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Site-specific incorporation of 5-methylaminomethyl-2-thiouridine and 2-thiouridine(s) into RNA sequences

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ABSTRACT

We present a reproducible protocol for the site-specific incorporation of 5-methylaminomethyl-2-thiouridine (mnm^5s^2U) into a model RNA fragment and, together with 2-methyladenosine (m^2A), into the native sequence of the *Escherichia coli* $tRNA^{Glu2}$ anticodon arm (*E. coli* ASL^{Glu2}). This approach is also utilized for the synthesis of oligomers modified with multiple 2-thiouridines.

2-Thiouridine (s^2U), its 2′-*O*-methyl analog (s^2Um), and variously 5-substituted 2-thiouridines (s^2U^*) are components of cytosolic and mitochondrial tRNAs sequences[1] and control fundamental cellular processes,[2] while their absence results in serious diseases.[3] The s^2U/s^2U^* modified tRNA fragments have been utilized, among others, in mutually related model studies on the impact of post-transcriptional modifications on RNA conformation/tertiary structure dynamics,[4] and on the effectiveness of homo- (RNA–RNA) and heterotropic (RNA–protein) biopolymer interactions with other partners of the ribosomal decoding machinery,[5,6] or of the HIV-1 RNA reverse transcription process.[7,8]

The superior hybridization and base discrimination abilities of oligoribonucleotides modified with s^2U/s^2Um compared to non-modified oligomers[9,10] has encouraged their use in 'microarray' construction and probing, in this way, the RNA 2-D structure,[11] and also for the development of potential therapies based on selective gene silencing.[12]

Polymer-supported synthesis of oligoribonucleotides by phosphoramidite chemistry necessitates a P(III) to P(V) oxidation step. Under typical oxidizing conditions, 2-thiouridine and its 5-substituted derivatives undergo oxidation into the 2-oxo-analogs and/or oxidative desulfurization into the respective 1-β-D-ribofuranosyl-1*H*-pyrimidin-4-one.[13–15] The scope and nature of these processes depend not only on the oxidant, but also on the type of 5-substituents.[13] Numerous protocols for the incorporation of s^2U/s^2U^* into oligomers by solid-phase synthesis have been reported,[10,11,14,16–20] however none of them offers a general approach.

Until now the most effective oxidation systems have been elaborated for incorporation of 5-methoxycarbonylmethyl-2-thiouridine (1 M solution of cumene hydroperoxide in toluene)[20] and 2′-*O*-methyl-2-thiouridine(s) (0.02 M solution of iodine in pyridine-H_2O).[14]

5-Methylaminomethyl-2-thiouridine (mnm^5s^2U) reveals significant sensitivity to the oxidation step. The mnm^5s^2U modified sequence of *Escherichia coli* ASL^{Lys} was prepared using a 1 M solution of *tert*-butyl hydroperoxide as oxidant in acetonitrile.[18] We were not able to repeat this synthesis, however, and a similar observation was reported by another laboratory.[21]

In this communication, we present a reliable approach for the incorporation of mnm^5s^2U into synthetic RNA sequences. The utility of this protocol has been confirmed by the synthesis of model oligomers modified with mnm^5s^2U, with site-specifically located s^2U unit(s) and the native sequence of *E. coli* ASL^{Glu2} ($mnm^5s^2U_{34}$, m^2A_{37}). It is noteworthy, that the mnm^5s^2U is located only in the tRNA sequences from prokaryotes. Thus, the synthetic *E. coli* ASL^{Glu2} and ASL^{Lys} fragments containing $mnm^5s^2U_{34}$ could be employed in the automated system for the selection of potential therapeutics against antibiotic-resistant *E. coli* strains.[22]

Considering the previously cited literature,[13,14,20] the stability of a monomeric unit derived from mnm^5s^2U (**1**) in a 0.02 M solution

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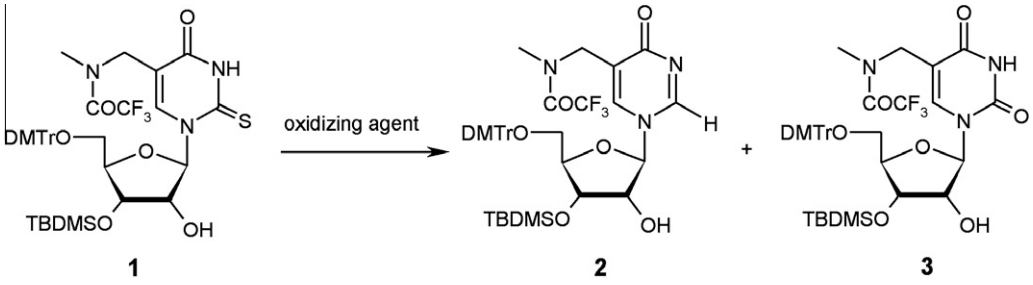

Scheme 1. The products of oxidation and/or oxidative desulfurization of mnm^{5}s^{2}U derivative **1**.

