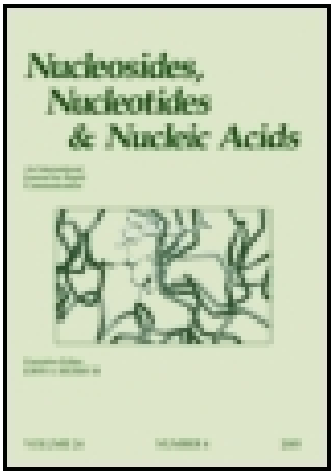

Nucleosides, Nucleotides and Nucleic Acids

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Synthesis and Properties of Uniquely Modified Oligoribonucleotides: Yeast Trna^{Phe} Fragments with 6-Methyluridine and 5,6-Dimethyluridine at Site-Specific Positions

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SYNTHESIS AND PROPERTIES OF UNIQUELY MODIFIED OLIGORIBONUCLEOTIDES: YEAST tRNA^{Phe} FRAGMENTS WITH 6-METHYLURIDINE AND 5,6-DIMETHYLURIDINE AT SITE-SPECIFIC POSITIONS

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ABSTRACT: The phosphoramidites of 6-methyluridine and 5,6-dimethyluridine were synthesized and the modified uridines site-selectively incorporated into heptadecamers corresponding in sequence to the yeast tRNA^{Phe} anticodon and TΨC domains. The oligoribonucleotides were characterized by NMR, MALDI-TOF MS and UV-monitored thermal denaturations. The 6-methylated uridines retained the *syn* conformation at the polymer level and in each sequence location destabilized the RNAs compared to that of the unmodified RNA. The decrease in RNA duplex stability is predictable. However, loss of stability when the modified uridine is in a loop is sequence context dependent, and can not, at this time, be predicted from the location in the loop.

INTRODUCTION: Nucleoside analogs with unusual chemical or physical properties afford the investigator the potential of perturbing nucleic acid structure and function[1]. Thus, the synthesis of base-modified nucleosides with unusual physicochemical properties and their incorporation into synthetic oligonucleotides at the site-specific positions is of increasing interest in studies of fundamental molecular biology and applicable to biomedicine[2,3]. The non-natural modified ribonucleosides 6-methyluridine (m^6U) and 5,6-dimethyluridine (m^5m^6U) are constrained to the unusual *syn* conformation about the N-glycosidic bond[4,5] while most pyrimidine nucleosides exist predominantly in the *anti* orientation[5]. The *syn* conformation of the 6-methylated uridines is due to the steric hindrance of the bulky methyl substituent. If incorporated into RNA at site-selected positions, m^6U and m^5m^6U could strongly influence base pairing properties and other non-covalent interactions. Although the synthesis of m^6U

had been described[4,6], neither of the modified uridines had been incorporated into RNA. A comparison of the properties of the modified RNA with that of the unmodified RNA of otherwise identical sequence allows one to draw conclusions concerning the importance of specific structural elements within the RNA. Here, we describe the synthesis of the 6-methyluridine and 5,6-dimethyluridine phosphoramidites (FIG.1), the incorporation of the modified uridines at site-specific positions in oligoribonucleotides with sequences corresponding to that of the yeast $tRNA^{Phe}$ anticodon (ASL) and TΨC (TSL) stem and loop domains (FIG.2) and analyses of these RNAs.

