Pergamon

Tetrahedron Letters, Vol. 36, No. 26, pp. 4637-4640, 1995
Elsevier Science Ltd
Printed in Great Britain
0040-4039/95 $9.50+0.00

0040-4039(95)00805-5

Incorporation Of 6-Thioinosine Into Oligoribonucleotides

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Abstract: *Preparation of S^6-cyanoethyl-6-thioinosine and its incorporation into oligoribonucleotides, using standard phosphoramidite methods, is described. Evidence for binding between the minimal RNA recognition sequence (minSLIIB) and the cognate HIV-I-GST- Rev protein was obtained from the changes in the unique circular dichroism band at 320 nm of 6-thioinosine.*

We are developing methods for the incorporation of sulphur-containing bases into oligoribonucleotides, recently reporting the incorporation of 4-thiouridine.[1] The replacement of a keto oxygen atom with sulphur provides a probe for the study of protein-nucleic acid and nucleic acid-nucleic acid interactions with 4-thiopyrimidines and 6-thiopurines being used in post-synthetic modification[2] and photo-cross-linking[3,4] studies. These bases absorb light in the region 320-350 nm which is well removed from the maxima of proteins and RNA/DNA, making them potentially useful as circular dichroism and uv spectral probes. The incorporation of 2'-deoxy-6-thioinosine into oligodeoxynucleotides has been previously reported.[4,5] Woisard *et al.*[4] demonstrated that 2'-deoxy-6-thioinosine could be photo-cross-linked to pyrimidines in good yield. Milton *et al.*[5] formed an interstrand disulphide bridge between 2'-deoxy-6-thioinosine and 4-thiothymidine in a self complementary oligodeoxynucleotide.

We report a synthesis of the phosphoramidite derivative of S^6-cyanoethyl-6-thioinosine and its incorporation into the 29 nucleotide minimal Rev-responsive element, known as minSLIIB (Figure 1).[6] This sequence encompasses the minimum recognition target for the HIV-I-GST Rev protein (Rev), which is a single-stranded "bubble" flanked by Watson-Crick base-paired stems. The circular dichroism spectra of a 6-thioinosine-containing variant (ORN-1) were recorded with and without Rev in order to determine the importance, if any, of position-73 in the bubble.

The scheme used to prepare the fully protected phosphoramidite derivative of 6-thioinosine (^{6S}I) (**2**) is shown in Figure 2. 6-Thioinosine was prepared in a similar manner to 2'-deoxy-6-thioinosine using the method described by Robins and Basom.[7] Treatment of 6-thioinosine with 3-bromopropionitrile and potassium carbonate in DMF afforded S^6-cyanoethyl-6-thioinosine in high yield.[3] Preparation of the fully protected phosphoramidite derivative was achieved using standard methods for tritylation[8] and silylation.[9,10] The phosphitylation reaction was carried out over 18 h in the absence of a catalyst, usually DMAP or *N*-

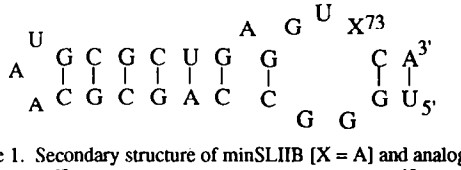
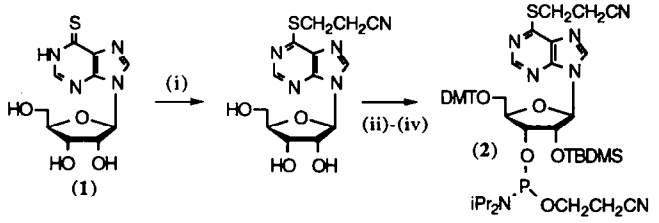


Figure 1. Secondary structure of minSLIIB [X = A] and analogues ^{6S}I-73 [X = ^{6S}I (ORN-1)] and dI-73 [X = dI (ORN-2)][19]

methylimidazole, affording **2** in high yield (90%). We observed no 2'→3' isomerisation of the TBDMS group under these conditions.[11] NMR data are listed in the References and Notes.[12]

Figure 2



(i) $BrCH_2CH_2CN$, K_2CO_3, DMF (ii) 4,4'-dimethoxytrityl chloride, pyridine
(iii) TBDMS-Cl, pyridine (iv) $iPr_2NP(Cl)OCH_2CH_2CN$, iPr_2NEt, THF, 18 h

In a preliminary experiment, 6-thioinosine was incorporated into the trimer $U^{6S}IU$ using the methods described below for ORN-**1**. An overall yield, after HPLC purification, of 360 μg (37%) was obtained. TBDMS deprotection was achieved with $NEt_3.3HF$, using TBAF (1 M in THF) gave a drastically reduced yield [*ca.* 30 μg (3%)]. The UV spectrum (Figure 3) and base composition analysis confirmed that the major product was $U^{6S}IU$.

