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Incorporation of methylated pyrimidine analogues into RNA

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Abstract

Routes for the preparation of 2'-silyl protected phosphoramidites of the modified nucleosides O^2-methyluridine and O^4-methyluridine are described. Methodology for the site-specific incorporation of these phosphoramidites into a 25 nucleotide long oligoribonucleotide sequence by solid-phase synthesis is reported.

Keywords: Nucleic acid analogues; Solid-phase synthesis; Pyrimidines.

Since the discovery of catalytic RNA in 1982 there has been considerable interest in structural and mechanistic studies of ribozymes particularly due to their potential as therapeutic agents[1, 2] and as a prebiotic candidate for the progenitor of biological catalysis[3]. Complimentary to X-ray crystallographic and NMR structural studies[4] in developing models of ribozyme action, is the use of nucleoside analogues to probe the role of individual functional groups critical for activity[1, 5]. Base-modified analogues are perhaps the most obvious target due to the density of accessible hydrogen bond donor and acceptor sites that reside on the heterocycle and such analogues have been used to comprehensively map the contributions of functional groups on conserved purines to hairpin ribozyme activity[6]. In contrast, the roles of the functional groups on conserved pyrimidines in this system have not been examined and in general, the number of reports of pyrimidine analogues used in such RNA structure-function analysis has been more limited than that described for purines.

One of the simplest strategies for the mutation of functional groups is methylation. This has the effect of removing a hydrogen bond donor site from nitrogen on either the heteroatom itself or at a conjugated site (in the case of lactam carbonyl methylation) whilst introducing minimal steric perturbation. O-Alkylation of deoxythymidine has been the focus of considerable study due to its potential carcinogenic effects and therefore the chemistry for site-specific incorporation of O^4- and O^2-alkylated pyrimidines into oligodeoxyribonucleotides is well developed. However, oligoribonucleotides containing the corresponding ribonucleoside analogues (O^4-methyluridine and O^2-methyluridine) have not (to the authors' knowledge) been previously reported. Herein we describe protocols for the synthesis of monomers of these analogues and their incorporation into RNA by phosphoramidite chemistry

O^4-Methylthymidine has been incorporated into oligonucleotides both as its

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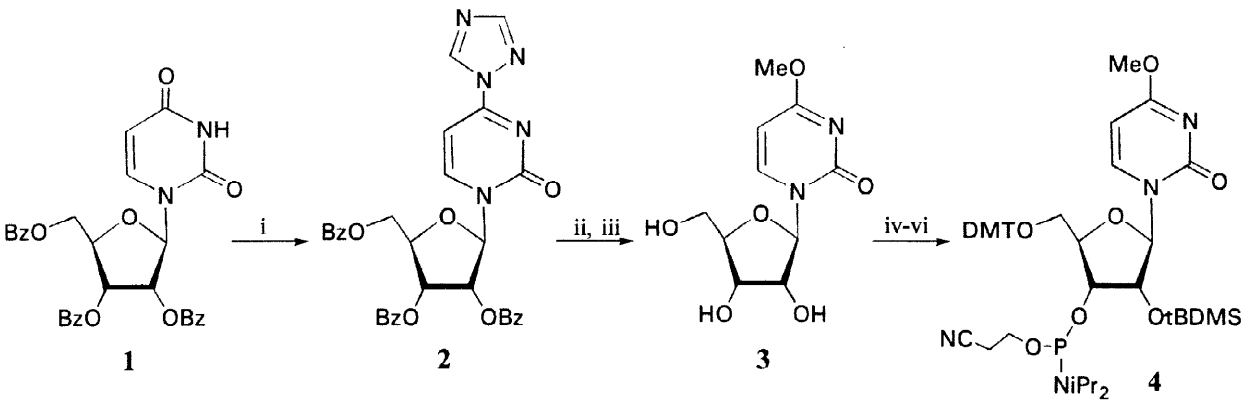