Table 1
Sensitivity of **1** toward oxidants (8 equiv) under various reaction conditions (concentration, solvent, time of reaction)

Entry	Conditions	Time (min)	Product[a] (%)		Recovery of 1 (%)
			2	**3**	
1	0.25 M *t*-BuOOH in toluene	40	2	2	90
2	0.5 M *t*-BuOOH in toluene	10	5	2	85
3	1 M *t*-BuOOH in toluene	2	5	2	84
4	0.05 M *t*-BuOOH in MeCN	2	nd	25	60
5	0.02 M I_2 in THF/H_2O/pyridine 7/1/2 (v/v/v)	2	50	nd	40
6	0.02 M I_2 in MeCN/H_2O/pyridine 7/1/2 (v/v/v)	2	70	nd	20

[a] Yield of isolated product. nd = not detected.

Table 2
MALDI-TOF data for the synthesized oligoribonucleotides and yield of the products synthesized on a 0.2 μmol scale

Oligonucleotide Sequences	Calcd. $[M-H]^-$	Found	Yield[b] [OD_{260}]
5′-UUUUUUUU-3′[a]	2385.2	2385.8	7.8
5′-UUUUUUU**s^{2}U**U-3′	2707.2	2709.1	7.9
5′-U**s^{2}U**UU**s^{2}U**UU**s^{2}U**U-3′	2739.2	2739.7	5.2
5′-UUUUUUU**mnm^{5}s^{2}U**UUUU-3′	3668.4	3671.0	4.2
5′-CCGCCCU**mnm^{5}s^{2}U**UC**m^{2}A**CGGCGG-3′	5422.8	5422.9	4.0

[a] Reference sequence.
[b] Yield of oligoribonucleotide was determined by measurement of the UV absorbance at 260 nm, using a 1 cm path length quartz cuvette.

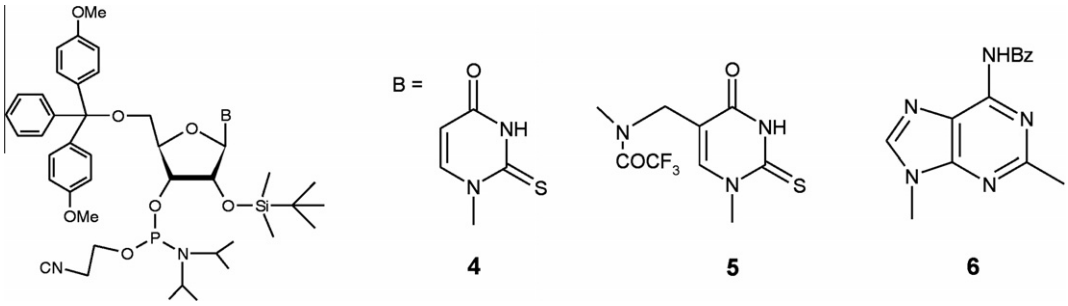

Figure 1. Structures of the fully protected 3′-*O*-phosphoramidites of s^{2}U (**4**), mnm^{5}s^{2}U (**5**) and m^{2}A (**6**).

of iodine in THF/H_2O/pyridine and, alternatively, in acetonitrile/H_2O/pyridine, as well as in the presence of various concentrations of *tert*-butyl hydroperoxide in acetonitrile or toluene, was tested (Scheme 1), with the results listed in Table 1. Compound **1** was obtained as a side-product during the preparation of fully protected mnm^{5}s^{2}U 3′-*O*-phosphoramidite.[23] Under oxidizing conditions, derivative **1** undergoes transformation into 2-oxo analog **3** and/or the product of oxidative desulfurization **2** (Scheme 1). Both compounds **2** and **3** were synthesized independently, and their structures were confirmed by standard methods.[24,25]

Treatment of **1** with 0.02 M I_2 in acetonitrile/H_2O/pyridine or in THF/H_2O/pyridine solution gave only the product of oxidative desulfurization **2**, and its yield was notably higher in more polar solvents (Table 1, entries 5 and 6).