Incorporation of **2** into ORN-**1** was carried out on an ABI 391PCR-Mate DNA synthesiser (Applied Biosystems). Phosphoramidites were from Chemgene Inc., Waltham, Mass., the bases being benzoyl-protected for C and A and isobutyryl-protected for G. CPG-uridine and -benzoyladenosine were from Millipore. Deprotection of the *S*-cyanoethyl group was achieved with 1 M DBU in acetonitrile for 100 min.[2,13,14] Cleavage from the support and deprotection of the exocyclic amino and *O*-cyanoethyl groups was 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 16 h. After evaporation to dryness, the residue was dissolved in DMSO (100 μl) and then 2'-*O*-TBDMS deprotected for 7 h with $NEt_3.3HF$ (1 ml).[15] DMSO was required as ORN-**1** was virtually insoluble in $NEt_3.3HF$, a result we have previously observed for a number of other oligoribonucleotides.[1,16] Deprotection with TBAF in THF gave none of the required oligoribonucleotide. The coupling yield of ^{6S}I was similar to that of the commercial phosphoramidites with the overall yield of ORN-**1**, based on trityl assays, being 65%. All oligoribonucleotides were purified by HPLC[16] using a linear gradient of acetonitrile from 2-10% over 40 min. Buffer A was 0.1 M ammonium acetate pH 6.5, buffer B 50% acetonitrile and 0.05 M ammonium acetate, pH 6.5. The desired oligoribonucleotide (ORN-**1**) was identified by its unique UV spectrum, with maxima at 258 and 324 nm, and base composition analysis.[17] The final yield, after HPLC purification, was *ca.* 600 μg (12%).

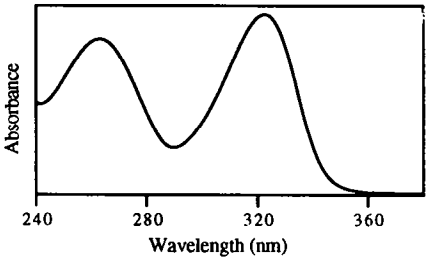


Figure 3. UV spectrum of $U^{6S}IU$ in HPLC buffers (*ca.* 2% acetonitrile in 0.1 M ammonium acetate)

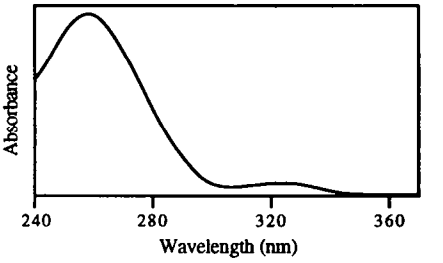


Figure 4. UV spectrum of ^{6S}I-73 minSLIIB variant (ORN-**1**) in HPLC buffers (*ca.* 2% acetonitrile in 0.1 M ammonium acetate)

The importance of A-73 (Figure 1) in the binding of Rev to minSLIIB has previously been studied by both gel retardation assays and circular dichroism spectroscopy.[19] In gel retardation assays, substitution of adenosine with 2'-deoxyadenosine has no effect on binding whereas substitution with 2'-deoxyinosine completely abolishes binding, which is consistent with a base-specific effect. However, a specific change at 260 nm in the circular dichroism spectra of each variant upon addition of Rev is observed. These data suggest that the ORN-2/Rev complex has a faster dissociation rate than the wild-type complex. The lack of a unique circular dichroism transition prevents a determination of the region of minSLIIB which has undergone conformational change. By substituting ^{6S}I at position 73 we are able to exploit its unique circular dichroism transition to determine whether this residue was in the region undergoing the conformational change.[20] As Figure 5 shows there is a small but clearly measurable change in the circular dichroism spectrum at 320 nm, indicating a change in the environment of ^{6S}I-73, this could come from either a direct contact to the protein or a protein-induced conformational change. We are currently preparing a number of other derivatives in an attempt to distinguish these possibilities.

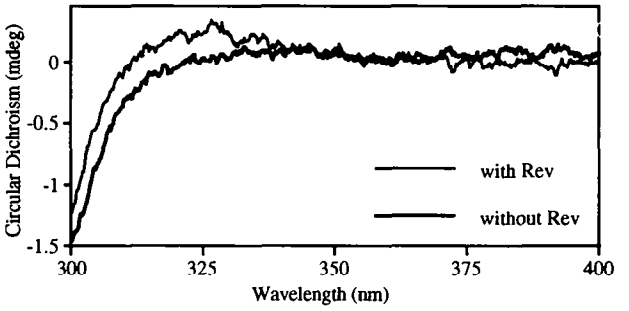


Figure 5. Circular dichroism spectra of ^{6S}I-73 minSLIIB variant in the presence and absence (bold) of Rev

We are currently utilising the photo-cross-linking properties of 6-thioinosine to probe RNA-RNA and protein-RNA interactions.

