4.33-4.36 (3H, m(overlap), H-3',4'), 5.30, 5.39* (2 × 1H, 2 × dd, *J* 7.5, 4.5 & 7.5, 5.0 Hz, H-2'), 5.74*, 5.92 (2 × 1H, 2 × d, *J* 7.5, 7.5 Hz, H-1'), 6.69-7.70 (18H, m, Ph), 8.02, 8.06* (2 × 1H, 2 × s, H-8) (* differentiates the two diastereoisomers, where possible). ^{31}P NMR δ ($CDCl_3$) 149.93, 152.94.
19. Phosphoramidites were from Chemgene Inc., Waltham, Mass., the bases being benzoyl-protected for C and A and isobutyryl-protected for G. CPG-benzoylC and -uridine were from Millipore.
20. The half-life for *S*-cyanoethyl deprotection of N^2-benzoyl-*S*-cyanoethyl-6-thioguanosine in 1 M DBU in acetonitrile, at 20°C, was determined spectrophotometrically to be 130 ± 1 min.
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22. Base composition analyses were performed with minor modifications as described by Eadie *et al.*[23] Shrimp alkaline phosphatase (USM) and phosphodiesterase (Boehringer Mannheim) were used. Dithiothreitol (0.5 mM) was essential during the enzyme digestion for detection of 6-thioguanosine. Elution times: C, 7.1; U, 10.4; G, 24.4; ^{6S}G, 27.6; A, 43.1 min.
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