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Synthesis and Biophysical Characterization of $tRNA^{Lys,3}$ Anticodon Stem-Loop RNAs Containing the mcm^5s^2U Nucleoside

Ashok C. Bajji and Darrell R. Davis*

Department of Medicinal Chemistry, University of Utah, Salt Lake City, Utah 84112

davis@adenosine.pharm.utah.edu

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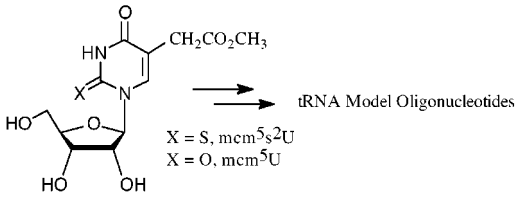
ABSTRACT



Phosphoramidite reagents of the naturally occurring modified nucleosides mcm^5s^2U and mcm^5U were synthesized and along with pseudouridine were incorporated into 17-nucleotide lysine tRNA anticodon stem-loop domains. Standard RNA phosphoramidite coupling chemistry allowed us to systematically investigate the thermodynamic effects of nucleoside modification and to correlate thermodynamic trends with qualitative structure effects seen by NMR spectroscopy.

Transfer RNAs are unique among natural RNAs in that a very high percentage of the nucleosides are chemically modified.[1,2] Despite the wide distribution of modified nucleosides, with few exceptions, the functional role of many of the modified nucleosides is unknown. Reviews published in recent years describe the known biochemical roles of the modified nucleosides,[3−5] but structural studies have been limited by the availability of suitably modified RNAs. Natural and unnatural modified nucleosides have also been used as tools to study RNA structure−function relationships. Current methods and a summary of applications have been recently reviewed.[6,7] Among the uridine modifications, 2-thiouridine (s^2U) and the C-5 modified 2-thiouridines are found predominantly at the wobble position of tRNAs.[1] For s^2U, it has been shown that sulfur substantially stabilizes the 3′-endo sugar conformation at the nucleoside and dinucleotide level.[3,8−10] It has also been shown that 2-thio,5-methyluridine (s^2T) stabilizes tRNAs isolated from thermophilic bacteria.[11−13]

A "modified wobble hypothesis" has been proposed to explain the role of 2-thiouridine and the 5-modified 2-thiouridines during translation.[14] The modified nucleoside 5-me-

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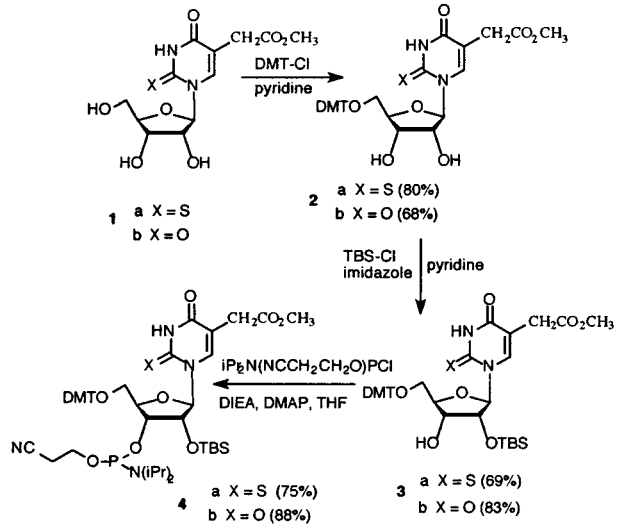
thylaminomethyl-2-thiouridine (mnm^5s^2U) has been shown to be important for reading frame maintenance in *Escherichia coli*,[15] and we have recently reported the structure of the tRNA anticodon domain containing mnm^5s^2U.[16] There is less known about the eukaryotic modified nucleoside 5-carbomethoxymethyl-2-thiouridine (mcm^5s^2U) than its close analogue mnm^5s^2U. An understanding of the biophysical and structural effects of mcm^5s^2U is particularly important because this nucleoside appears to be directly involved in RNA and protein recognition during HIV-1 reverse transcription initiation.[17,18] The present study describes the synthesis of protected nucleoside phosphoramidites of mcm^5s^2U and 5-carbomethoxymethyluridine (mcm^5U) and describes their incorporation, along with pseudouridine, into the anticodon stem loop (ASL) domain of human $tRNA^{Lys,3}$.