Nucleoside **1** remained unchanged for a relatively long time in a 0.25 M solution of *tert*-butyl hydroperoxide in toluene, but at higher oxidant concentrations the yield of **2** increased (Table 1, entries 1–3). It is noteworthy that even a dilute solution of *tert*-butyl hydroperoxide in acetonitrile gave **3** as the sole product with a notable yield of 25% (Table 1, entry 4).

The results of these model studies favor a dilute solution of *tert*-butyl hydroperoxide in toluene as the most suitable oxidant for solid-phase supported incorporation of mnm^{5}s^{2}U into oligoribonucleotides using phosphoramidite methodology.

Some aspects of the mechanism of s^2U/s^2U^* oxidation/desulfurization have been studied, but only the pathway involving selective desulfurization of s^2U upon treatment with 2-phenylsulfonyloxaziridine has been investigated in detail and discussed so far.[15] Based on literature and the results of model studies, the degrees of sensitivity of s^2U/s^2U^* to oxidizers could be arranged in the ascending order as follows: $m^5s^2U < mcm^5s^2U \leqslant s^2U \ll mnm^5s^2U < mo^5s^2U$. This ranking may reflect the tendency of s^2U derivatives toward enolization. A shift toward the enol form has been recorded for mo^5U[26] and mo^5s^2U,[27] while mnm^5s^2U crystallizes as a zwitterion.[28] Protonation of the side-chain amine function of mnm^5s^2U under neutral conditions was postulated, and, based on $pK_a < 8$ estimated for mnm^5s^2U, it can be expected that a considerable fraction of this nucleoside is ionized under physiological conditions.[29] Blocking the side-chain amine function of **1** with a strong electron-withdrawing $CF_3C(O)$-group can induce a charge distribution in the heterocyclic moiety similar to that originating from the above-mentioned protonation process. This effect can be responsible for the enhanced tendency of the mnm^5s^2U-derived monomeric unit to enolization, and consequently to oxidation/oxidative desulfurization processes during the P(III) to P(V) oxidation step. The substantial reduction of *tert*-butyl hydroperoxide degradation to the active oxygen species in toluene compared to CH_2Cl_2,[30] and the diminished tendency of **1** to ionization in a non-polar aprotic solvent would be expected to decrease the sensitivity of nucleoside **1** to oxidation.

A solution of 0.25 M *t*-BuOOH in toluene was successfully applied to the solid-phase supported synthesis (phosphoramidite methodology) of model oligoribonucleotides modified with mnm^5s^2U or s^2U unit(s) and double modified *E. coli* ASL^{Glu2}, with site-specifically located $mnm^5s^2U_{34}$ and m^2A_{37} (Table 2).

The 5′-*O*-DMTr, 2′-*O*-TBDMS protection strategy for the phosphoramidites of s^2U (**4**), mnm^5s^2U (**5**) and m^2A (**6**) was employed (Fig. 1). Compounds s^2U and mnm^5s^2U were synthesized according to previously reported methods.[31,32] 2-Methyladenosine (m^2A) was prepared as follows: peracetylated guanosine was chlorinated to give 2-amino-6-chloro-9-(2′,3′,5′-tri-*O*-acetylribofuranosyl) purine,[33] and this compound was converted into 2-iodoadenosine.[34] Subsequently, 2-iodoadenosine was alkylated with trimethylaluminium in the presence of a palladium catalyst to give 2-methyladenosine (m^2A).[35] The side-chain amine function of m^2A was protected with a benzoyl group as previously described.[36] Standard procedures were employed for final protection of the 5′-*O*- and 2′-*O*-functions with DMTr and TBDMS groups, respectively, as well as for 3′-*O*-phosphitylation.[18,36,37] The analytical and spectral data of monomer units **4–6** were identical with those reported in the literature.[36,38]

The synthesis of the RNA sequences was conducted automatically on a 0.2 μmol scale using commercial Pac(tac)-protected phosphoramidites (Proligo®) and standard coupling chemistry with the exception of *t*-BuOOH oxidation.[39] Oligoribonucleotides were deprotected according to a slightly modified Sproat protocol.[40] The fully deprotected oligomers were precipitated and purified by anion-exchange HPLC (see Supplementary data). The integrity of the synthesized oligoribonucleotide sequences was confirmed by analytical HPLC and MALDI-TOF mass spectrometry (Table 2).