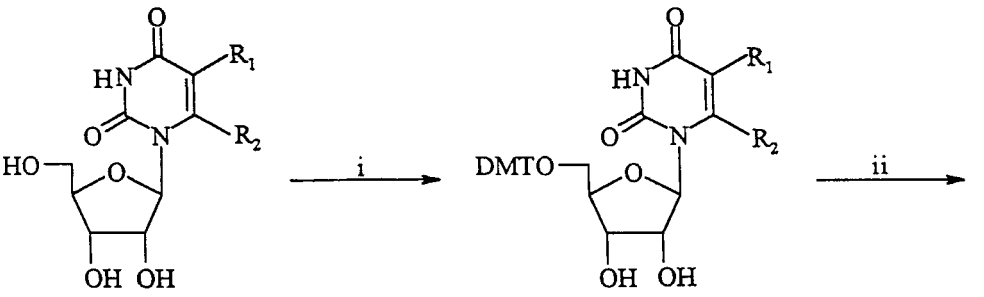
RESULTS AND DISCUSSION

Synthesis of modified nucleoside phosphoramidites and oligoribonucleotides

Synthesis of the methyl derivatives of uridine: 6-methyluridine (m^6U) and 5,6-dimethyluridine (m^5m^6U) was achieved by coupling the bissilyl derivatives of 6-methyluracil and 5,6-dimethyluracil to 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose[6,7]. The acid catalyst for this condensation was trimethylsilyl-trifluoromethanesulfonate. The desired N_1-$C_{1'}$ products were obtained in sufficient yields, 48% for m^6U and 54 % for m^5m^6U, to proceed with protection and phosphoramidite syntheses.

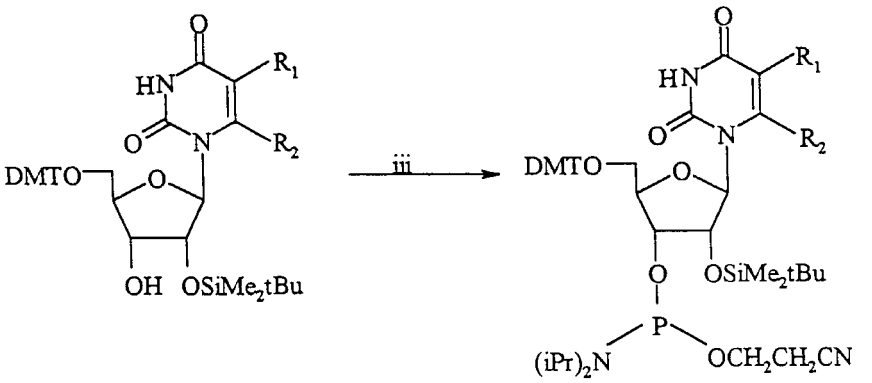
Protections of the ribose 2'OH and 5'OH of m^6U and m^5m^6U and their phosphoramidite synthesis (FIG.1) were accomplished with known standard procedures[8]. The 5'-hydroxyl was protected by dimethoxytritylation using DMT-Cl in pyridine to give compounds **3** and **4** in very good yield. Treatment of **3** and **4** with *tert*-butyldimethylsilyl chloride in THF in the presence of silver nitrate gave a mixture of 2',3'-*O*-bissilyl, 2'-*O*- and 3'-*O*-*tert*-butyldimethylsilyl derivatives, which on purification by flash column chromatography yielded the pure 2'-isomer **5** in 37% and **6** in 35% yields. Reaction of **5** and **6** with 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite in the presence of diisopropylethylamine in methylene chloride for 3 h at room temperature resulted in the desired m^6U and m^5m^6U phosphoramidites, **7** and **8** in 75% and 80% yields, respectively.

The phosphoramidites **7** and **8** were used for incorporation of the methylated uridines at site-selected positions of important functional domains of yeast $tRNA^{Phe}$. The sequences synthesized in this study were heptadecamer analogs of the anticodon (ASL) and TΨC



1 $R_1 = H$; $R_2 = CH_3$
2 $R_1 = CH_3$; $R_2 = CH_3$

3 $R_1 = H$; $R_2 = CH_3$
4 $R_1 = CH_3$; $R_2 = CH_3$



5 $R_1 = H$; $R_2 = CH_3$
6 $R_1 = CH_3$; $R_2 = CH_3$

7 $R_1 = H$; $R_2 = CH_3$
8 $R_1 = CH_3$; $R_2 = CH_3$

FIG.1 Synthetic route for the preparation of m^6U and m^5m^6U phosphoramidites
i) 4,4'-dimethoxytrityl chloride / pyridine; ii) *tert*-butyldimethylsilyl chloride /$AgNO_3$/THF; iii) 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite / N,N-diisopropylethylamine / CH_2Cl_2.