The mcm^5s^2U and mcm^5U nucleosides[19] were prepared in good yields by the coupling of the bis-silyl derivative of 5-carbomethoxymethyl-2-thiouracil and 5-carbomethoxyuracil with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose following the procedure of Vorbruggen.[20] The nucleoside sugar protection and phosphoramidite synthesis follows a standard literature protocol for RNA oligonucleotide chemistry (Scheme 1).[21] The 5′-hydroxyl groups of **1a** and **1b**

Scheme 1



were protected as their dimethoxytrityl ethers using DMT-Cl to give compounds **2a** and **2b** in 80% and 68% yields, respectively. Treatment of **2a** and **2b** with TBS-Cl gave a mixture of 2′− and 3′-TBS derivatives, which after chromatographic separation, isomerization of the 3′-TBS, and subsequent chromatographic separation (repeated twice) yielded the pure 2′-isomers **3a** and **3b** in 69% and 83% yields, respectively. The 5′-DMT and 2′-TBS nucleosides **3a** and **3b** were then reacted with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite for 3 h in the presence of diisopropylethylamine with DMAP catalyst to afford phosphoramidites **4a** and **4b** in 75% and 88% yields, respectively.

The modified $tRNA^{Lys,3}$ anticodon domain in Figure 1 was synthesized using commercial PAC-protected phosphoramidites (Glen Research) and standard coupling chemistry[21] with t-BuOOH oxidation.[22−24] Trityl assays indicated >98% coupling for all the phosphoramidites; this was verified by HPLC and MALDI/MS of the crude deprotected RNA. The deprotection was carried out by treating column-bound RNA with 10% DBU in methanol for 12 h at 25 °C.[25] After DBU/methanol treatment, the RNA solution was dried and then treated with $Et_3N{\cdot}3HF$[26] to remove the TBS ethers. The fully deprotected RNA was then purified by HPLC,[27] and MALDI-MS was used to verify that the purified oligonucleotides had the correct molecular weight. The presence of the correct modified nucleosides was further verified by digesting the RNA to mononucleosides and then analyzing the digest by combined LC/MS[28,29] (Supporting Information). The ^1H NMR spectrum in Figure 2 shows strong peaks at 13.20, 12.30, and 11.60 from the central base pairs of the stem.

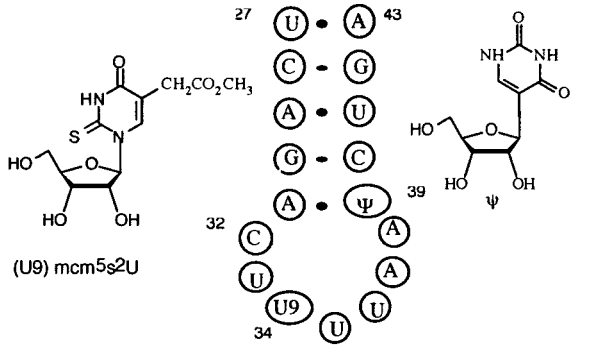


Figure 1. Secondary structure of the 17-nucleotide anticodon stem-loop domain of human $tRNA^{Lys,3}$. Structures of the modified nucleosides at positions 34 and 39 (tRNA numbering) are shown. The native human tRNA also has the hypermodified ms^2t^6A nucleoside at position 37.

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Table 1. Thermodynamic Effects of Nucleoside Modification in the Anticodon of Lysine tRNA[a]