In conclusion, under oxidizing conditions, the heterocyclic moieties of 2-thiouridine derivatives undergo side-reactions, and consequently the P(III) to P(V) oxidation is a critical step for the solid-phase supported synthesis of oligoribonucleotides containing s^2U/s^2U^*. Protected 5-methylaminomethyl-2-thiouridine appeared to be very sensitive to the standard oxidants used in the phosphoramidite methodology, however it remains intact in a dilute solution of *tert*-butyl hydroperoxide in toluene. Based on these observations, a reliable approach to the synthesis of model oligoribonucleotides modified with mnm^5s^2U as well as the native sequence of the *E. coli* $tRNA^{Glu2}$ anticodon arm bearing mnm^5s^2U and m^2A have been elaborated. The same procedure can be used for the synthesis of tRNA fragments modified with mnm^5s^2U in the presence of t^6A, as well as for the incorporation of several 2-thiouridines into oligoribonucleotide sequences.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2011.12.079.

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23. 5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-5-(*N*-trifluoroacetyl)methylaminomethyl-2-thiouridine (**1**). Readily available 2-thiouridine[31] was converted into 2′,3′-*O*-isopropylidene-5-methylaminomethyl-2-thiouridine via the 5-chloromethyluridine derivative, as described previously.[32a] The heterobase side chain was efficiently protected with a trifluoroacetyl group and the 2′,3′-*O*-acetonide was removed under mild acidic conditions to give an N-protected nucleoside. The 5′-*O*-DMTr, 3′-*O*-TBDMS and 5′-*O*-DMTr, 2′-*O*-TBDMS masked nucleosides were then synthesized according to the standard procedure[18] and separated by silica gel chromatography. 5′-*O*-DMTr, 3′-*O*-TBDMS isomer: TLC, $R_f = 0.71$ (CH_2Cl_2/acetone 9:1 v/v); ^1H NMR ($CDCl_3$, 250 MHz): $\delta = -0.13$ (s, 3H, $SiCH_3$), 0.00 (s, 3H, $SiCH_3$), 0.81 (s, 9H, $Si(CH_3)_3$), 3.22 (s, 3H, NCH_3), 3.45–3.59 (m, 4H, 5-CH_2, H5′, H5″), 3.78 (s, 6H, 2xOCH_3), 4.17–4.20 (m, 3H, H2′, H3′, H4′), 6.66 (d, 1H, $J = 2.5$ Hz, H1′), 6.80–7.48 (m, 13H, Ar), 8.02 (s, 1H, H6); FAB MS *m*/*z* for $C_{40}F_3H_{48}N_3O_8SiS$ calcd 815, found 814.3 $[M-H]^-$. 5′-*O*-DMTr, 2′-*O*-TBDMS isomer: TLC $R_f = 0.80$ (CH_2Cl_2/acetone 9:1 v/v); ^1H NMR ($CDCl_3$, 250 MHz): $\delta = 0.12$ (s, 3H, $SiCH_3$), 0.14 (s, 3H, $SiCH_3$), 0.91 (s, 9H, $Si(CH_3)_3$), 3.21 (s, 3H, NCH_3), 3.45–3.59 (m, 9H, 5-CH_2, 2xOCH_3, H5′), 4.09–4.22 (m, 2H, H3′, H4′), 4.40–4.44 (m, 1H, H2′), 6.75 (d, 1H, $J = 4.75$ Hz, H1′), 6.81–7.48 (m, 13H, Ar), 7.98 (s, 1H, H6).
24. 5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-1-(β-D-ribofuranosyl)-5-(*N*-trifluoroacetyl)methylaminomethyl-4-pyrimidinone (**2**). Nucleoside **1** (50 mg, 0.061 mmol) was dissolved in a mixture of anhydrous CH_2Cl_2 (500 μl) and anhydrous pyridine (120 μl) and then 0.2 M *m*CPBA in CH_2Cl_2 (602 μl) was added. The solution was stirred at room temperature for 4 h. After TLC analysis ($R_f = 0.12$; CH_2Cl_2/acetone 9:1 v/v), the mixture was washed with 5% aq. $NaHCO_3$ (500 μl), dried over $MgSO_4$ and evaporated under reduced pressure. The crude product was