(TSL) stem and loop domains (FIG.2). Unsubstituted ASL and TSL domains, as well as the ASLs and TSLs containing the modified uridines, were synthesized using standard solid phase chemistry for RNA synthesis[9]. Coupling efficiency was estimated by monitoring the released trityl group. Although there was some noticeable decrease (approximately 10%) in coupling at the time of incorporation of the modified unit, the

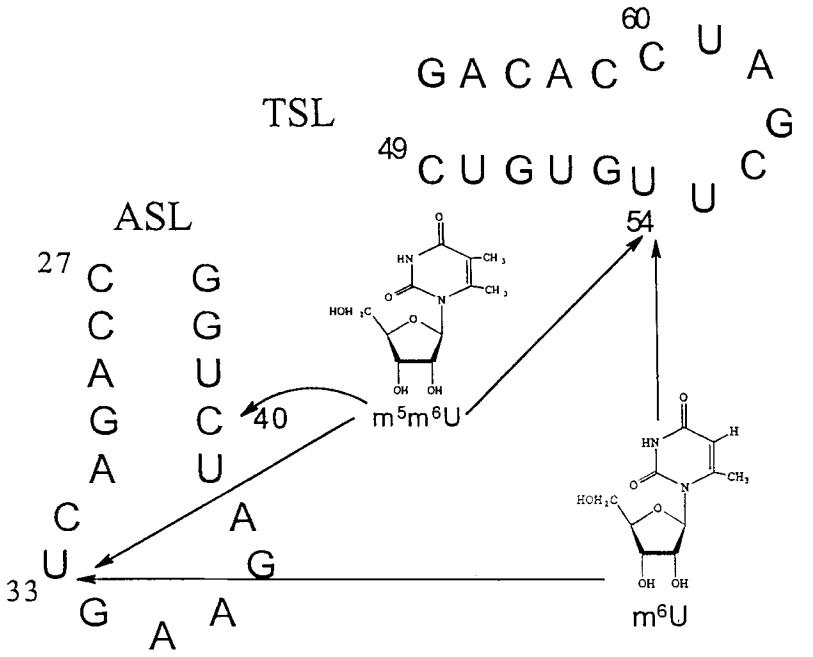


FIG.2 Nucleoside sequences of the yeast unmodified $tRNA^{Phe}$ anticodon (ASL) and TΨC (TSL) stem and loop domains. Modified and unmodified ASL and TSL sequences were synthesized. Arrows are used to identify the site-specific placement of m^6U and m^5m^6U.

average efficiency was approximately 98%. Removal of the oligoribonucleotides from the support and deprotections were accomplished with published protocols[10-12]. The oligonucleotides were purified by anion exchange HPLC. Chromatograms of the purification of the TSLs and ASLs with m^6U and m^5m^6U were representative of that observed for other heptadecamer oligoribonucleotides. Purity of each oligonucleotide was confirmed by gel electrophoresis and nucleoside composition. The nucleosides composition of each RNA was determined by enzymatic hydrolysis of the RNA followed by reversed-phase HPLC of the constituent nucleosides. The retention times and UV-spectra (on-the-fly) of the nucleosides compared favorably to standards. All oligomers were analysed by MALDI-TOF MS and the measured molecular weights were in good agreement with calculated ones (TABLE 1).

TABLE 1. Calculated and measured molecular masses of ASL and TSL analogs

	M - H^{1-} - calculated	M - H^{1-} - measured
ASL - unmodified	5449.34	5448.9
ASL - m^6U_{33}	5464.35	5462.3
ASL - $m^5m^6U_{40}$	5478.38	5476.8
TSL - unmodified	5362.25	5360.9
TSL - m^6U_{54}	5376.28	5375.3
TSL - $m^5m^6U_{54}$	5390.30	5390.8