tRNA ASL[b]	Mg (mM)	T_m (°C)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (eu)	$\Delta G°$ (kcal/mol)	ΔT_m (°C)	$\Delta\Delta G°$ (kcal/mol)
$tRNA^{Lys,3}$	0	50.0 ± 0.1	−44 ± 1	−136 ± 2	−1.8 ± 0.1	0	0
$tRNA^{Lys,3}$ ψ	0	55.3 ± 0.2	−42 ± 1	−127 ± 2	−2.3 ± 0.1	+5.3[c]	−0.5[c]
$tRNA^{Lys,3}$ U9	0	49.4 ± 0.2	−43 ± 1	−132 ± 1	−1.6 ± 0.2	−0.6[c]	+0.2[c]
$tRNA^{Lys,3}$ U9	10	55.8 ± 0.1	−43 ± 2	−131 ± 1	−2.4 ± 0.2	+6.4[d]	−0.8[d]
$tRNA^{Lys,3}$ U7	0	51.6 ± 0.3	−37 ± 1	−114 ± 1	−1.7 ± 0.1	+1.6[c]	+0.1[c]
$tRNA^{Lys,3}$ U7	10	55.6 ± 0.2	−44 ± 2	−133.8 ± 0.5	−2.4 ± 0.2	+4.0[d]	−0.7[d]
$tRNA^{Lys,3}$ U9,ψ	0	54.6 ± 0.1	−42.8 ± 0.5	−131 ± 2	−2.3 ± 0.1	+4.6[c]	−0.5[c]
$tRNA^{Lys,3}$ U9,ψ	5	60.2 ± 0.1	−43 ± 1	−130 ± 1	−3.2 ± 0.1	+5.6[d]	−0.9[d]
$tRNA^{Lys,3}$ U9,ψ	10	64.2 ± 0.1	−36 ± 2	−106 ± 1	−2.8 ± 0.1	+9.6[d]	−0.6[d]
$tRNA^{Lys,3}$ U7,ψ	0	55.3 ± 0.2	−43 ± 2	−132 ± 2	−2.4 ± 0.3	+5.3[c]	−0.6[c]
$tRNA^{Lys,3}$ U7,ψ	5	59.9 ± 0.3	−45 ± 1	−136 ± 2	−3.2 ± 0.1	+4.6[d]	−0.8[d]
$tRNA^{Lys,3}$ *U7*,ψ	10	60.4 ± 0.2	−42.2 ± 0.3	−127 ± 1	−2.9 ± 0.1	+5.1[d]	−0.5[d]

[a] Oligonucleotide stability was measured on samples containing 10 μM RNA dissolved in 10 mM sodium phosphate, pH 7.0, containing, 50 mM KCl and 50 mM NaCl. To the parent buffer was added Mg^{2+} in the concentration indicated. Reported values are the average of three independent measurements.[b] The tRNA ASLs have the sequence shown in Figure 1 and the modifications indicated. $tRNA^{Lys,3}$ U7 for example has the U7 (mcm^5U) modification at position 34 and unmodified ribonucleosides at the other positions. [c] The modification effect is compared to unmodified $tRNA^{Lys,3}$. [d] ΔT_m and $\Delta\Delta G°$ depict the effect of 5 or 10 mM $MgCl_2$ compared to the measurements on the same oligonucleotide at 0 mM $MgCl_2$.

The far downfield shifted resonance is for the N3-H proton of ψ39, which forms a Watson−Crick base pair with A31.[30,31] The resonance at 10.50 is the ψ39N1-H proton. The peaks at 13.50 and 10.50 are absent in the NMR spectra for $tRNA^{Lys,3}$-mcm^5s^2U34 and $tRNA^{Lys,3}$-mcm^5U34 which do not have the ψ39 modification (not shown). The ^{31}P spectrum (Figure 2b) shows a downfield-shifted phosphorus assigned

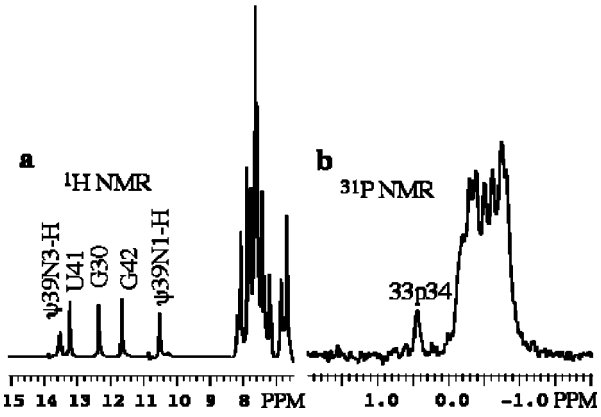

Figure 2. NMR spectra of the human $tRNA^{Lys,3}$ ASL in Figure 1, containing mcm^5s^2U34, ψ39, and unmodified nucleosides at the remaining sequence locations. NMR spectra were acquired at 500 MHz on a 2 mM RNA sample in 10 mM phosphate buffer, pH 7.4, containing 50 mM NaCl, 50 mM KCl, at 5 °C: (a) ^1H NMR spectrum of the downfield region; (b) ^{31}P NMR spectrum.