Scheme 1. Reagents and conditions: i) 15 eq. 1,2,4-triazole, 3 eq. $POCl_3$, 18 eq. Et_3N, MeCN, 0°C addition, r.t. 1.5h; ii) 20 eq. MeOH, 1.1 eq. DBU, MeCN, 0°C addition, r.t. 40 min 59% from **1**; iii) 3.2 eq. NaOMe, MeOH, r.t. 80 min 72%; iv) 1.6 eq. DMT-Cl, pyridine/DCM 2.4/1, r.t. 30 min, 88%; v) 1.8 eq. tBDMS-Cl, 1.4 eq. $AgNO_3$, THF/pyridine 10/1, r.t. 1.75h, 56%; vi) 1.75 eq. 2-cyanoethyl *N,N*-diisopropyl chlorophosphoramidite, 4 eq. DIPEA, r.t. O/N, 79%.

phosphoramidite[7-9] and by post-synthetic substitution of a triazolide[10, 11]. We adopted the former strategy and applied the same basic chemistry described by Xu and Swann for the synthesis of the deoxythymidine monomer[8] to that of the suitably protected O^4-methyluridine (O^4MeU) phosphoramidite (**4**) (Scheme 1).

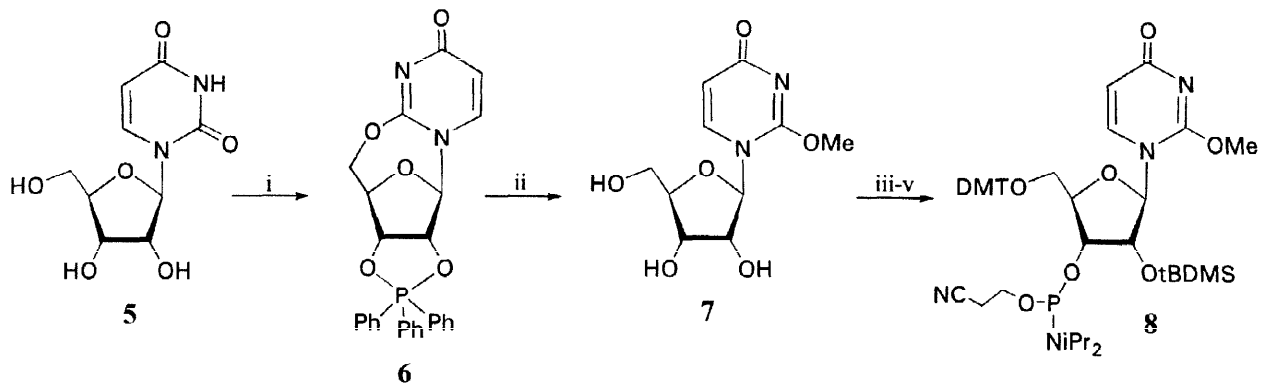
Thus, the fully ribose protected 2',3',5'-tri-*O*-benzoyluridine (**1**) was derivatised at the 4 position with triazole which was then displaced with methanol/DBU. The use of base-labile protecting groups in this procedure, rather than the silyl ethers described for dT, led to some reduction in yield due to debenzoylation at this stage and also in their subsequent removal by sodium methoxide to give **3** due to hydrolysis of the O^4-methyl moiety. The susceptibility of O^4-methyl derivatised pyrimidines to alkali hydrolysis is well documented[7, 9] and care therefore had to be taken to maintain anhydrous conditions during manipulations involving base - particularly quenching of the excess methoxide (Dowex - H^+ was washed with dry MeOH and stored over silica gel *in vacuo*). **3** was isolated with contaminating uridine (4%) due to this hydrolysis but this impurity was readily removed by silica gel chromatography following tritylation[3]. It is anticipated that performing displacement and deprotection steps simultaneously using excess sodium methoxide should improve the overall yield of **3** from **1**. Tritylation of **3** by dropwise addition of DMT-Cl in DCM/pyridine[12], silver mediated silylation[4][13] and phosphitylation[14] gave the appropriate phosphoramidite (**4**)[5].

