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INCORPORATION OF A FLUORESCENT NUCLEOTIDE INTO OLIGORIBONUCLEOTIDES

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Abstract: The fluorescent nucleoside 2-pyrimidinone-1-β-D-riboside (^{4H}C) has been incorporated into oligoribonucleotides using standard cyanoethyl phosphoramidite methods. This base provides a useful probe for the exocyclic amino function in cytidine. Cleavage of hammerhead ribozymes using GCGCCG-AAACACCGUGUCUCGAGC as the substrate and GGCUCGA[^{4H}C]UGAUGAGGCGC as a modified ribozyme resulted in a cleavage rate 17.5-fold slower than the unmodified analogue. The strand GGCUCG-ACUGA[^{4H}C]GAGGCGC represents an oligoribonucleotide in which a fluorescent base is introduced into a site non-essential for ribozyme function but is centrally located in the ribozyme's core of conserved nucleotides, thus providing a possible physical probe for ribozyme cleavage.

Introduction of modified nucleotides into oligoribonucleotides offers an opportunity to probe protein-oligoribonucleotide and oligoribonucleotide-oligoribonucleotide interactions.[1,2] 2-Pyrimidinone-1-β-D-riboside [^{4H}C (**1**)] differs from cytidine by substitution of 4-H for the exocyclic amine. In the case of uridine, 4-H is replaced by a carbonyl group and the adjacent nitrogen atom is deprotonated. The nett effect is a reversal of the H-bonding properties at these positions.

Both ^{4H}dC and 5-methyl-2-pyrimidinone-1-β-D-riboside (^{4H}T) are fluorescent.[3,4a] The analogous purine nucleoside 2-aminopurine-1-β-D-(2'-deoxyriboside), in which the 6-keto oxygen from 2'-deoxyguanosine has been replaced by a hydrogen atom, has been incorporated into oligodeoxynucleotides. Fluorescence studies yielded information on structure and molecular dynamics.[5,6]

McLaughlin *et al*[7] recently found that 2-pyrimidinone-2'-deoxynucleosides underwent facile glycosidic bond cleavage under mildly acidic conditions (pH 3.0) and ambient temperature. When incorporated into oligodeoxynucleotides an apyrimidinic/apurinic (abasic) site could be introduced at a preselected position.

This paper reports the synthesis of 2-pyrimidinone-1-β-D-(2'-*O*-*t*-butyldimethylsilyl-5'-*O*-dimethoxytritylriboside 3'-phosphoramidite) (**2**) and its incorporation into a 19-mer ribozyme at either positions 3 or 7 (Fig. 1) to give GGCUCGACUGA[^{4H}C]GAGGCGC (ORN-3) and GGCUCGA[^{4H}C]UGAUGAGGCGC (ORN-7). The rates of cleavage of the ribozyme substrate GCGCCGAAACACCGUGUC-UCGAGC by these modified ribozyme strands are reported and compared with the wild-type analogue. The fluorescence emission spectrum of ORN-7 is reported.

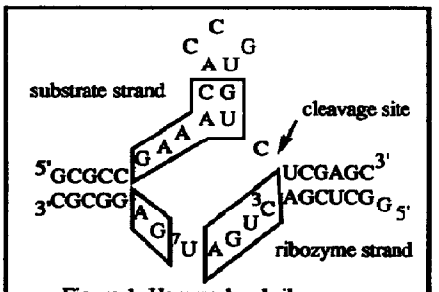
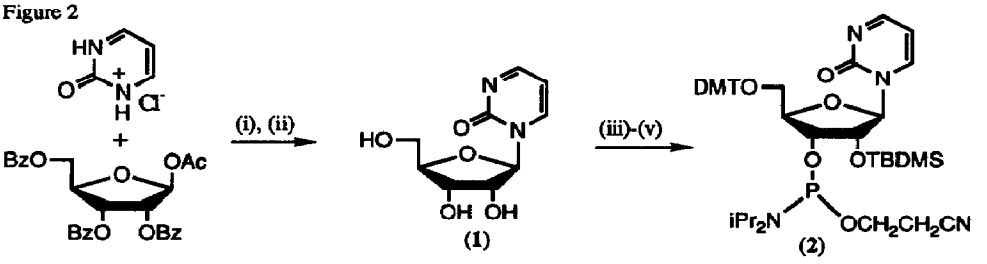