to the U33-p-mcm^5s^2U34 phosphate.[16,32−34] There was no downfield-shifted phosphorus observed for $tRNA^{Lys,3}$-mcm^5-U34,ψ39, indicating that the 2-thio modification has a similar effect on the anticodon structure of the human $tRNA^{Lys,3}$ as that found for mnm^5s^2U34 in the *E. coli* $tRNA^{Lys}$.[16]

To quantitate the modification effects, temperature-dependent UV spectroscopy was used to measure the melting transition (T_m) and to derive thermodynamic parameters for the $tRNA^{Lys,3}$ ASL oligonucleotides. The T_m's were measured at pH 7.0 for $tRNA^{Lys,3}$ ASLs containing different combinations of three naturally occurring modified nucleosides mcm^5s^2U34, mcm^5U34, and ψ39. The modification effects were compared with data obtained previously on unmodified $tRNA^{Lys.3}$ and $tRNA^{Lys,3}$-ψ39 (Table 1).

A comparison of unmodified $tRNA^{Lys,3}$, $tRNA^{Lys,3}$-ψ39, $tRNA^{Lys,3}$-mcm^5s^2U34, and $tRNA^{Lys,3}$-mcm^5s^2U34,ψ39 shows that the wobble modification has a minimal effect on the overall thermodynamic stability. This indicates that although the mcm^5s^2U modification stabilizes the U-turn as seen in the *E. coli* tRNA, any overall gain in base stacking within the loop has a minimal global thermodynamic effect. In contrast, the stabilization in base stacking upon ψ39 modification is substantial (∼5 °C T_m increase) as has been seen in other tRNA ASLs.[16,30,35] Furthermore, a comparison of mcm^5U34- and mcm^5s^2U34-modified RNAs serves to emphasize that the sulfur has little effect on thermodynamic stability. However, the ^{31}P NMR spectra clearly show that sulfur modification in combination with the side chain is necessary to affect the RNA backbone conformational change reflected by the downfield-shifted 33p34 resonance. The backbone conformation is also stabilized by Mg^{2+} as shown by a further downfield shift in the ^{31}P NMR resonance when going from 0 to 10 mM $MgCl_2$ (not shown).

A noticeable difference is seen in the effect Mg^{2+} has on the T_m's for $tRNA^{Lys,3}$-mcm^5U34,ψ39 and $tRNA^{Lys,3}$-mcm^5s^2U34,ψ39. The sulfur-modified $tRNA^{Lys,3}$ ASL is the only case where we have seen a substantial increase in T_m upon

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increasing the Mg^{2+} concentration from 5 to 10 mM. This effect is significant and may indicate that the conformational change caused by sulfur modification provides a discrete millimolar K_D binding site for Mg^{2+} which stabilizes the $tRNA^{Lys,3}$ anticodon structure.

We have described an efficient approach for the synthesis of the mcm^5s^2U and mcm^5U nucleoside phosphoramidites. The synthetic methodology allowed for facile integration of these nucleosides into $tRNA^{Lys,3}$ model oligonucleotides, and we established deprotection conditions enabling us to obtain quantities of modified RNA necessary for biophysical and structural investigation. The presence of mcm^5s^2U at the tRNA wobble position, along with ψ at position 39, significantly alters the local structure and the thermodynamic stability of these model oligonucleotides. The versatile synthetic protocol will enable us to pursue high-resolution structural studies and to investigate the biochemical effects of nucleoside modification in $tRNA^{Lys,3}$. These synthetic tools will aid in our investigations to understand the established role of nucleoside modification in the HIV-1 transcription initiation complex.

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Supporting Information Available: HPLC traces, MALDI-MS spectra, and LC/MS nucleoside analysis for modified oligonucleotides. Experimental procedures and ^1H NMR data for nucleosides and phosphoramidites. This material is available free of charge via the Internet at http://pubs.acs.org.

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