Automated chemical synthesis has the advantage of site-selected placement of nucleosides. In order to take full advantage of this property of the synthetic method, we chose to replace pyrimidines that are known to be important to the structure of yeast tRNA^{Phe} with m^6U and m^5m^6U. The nucleosides m^6U and m^5m^6U were introduced at position 33 of the tRNA within the ASL. In the crystal structure of yeast tRNA^{Phe}, the unmodified uridine at position 33 facilitates an abrupt change in the direction of the polynucleotide backbone of the anticodon loop, the U-turn[13]. 6-Methyluridine was also incorporated at position 54 of the TSL replacing the naturally modified m^5U (riboT). In the native molecule m^5U is involved in a tertiary structure, reverse Hoogsteen, base pair with m^1A at position 58 across the loop. The dimethyluridine, m^5m^6U, a close analog of m^5U, also was introduced at position 54 of the TSL. In addition, m^5m^6U was incorporated into the stem region of the ASL at position 40. The naturally modified 5-methylcytidine is found at position 40 in the tRNA. Methylation of C_{40} contributes to enhanced base stacking within the anticodon stem of yeast tRNA^{Phe} (Guenther and Agris, personal communication) and its DNA analog[14].

Analysis of methylated nucleoside conformation and effect on RNA thermal stability

The conformation around the N-glycosidic linkage of the 6-methylated uridine is *syn*[4,5]. If this conformation is retained in the oligonucleotides it could impact on the structure and dynamics of the RNA in which it was incorporated. The torsion angle of the N-glycosidic bond (χ) of m^6U_{33} in the ASL was analyzed by NMR (FIG.3). In the determination of nucleoside conformation for structural calculations, the ribose H1' of

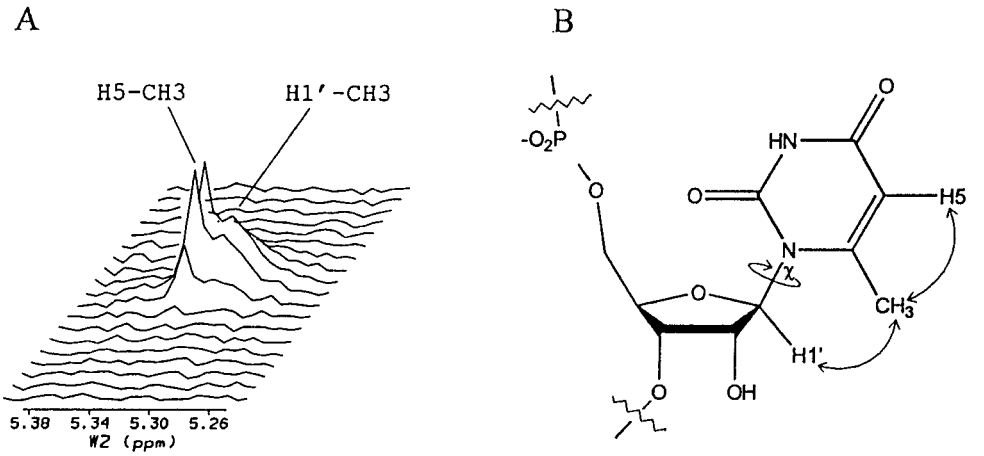


FIG.3 Determination of the N-glycosidic torsion angle χ of m^6U_{33} of the ASL

The N-glycosidic torsion angle χ was determined to be *syn* by comparing the NOE between the C1'-H and the methyl to that between the H5 and the methyl. (A) A stacked-plot of a region of the NOESY spectrum of ASL with m^6U_{33}. The region of the NOESY spectrum that contained the H5-CH_3 and H1'-CH_3 cross peaks of m^6U_{33} was expanded. The relatively similar intensities of these cross peaks indicate that 6-methyluridine takes the *syn* conformation in the polymer. (B) The *syn* conformer of m^6U. Arrows in the figure of m^6U_{33} in the syn conformation denote the NOEs measured and the angle χ.

pyrimidine nucleosides exhibit NOEs to the base H6 that typically constrain χ as *anti*. A stacked-plot expansion of the region of the NOESY spectrum of the m^6U_{33} ASL (FIG.3) shows the H5-CH_3 and H1'-CH_3 cross peaks of m^6U_{33}. Although these peaks are overlapped, peak-fitting procedures revealed that the integral of the H5-CH_3 cross peak is greater than the H1'-CH_3 cross peak by ca. 2-fold. Regardless of sugar conformation, the integral of the H1'-CH_3 cross peak as compared to that of the H5-CH_3 allows us to estimate the torsion angle χ. In the *syn* conformer, the two peaks will be of comparable intensity. In the *anti* conformer a weak H1'-CH_3 cross peak will be observed with approximately 10% of the H5-CH_3 intensity due to the increased CH_3-H1' distance. Here the strength of the H1'-CH_3 cross peak relative to that of the H5-CH_3

indicates that the N-glycosidic linkage is *syn*. In addition, the 6-methylated uridine sugar pucker was determined to be C3'-endo via the C1'H-C2'H coupling. Determination of sugar pucker first involved assignment of the ribose C1' proton signal. However, the modified uridines were site-selected markers helping to overcome this problem through unambiguous C1' to methyl NOEs.