co-evaporated with anhydrous toluene and purified by silica gel column chromatography, using $CHCl_3$ as the eluent. Yield 82%. ^{1}H NMR (C_6D_6, 700 MHz): δ = 0.04 (s, 3H, $SiCH_3$), 0.15 (s, 3H, $SiCH_3$), 0.91 (s, 9H, $Si(CH_3)_3$), 2.93 (s, 3H, NCH_3), 3.35 (s, 6H, 2xOCH_3), 3.37 (dd, 1H, J = 3.5 Hz, 11.2 Hz, H5''), 3.65 (dd, 1H, J = 3.5 Hz, 11.2 Hz, H5′), 3.87 (d, 1H, J = 14.0 Hz, 5-CH_2), 3.89 (d, 1H, J = 14.0 Hz, 5-CH_2), 4.09–4.11 (m, 1H, H4′), 4.10 (dd, 1H, J = 3.5 Hz, 6.3 Hz, H3′), 4.35–4.36 (m, 1H, H2′), 5.14 (d, 1H, J = 6.3 Hz, H1′), 6.85–7.65 (m, 13H, Ar), 7.81 (s, 1H, H6), 8.05 (br s, 1H, H2); FAB MS m/z for $C_{40}F_3H_{48}N_3O_8Si$ calcd 783, found 784.5 $[M+H]^+$; 782.3 $[M-H]^-$.

25. 5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-5-(*N*-trifluoroacetyl)-methylaminomethyluridine (**3**) was prepared according to the previously described method[36] and protected in the same way as the 2-thio analog **1**. TLC, R_f = 0.33 (CH_2Cl_2/acetone 9:1 v/v); ^{1}H NMR (C_6D_6, 700 MHz): δ = −0.09 (s, 3H, $SiCH_3$); −0.01 (s, 3H, $SiCH_3$), 0.77 (s, 9H, $Si(CH_3)_3$), 2.85 (s, 3H, NCH_3), 3.34 (s, 6H, 2xOCH_3), 3.44 (dd, 1H, J = 4.0 Hz, 11.25 Hz, H5''), 3.47 (d, 1H, J = 14 Hz, 5-CH_2), 3.63 (dd, 1H, J = 2.8 Hz, 11.25 Hz, H5′), 3.74 (d, 1H, J = 14.0 Hz, 5-CH_2), 4.13–4.15 (m, 1H, H4′), 4.19 (dd, 1H, J = 4.2 Hz, 8.4 Hz, H3′), 4.43–4.45 (m, 1H, H2′), 5.14 (d, 1H, J = 3.5 Hz, H1′), 6.81–7.70 (m, 13H, Ar), 7.89 (s, 1H, H6); FAB MS m/z for $C_{40}F_3H_{48}N_3O_9Si$ calcd 799, found 798.3 $[M-H]^-$.
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39. The oligoribonucleotides were synthesized on a Gene World DNA synthesizer on a 0.2 μmol scale using 0.1 M acetonitrile solutions of commercially available 5'-*O*-DMTr and 2'-*O*-TBDMS phosphoramidites of U, A, C and G, with tac protection of the side-chain amine functions (Proligo®). Typical rU/rG(tac)-succinyl-CPG (Proligo®) supports were utilized. The detritylation reagent was a 3% solution of dichloroacetic acid in methylene chloride. Modified units as well as A, C, G and U monomers were coupled in 10 M excess for 10 min in the presence of a 0.25 M solution of 5-(benzylmercapto)-1*H*-tetrazole in acetonitrile. Capping was performed with tac anhydride (35 s) followed by oxidation using 0.25 M *t*-BuOOH solution in toluene for 100 s (8 equiv). The oxidizing agent was prepared from a commercial 6 M solution of *t*-BuOOH in decane (Aldrich®) and anhydrous toluene.
40. (a) Sproat, B. *Methods Mol. Biol.* **2005**, *288*, 17. (b) Upon completion of the synthesis, the oligonucleotides were cleaved from the solid support and deprotected by treatment with concentrated NH_3: 8 M methylamine in EtOH (1:1 v/v) for 30 min at 65 °C. The TBDMS protecting groups were removed by treatment with $Et_3N{\cdot}3HF$ in DMF for 2.5 h at 60 °C. The oligomers were then precipitated in isopropyl trimethylsilyl ether. The fully deprotected RNAs were purified by semipreparative anion-exchange HPLC (Source 15Q 4.6/100PE®, eluted with a linear gradient of $NaClO_4$ (0.01–0.6 M) in sterile 50 mM Tris HCl buffer at pH 7.6 containing 50 μM EDTA and 10% of acetonitrile for 55 min). The desired products were desalted on a C18 cartridge (Sep-Pak®, Waters) and the integrity of the synthesized oligoribonucleotides was confirmed unambiguously by analytical HPLC and MALDI-TOF mass spectrometry.