Figure 1 Hammerhead ribozyme
Nucleotides in boxes are essential for function

The 2'-deoxy analogues of ^{4H}C, namely ^{4H}dC and ^{4H}T, have been incorporated into oligodeoxyribonucleotides using triester[8,9] and phosphoramidite[4,10] methods. Two approaches to the synthesis of ^{4H}dC and ^{4H}T have been reported. The first involves glycosylation of the modified base,[11,12] while the second involves modifications of 2'-deoxycytidine[10] (or 2'-deoxyuridine[13,14]) and 2'-deoxythymidine[4,10,13] to afford ^{4H}dC and ^{4H}T, respectively.

Figure 2 shows the reaction scheme used to prepare the required phosphoramidite derivative **2**. The nucleoside ^{4H}C (**1**) was prepared from 2-pyrimidinone-1-β-D-(2',3',5'-tribenzoylriboside) which, in turn, was prepared from β-D-ribofuranose 1-acetate 2,3,5-tribenzoate and 2-hydroxypyrimidine hydrochloride using the one-pot glycosylation method (hexamethyldisilizane, TMS-Cl, $SnCl_4$, CH_3CN, 74%) described by Vorbrüggen and Bennua.[15] Deprotection of the benzoyl groups was achieved with either saturated (0°C) methanolic ammonia (sealed container, 0°C, 15 h, 87%) or methanolic NaOMe (RT, 2 d, 84%). Dimethoxytritylation, silylation and phosphitilation using standard procedures[16,17] afforded the corresponding phosphoramidite derivative **2** (52% yield from **1**).[18]

Figure 2



(i) Hexamethyldisilizane, TMS-Cl, $SnCl_4$, CH_3CN (ii) NH_3, MeOH (iii) 4,4'-dimethoxytrityl chloride, pyridine (iv) TBDMS-Cl, pyridine (v) $iPr_2NP(Cl)OCH_2CH_2CN$, DMAP, iPr_2NEt, CH_2Cl_2

Incorporation of **2** into the 19-mer 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-benzoylC was from Millipore. Cleavage from the support and deprotection of the exocyclic amine protecting 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 24 h. After evaporation the 2'-*O*-TBDMS groups were deprotected with NEt3.3HF at 30°C for 30 h.[19] Based on trityl assays the overall yields of the modified 19-mers were 62 and 65%, with average stepwise yields of 97.4 and 97.7% for ORN-3 and ORN-7, respectively. Incorporation of ^{4H}C was the similar [98.5 % (ORN-3), 99.2 % (ORN-7)] to that observed for the standard phosphoramidites. Both strands were purified by HPLC[20] 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. In both cases two major products were isolated with the required modified strands eluting last. The other product has not been identified but it may be due to 5,6-saturation of the 2-pyrimidinone by the addition of NH_3 across the double bond.[4a] It is possible that the use of faster base-deprotecting PAC (Pharmacia)[21] or FOD (ABI)[22] phosphoramidites may limit this problem thereby producing better yields of the required strands. No separation of the two major products was observed using PAGE. The final yields after HPLC purification of the modified 19-mers were 780 μg (19%) and 840 μg (20% based on the final trityl assay) for ORN-3 and ORN-7, respectively.

The UV spectra of the modified 19-mers contained small shoulders near 308 nm, which is characteristic of the 2-pyrimidinone.[11] Compositions of the modified 19-mers were confirmed by base composition analysis[23] with the modified nucleoside (**1**) eluting with a retention time identical to that of an authentic sample.

A number of modified oligoribonucleotides have been prepared in order to study the mechanism of hammerhead ribozyme cleavage.[24] Single turnover reactions, carried out in triplicate, were performed at 37°C in a volume of 40 μL 50 mM Tris-HCl, pH 7.3, with 5'-^{32}P-labelled substrate at a concentration of 0.2 μM and a ribozyme concentration of 1 μM. Cleavage was intiated by the addition of $MgCl_2$ to a final concentration of 10 mM. Aliquots of 4 μL were removed at 1 min intervals and quenched with 5 μL of 8 M urea and 50 mM EDTA.