O^2-Methyluridine (O^2MeU) has previously been prepared from 5'-deoxy-5'-iodo-2',3'-isopropylideneuridine by silver acetate treatment followed by base mediated methanolysis of the resultant $O^{2,5'}$-cyclonucleoside and deprotection of the isopropylidene moiety[15] and a similar strategy has been employed by Xu and Swann for the synthesis of O^2-methyldeoxythymidine[16]. However, we used the more succinct route directly from uridine

[3] A small sample of **3** was purified by silica gel chromatography and fully characterised to provide an authentic sample for enzymatic digestion. **3**: ^{1}H NMR (d_6-DMSO) δ, 8.33 (1H, d, H6), 6.07 (1H, d, H5), 5.78 (1H, d, H1'), 5.50 (1H, d, OH), 5.21 (1H, t, 5'OH), 5.06 (1H, d, OH), 3.97 (2H, m, H2', H3'), 3.90 (1H, m, H4'), 3.84 (3H, s, CH_3O), 3.79 - 3.52 (2H, m, H5', H5"), ^{13}C NMR (d_6-DMSO) δ, 171.55, 155.45, 144.73, 95.05, 90.31, 84.63, 74.94, 69.28, 60.44, 54.28, FAB+ ms e/z 259 (98%, M+H), 127 (base +2H) ,UV (H_2O) λ max. 275, 204 nm

[4] ^{1}H NMR δ ($CDCl_3$), 8.28 (1H, d, H6), 7.43 - 7.20 (9H, m, ArH), 6.83 (4H, d, ArH *o* to OMe), 5.86 (1H, s, H1'), 5.48 (1H, d H5), 4.35 (1H, m, H3'), 4.27 (1H, m, H2'), 4.06 (1H, m, H4'), 3.93 (3H, s, CH_3O-U), 3.79 (6H, s, 2x CH_3O-Ar), 3.60 - 3.46 (2H, m, H5', H5"), 2.38 (1H, d, OH), 0.92 (9H, s, $(CH_3)_3C$), 0.31 (3H, s, CH_3Si), 0.19 (3H, s, CH_3Si), FAB+ ms e/z 675 (M+H)

[5] **4**: ^{31}P NMR δ ($CDCl_3$), 150.58, 149.47; FAB+ ms e/z 875 (M+H)



Scheme 2. Reagents and conditions: i) 3 eq. Ph_3P, 3eq. DEAD, THF, r.t. 1.5h,; ii) MeOH reflux, 13h, 61% from **5**; iii) 1.5 eq. DMT-Cl, pyridine/DCM 2.5/1, r.t. 30 min, 83%; iv) 2.5 eq. tBDMS-Cl, 9 eq. Imidazole, DMF, r.t. 5.5h; 52% v) 2.6 eq. 2-cyanoethyl bis(*N,N*-diisopropylamino)phosphoramidite, 1 eq. 1H-tetrazole, r.t., 36h, 82%.

described by Kimura *et al.*[17] (Scheme 2): under Mitsunobu conditions, uridine is simultaneously protected at the 2'- and 3'-hydroxyl groups and cyclised to form the $O^{2,5'}$-cyclonucleoside phosphorane (**6**). As described in this paper, we found no need for exogenous base to effect nucleophilic attack of the alcohol at the 2-position to give **7**[6].

Tritylation[12] of **7** proceeded smoothly, although subsequent silylation under the same conditions used for the O^4-methylated nucleoside[13] gave over 60% demethylation (of recovered material) and the products could only be resolved to a limited extent by silica gel chromatography. Subsequent work has shown that this degradation occurs in the absence of silver ions but not without tBDMS-Cl and is inhibited by triethylamine. No demethylation was encountered using imidazole mediated silylation in DMF[7][14]. Trial reactions on the 5'- and 2'-protected O^2MeU revealed that Hunig's base in the absence of phosphitylating agent gave significant silyl isomerisation although when using 2-cyanoethyl bis(*N,N*-diisopropylamino)phosphoramidite and tetrazole[18] no isomeric phosphoramidite was observed in the purified product (**8**)[8].

Following these results, the susceptibilities to demethylation of both bases (O^4-methyluracil and O^2-methyluracil) under the conditions of RNA synthesis were investigated by exposing the tritylated nucleosides to deblocking and oxidation reagents and monitoring the reactions by tlc. No demethylation was observed overnight at room temperature. However, studies on the desilylation of the fully protected nucleosides with TBAF and $Et_3N.3HF$ indicated that both positions were subject to greater demethylation in 1M TBAF in THF compared to $Et_3N.3HF$ and the O^2MeU analogue appeared more susceptible to both reagents as has previously been observed for thiophenolate mediated demethylation[7].