This work was supported by grants from the SERC and the Wellcome Trust. We would also like to thank Dr. J Fisher and Dr. J. R. P. Arnold for assistance in obtaining NMR spectra.

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9. Silylation (1.1 eq TBDMS-Cl, 1.1 eq imidazole, pyridine, 18 h)[10] affords a mixture of the 2'- and 3'-OTBDMS derivatives. Chromatographic separation (diethyl ether/light petroleum 3:1) elutes most of the 2'-OTBDMS derivative followed by a mixture of 2'-OTBDMS and 3'-OTBDMS derivatives (ca. 1:8). This mixture can be isomerised[10] (2% NEt_3 in MeOH for 24 h produces a 1:1 equilibrium mixture) to afford more of the 2'-OTBDMS derivative.
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12. NMR data were recorded on either General Electric QE 300, Brucker AM 400 or Varian Unity 500 spectrometers. *S*^{6}-cyanoethyl-6-thioinosine: ^{1}H NMR δ (d^{6}-DMSO) 3.01 (2H, t, *J* 6.3 Hz, CH_2CN), 3.64 (2H, t, *J* 6.3 Hz, SCH_2), 3.70, 3.85 (2 × 1H, 2 × d, *J* 11.7, 11.4 Hz, 5'-H), 4.15, 4.34, 5.34 (3 × 1H, 3 × s, OH), 4.65, (1H, d, *J* 5.1 Hz, 4'-H), 5.0 (1H, m, 3'-H), 5.44 (1H, d, *J* 5.7 Hz, 2'-H), 6.09 (1H, d, *J* 5.1 Hz, 1'-H), 8.62, 8.69 (2 × 1H, 2 × s, 2,8-H). ^{13}C NMR δ (d^{6}-DMSO) 18.38 (t, $\underline{C}H_2CN$), 24.04 (t, SCH_2), 61.65 (t, 5'-C), 70.67 (d, 2'-C), 74.47 (d, 3'-C), 86.23 (d, 4'-C), 88.98 (s, 1'-C), 118.35 (s, C≡N), 131.67 (s, 5-C), 143.32 (d(br), 8-C), 148.04 (s, 4-C), 151.07 (d(br), 2-C), 158.43 (s, 6-C). 5'-*O*-dimethoxytrityl-*S*^{6}-cyanoethyl-6-thioinosine: ^{1}H NMR δ ($CDCl_3$) 2.90 (2H, t, *J* 7.1 Hz, CH_2CN), 3.35, 3.44 (2 × 1H, 2 × dd, *J* 11, 3 and 11, 2.7 Hz, 5'-H), 3.57 (t, *J* 7.2 Hz, SCH_2), 3.75 (6H, s, OCH_3), 4.39 (1H, s(br), 4'-H), 4.48 (1H, s(br), 3'-H), 4.83 (1H, t, *J* 5 Hz, 2'-H), 6.06 (1H, d, *J* 5.4 Hz, 1'-H), 6.74 (4H, d *J* 8.7 Hz, Ph), 7.18-7.32 (9H, m, Ph), 8.25, 8.63 (2 × 1H, 2 × s, 2,8-H). ^{13}C NMR δ ($CDCl_3$) 18.61 (t, $\underline{C}H_2CN$), 24.42 (t, SCH_2), 55.16 (q, OCH_3), 63.35 (t, 5'-C), 72.07 (d, 2'-C), 75.52 (d, 3'-C), 85.53 (d, 4'-C), 86.56 (s, $O\underline{C}Ar_3$), 90.16 (d, 1'-C), 113.06 (d, DMT arom. C), 117.99 (s, C≡N), 126.86-129.86 (4 × d, DMT Ph), 131.64 (s, 5-C), 135.30, 135.33 (2 × s, DMT ipso arom. C), 141.74 (d, 8-C), 144.18 (s, DMT ipso arom. C), 147.84 (s, 4-C), 151.35 (d, 2-C), 158.42 (s, 2 × DMT arom. ipso C), 159.33 (s, 6-C). 2'-*O*-*t*-butyldimethylsilyl-5'-*O*-dimethoxytrityl-*S*^{6}-cyanoethyl-6-thioinosine: ^{1}H NMR δ ($CDCl_3$) -0.14, 0.00 (2 × 3H, 2 × s, $Si(CH_3)_3$), 0.84 (9H, s, $SiC(CH_3)_3$), 2.94 (2H, t, *J* 7.2 Hz, CH_2CN), 3.39, 3.52 (2 × 1H, 2 × dd, *J* 11, 3.6 and 11, 2.7 Hz, 5'-H), 3.60 (t, *J* 7.2 Hz, SCH_2), 3.78 (6H, s, OCH_3), 4.28 (1H, q, *J* 3 Hz, 4'-H), 4.37 (1H, q, *J* 3 Hz, 3'-H), 5.01 (1H, t, *J* 5.1 Hz, 2'-H), 6.09 (1H, d, *J* 