The samples were analysed by PAGE on 20% denaturing gels and the resulting autoradiographs subjected to laser scanning densitometry. The half-life for cleavage of the modified oligoribonucleotides ORN-3 and ORN-7 were 4300 ± 100 and 300 ± 20 s, respectively. The unmodified strand cleaved with a half-life of 250 ± 20 s (Fig. 3). Previous studies have shown the importance of purine exocyclic amines by systematic substitution of guanosine and adenosine with inosine and purine riboside, respectively. The similar cleavage rates observed for ORN-7 and the native strand are consistent with other[25] results which showed that the 7-position is non-conserved. However, the slow cleavage rate observed for ORN-3 indicates the importance of the exocyclic amine in the cleavage reaction. It is likely that this amino group plays an important part in a hydrogen-bonding network. When the other natural nucleotides, which involve grosser changes than that for ^{4H}C, are incorporated into this position reductions in the cleavage rate were also observed (50-or 77-fold[26] for U; 250-fold for A; no cleavage for G).[27]

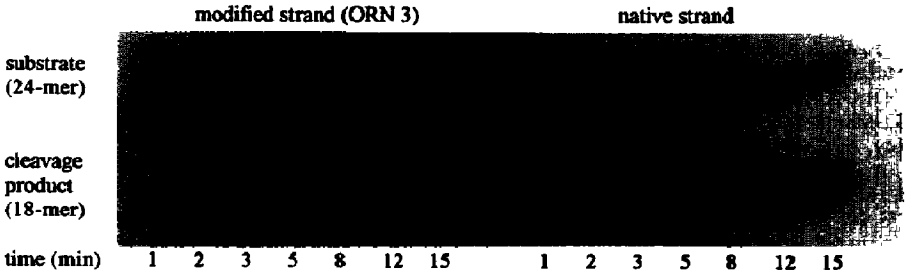


Figure 3. Autoradiograph of the cleavage of ORN-3 and the wild-type strand.

As mentioned, ORN-7 was cleaved at a rate similar to that observed for the native strand. This result combined with the proximity of ^{4H}C (1) to the conserved single-stranded residues of the central core makes ^{7}U a prime target for investigating possible fluorescence changes. Fluorescence emission spectra of unmodified and modified ORN-7 are shown in Figure 4. Oligoribonucleotides were excited at 298 nm. An emission maximum of 367 nm was observed for ORN-3. This value is similar to those recorded for ^{4H}dC and ^{4H}T.[3,4a] No change was observed in the fluorescence intensity of ORN-7 with addition of the 24-mer substrate. After annealing at 90°C for 5 min and cooling to 20°C a small (*ca.*10%) increase in the fluorescence intensity was observed. However, no significant change in wavelength was observed. The slight increase in quantum yield is consistent with a small decrease in the polarity of the environment surrounding ^{4H}C.[28]

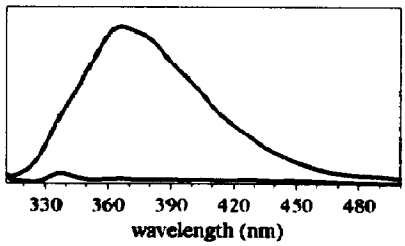


Figure 4. Fluorescence spectra of GGCUCGACUGA[^{4H}C]GAGGCGC (ORN-3) and GGCUCGACUGACGAGGCGC (12 μM solution of oligoribonucleotide in 100mM ammonium acetate)

In conclusion, the fluorescent 2-pyrimidinone-1-β-D-riboside (^{4H}C) has been incorporated into positions 3 and 7 in the ribozyme strand GGCUCGACUGAUGAGGCGC using standard phosphoramidite techniques. The modified nucleoside ^{4H}C, along with inosine and purine riboside, provide means by which exocyclic amine function can be examined. The 7-modified strand has little effect on the cleavage rate of the 24-mer

GCGCCGAAACACCGUGUCUCGAGC, whereas cleavage of the 3-modified strand was 17.5-fold slower than the native strand.