Sequence **1** (**S1** - Figure) is a 25-mer oligoribonucleotide which forms half of loop B of a 3-stranded *trans*-cleaving hairpin ribozyme with the sites of conserved pyrimidines labelled (**X**, **Y**). The phosphoramidite analogues **4** and **8** were incorporated at these sites using standard

[6] **7**: ^{1}H NMR (d_6-DMSO) δ, 8.01 (1H, d, H6), 5.86 (1H, d, H5), 5.71 (1H, d, H1'), 5.53 (1H, d, OH), 5.21 - 5.14 (2H, m, 2x OH), 4.07 (1H, m , H2') 3.97 (1H, m, H3'), 3.90 - 3.84 (1H, m, H4'), 3.88 (3H, s, CH_3O), 3.73 - 3.50 (2H, m, H5', H5"); ^{13}C NMR (d_6-DMSO) 170.22, 156.26, 138.79, 108.38, 89.93, 85.65, 74.65, 70.06, 61.02, 55.79; uv (H_2O) λ max. 252, 228; FAB+ ms e/z 259 (100%, M+H)

[7] ^{1}H NMR δ ($CDCl_3$), 7.86 (1H, d, H6), 7.40 - 7.20 (9H, m, ArH), 6.83 (4H, d, ArH *o* to OMe), 5.91 (1H, d, H1'), 5.67 (1H, d, H5), 4.39 (1H, m, H3'), 4.30 (1H,m, H2'), 4.16 (1H, m, H4'), 4.00 (3H, s, CH_3O-U), 3.79 (6H, s, 2x CH_3O-Ar), 3.53 - 3.45 (2H, m, H5', H5"), 2.61 (1H, d, OH), 0.90 (9H, s, $(CH_3)_3C$), 0.10 (3H, s, CH_3Si), 0.03 (3H, s, CH_3Si), FAB+ ms e/z 675 (M+H)

[8] **8**: FAB+ ms e/z 875 (M+H); ^{31}P NMR δ ($CDCl_3$), 151.18, 150.18

5' UCA GGU CGU GGU ACA **XYA** CCU GGU A 3'

Figure. Sequences synthesised using modified and unmodified bases.
S1..**X**=**Y**=U; **S2**..**X**= O^4MeU, **Y**=U; **S3**..**X**=U, **Y**= O^4MeU; **S4**..**X**= O^2MeU, **Y**=U; **S5**..**X**=U, **Y**= O^2MeU.

RNA chemistry to give mutated RNA sequences **S2** - **S5**.[9]

Several O-methylated DNA and RNA sequences have previously been prepared and the use of DBU/methanol is well established for the initial deprotection of such oligonucleotides and this procedure was also adopted for **S2** - **S5**. However, the use of aqueous neutralisation conditions[7, 14] was found to give significantly greater hydrolysis products (especially the O^2 demethylated sequences) than Dowex-50 - H^+ which had been washed with dry methanol and stored in a desiccator over silica gel. Following such treatment, standard desilylation and isolation protocols[19] were followed and the crude oligonucleotides purified initially by PAGE and then RP-HPLC[10] in order to remove contaminating **S1** which was observed in all other sequences and is assumed to be a result of hydrolysis of the modified base. **S2** - **S5** were characterised by nucleoside composition analysis and coinjection of the enzyme digest with standard samples of O^2MeU or O^4MeU. The effect of these substitutions on the activity of the hairpin ribozyme is currently under investigation.

In conclusion, nucleobase functional group mutations have been shown to be provide insight into the locations and roles of functionalities critical to the activity of catalytic RNA sequences and as a consequence, this powerful technique is being widely applied. It is envisaged that the introduction of the O^2-methyluridine and O^4-methyluridine into such sequences should expand the range of structure-function studies that can be performed.

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[9] Syntheses were performed on a 1μmol scale with double coupling at the site of modification. On-line trityl monitoring was performed at every step and average step-wise coupling yields ranged from 97 - 99% for all additions except that of the O^2MeU phosphoramidite which only coupled at 65 - 67% efficiency at both X and Y positions.

[10] Yields: **S1** 0.097 μmol; **S2** 0.011 μmol; **S3** 0.007 μmol; **S4** 0.005 μmol; **S5** 0.005 μmol; further data available on request.