5.1 Hz, 1'-H), 6.81 (4H, d *J* 8.7 Hz, Ph), 7.21-7.46 (9H, m, Ph), 8.23, 8.63 (2 × 1H, 2 × s, 2,8-H). ^{13}C NMR δ ($CDCl_3$) -5.18, -4.96 (q, $SiCH_3$), 17.84 (s, $Si\underline{C}(CH_3)_3$), 18.64 (t, $\underline{C}H_2CN$), 24.42 (t, SCH_2), 25.50 (q, $SiC(\underline{C}H_3)_3$), 55.16 (q, OCH_3), 63.25 (t, 5'-C), 71.45 (d, 2'-C), 75.66 (d, 3'-C), 84.19 (d, 4'-C), 86.61 (s, $O\underline{C}Ar_3$), 88.28 (d, 1'-C), 113.12 (d, DMT arom. C), 118.04 (s, C≡N), 126.88-129.95 (4 × d, DMT arom. C), 131.70 (s, 5-C), 135.43-135.46 (2 × s, DMT ipso arom. C), 141.88 (d, 8-C), 144.42 (s, DMT ipso arom. C), 148.54 (s, 4-C), 151.85 (d, 2-C), 158.46 (s, 2 × DMT ipso arom. C), 158.88 (s, 6-C). **2**: ^{1}H NMR δ ($CDCl_3$) -0.21*, -0.20 and -0.04*, -0.01 (4 × s, $SiCH_3$), 0.76 (s, $Si\underline{C}(CH_3)_3$), 1.05*, 1.17, 1.18*, 1.20 (4 × d, *J* 6.5, 6.5, 7.0, 6.5 Hz, $NH(C\underline{H}_3)_2$), 2.30, 2.64* (2 × q, *J* 5.8, 5.7 Hz, $OCH_2C\underline{H}_2CN$), 2.95 (m, $SCH_2C\underline{H}_2CN$), 3.31, 3.39* (2 × dd (overlapping), *J* 11, 4 and 11, 4 Hz, 5',5"-H), 3.58-3.68 (m, $POCH_2$, SCH_2 and $N\underline{H}(CH_3)_2$, 3.79 (s, OCH_3), 3.87-3.90*, 3.94-3.98* (2 × m, $POCH_2$), 4.35-4.44 (m, 3',4'-H), 5.05, 5.05* (overlapping) (t, *J* 5.0 Hz, 2'-H), 6.03, 6.09* (2 × d, *J* 6.0, 6.5 Hz, 1'-H), 6.80*, 6.81 (2 × d, *J* 8.5, 8.0 Hz, Ph), 7.34*, 7.35 (2 × d (overlapping) *J* 9, 8.5 Hz, Ph), 7.45*, 7.46 (2 × d (overlapping) *J* 8, 7.5 Hz, Ph), 8.20*, 8.23, 8.60, 8.61* (4 × s, 2,8-H) (* differentiates the two diastereoisomers, where possible). ^{31}P NMR δ ($CDCl_3$) 150.2, 152.3.

13. The half-life for *S*-cyanoethyl deprotection of *S*-cyanoethyl-6-thioinosine in 1 M DBU in acetonitrile, at 20°C, was determined spectrophotometrically to be 41 ± 1 min.

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17. Base compostion analyses were performed with minor modifications as described by Eadie *et al.*[18] Shrimp alkaline phosphatase (USM) and phosphodiesterase (Boehringer Mannheim) were used. 2-Mercaptoethanol (1 mM) was essential during the enzyme digestion for detection of 6-thioinosine. A single peak was observed at 318 nm whose retention time corresponded to an authentic sample of 6-thioinosine.

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20. Circular dichroism spectra were measured using a JASCO model J-600 spectropolarimeter. RNA concentrations were 20 μM. Samples were prepared in 10 mM HEPES, pH 7.6, 150 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 10% (v/v) glycerol. Spectra were recorded using a 0.5 cm path-length cell at 20°C between 400 and 300 nm. GST-Rev was added to a two-fold molar excess. Any small effects that the addition of protein had were subtracted using the appropriate controls.

(*Received in UK* 27 *March* 1995; *accepted* 4 *May* 1995)