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18. Selected spectroscopic data: 2-Pyrimidinone-1-β-D-(5'-*O*-dimethoxytritylriboside): ^{1}H NMR δ ($CDCl_3$) 3.52 (2H, m, 5'-H), 3.78 (6H, s, OCH_3), 4.32, 4.34, 4.42 (3H, 2',3',4'-H), 5.92 (1H, s, 1'-H), 6.06 (1H, m, 5-H), 6.81-7.37 (13H, DMT PhH), 8.55 (2H, m, 4,6-H). ^{13}C NMR δ ($CDCl_3$) 55.20 (q, OCH_3), 61.73 (t, 5'-C), 69.83 (d, 2'-C), 75.83 (d, 3'-C), 84.58 (d, 4'-C), 86.90 (s, DMT O<u>C</u>Ar3), 93.01 (d, 1'-C), 104.71 (d, 5-C), 113.19, 113.48, 126.98, 127.91, 128.02, 129.97, 130.01 (d, DMT arom. C), 135.04, 135.25 (s, DMT. ipso arom. C), 144.18 (d, 6-C) 144.23 (s, DMT ipso arom. C), 156.43 (s, 2-C), 158.53 (s, DMT ipso arom. C(OMe)), 165.79 (d, 4-C). 2-Pyrimidinone-1-β-D-(2'-*O*-*t*-butyldimethylsilyl-5'-*O*-dimethoxytritylriboside): ^{1}H NMR δ ($CDCl_3$) 0.23, 0.37 (6H, 2 × s, $SiCH_3$), 0.94 (9H, s, Si{$C(CH_3)_3$}), 3.60 (2H, m, 5'-H), 3.80 (6H, s, OCH_3), 4.12 (1H, d, J 8.4 Hz, 4'-H), 4.31 (1H, d, J 4.2 Hz, 2'-H), 4.41 (1H, dt, J 8.4, 4.2 Hz, 3'-H), 5.86 (1H, s, 1'-H), 5.88 (1H, dd, J 6.6, 3.6 Hz, 5-H), 6.85 (4H, d, J 6.6 Hz, arom. H), 7.25-7.43 (9H, m, arom. H), 8.50 (1H, dd, J 3.6, 2.4 Hz, 6-H), 8.65 (1H, dd, J 6.6, 2.4 Hz, 4-H). ^{13}C NMR δ ($CDCl_3$) -5.49, -4.32 (q, $SiCH_3$), 18.05 (s, Si<u>C</u>$(CH_3)_3$), 18.32 (t, <u>C</u>H_2CN), 55.19 (q, OCH_3), 60.85 (t, 5'-C), 68.53 (d, 2'-C), 76.42 (d, 3'-C), 83.03 (d, 4'-C), 86.98 (s, DMT O<u>C</u>Ar3), 91.10 (d, 1'-C), 103.84 (d, 5-C), 113.20, 127.03, 127.93, 128.10, 130.03, 130.09 (d, DMT arom. C), 135.03, 135.29 (s, DMT. ipso arom. C), 143.94 (d, 6-C) 144.24 (s, DMT ipso arom. C), 155.31 (s, 2-C), 158.55, 158.58 (s, DMT ipso arom. C(OMe)), 165.78 (d, 4-C). 2-Pyrimidinone-1-β-D-(2'-*O*-*t*-butyldimethylsilyl-5'-*O*-dimethoxytritylriboside 3'-phosphoramidite (2): ^{1}H NMR δ ($CDCl_3$) ^{1}H NMR: δ ($CDCl_3$) 0.18, 0.31 (6H, 2 × s, $SiCH_3$), 0.92 (9H, s, Si{$C(CH_3)_3$}), 1.10, 1.15 (12H, 2 × d, J 6.9 Hz, NCH($C\underline{H}_3$)$_2$), 2.42 (2H, t, J 6.5 Hz, CH_2CN), 3.50-3.89 (m, 5'-H, OCH_2, NC<u>H</u>$(CH_3)_2$), 3.81 (6H, s, OCH_3), 4.32-4.41 (3H, m, 2',3',4'-H), 5.80 (1H, s, 1'-H), 5.87 (1H, dd, J 4.2, 6.6 Hz, 5-H), 6.86 (4H, d, J 8.4 Hz, PhH), 7.27-7.50 (9H, m, PhH), 8.52 (1H, dd, J 4.2, 2.4 Hz, 6-H), 8.72 (1H, dd, J 6.6, 2.4 Hz, 4-H).
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