The effect of site-specific incorporations of the methylated uridines on thermal stability of each modified ASL or TSL was analyzed by monitoring thermal denaturations and re-naturations with UV spectroscopy (FIG.4). A summary of the thermodynamic parameters derived from analyses of the thermal denaturations is found in Table 2. The most significant effect on structure occurred when a *syn* nucleotide was place in the middle of a helical stem region of a hairpin. Placement of m^5m^6U at position 40 in the stem region of the ASL significantly reduced the stability of the structure (FIG.4). The naturally-occurring m^5C at position 40 in the middle of the ASL stem is involved in base stacking on the 3'-side of the stem and hydrogen bonding to G_{30}. Though a G-U base pair within a stem is not only feasible but would also be favored to occur, the *syn* conformation of m^5m^6U was expected to disrupt base pairing to G_{30}. In addition, base stacking interactions would be disrupted by the intervening *syn* conformer. The observed increase in free energy, over 3 kcals/mole, is consistent with the stability lost when two base pairs that close a loop into a stem, as well as a loop-closing interaction, are disrupted[16]. The structural restraints of the helix do not appear to provide for the formation of alternative stabilizing interactions. The effect of a *syn* nucleotide when placed at the base of a stem (i.e. position 39) should have considerably less impact. In order to better understand the extent of the *syn* perturbation a longer helix, possibly similar to the system used to study base mismatches[17], would be required.

The thermodynamic contributions for loop closing for various combinations of major nucleotides have been determined[16]. Incorporation of m^5m^6U and m^6U at position 54 of the TSL lowered the Tm as compared to the unmodified oligomer. The nucleoside at position 54 is adjacent to the 5'-side of the TSL stem and in the crystal structure it is involved in stacking interactions, as well as the tertiary structure base pair across the loop. One or both of these stabilizing contributions may have been disrupted when m^5U was replaced with the *syn* conformers, m^5m^6U and m^6U. The observed 0.9

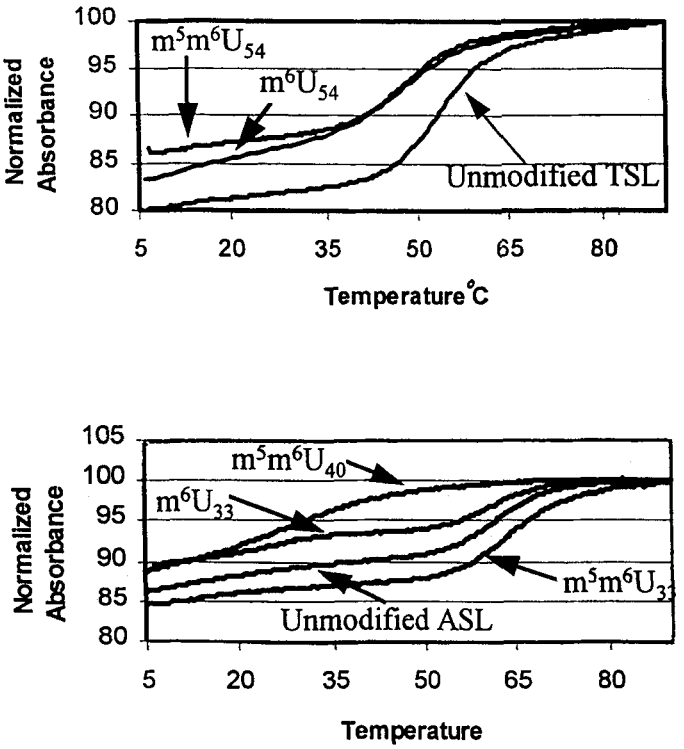


FIG.4 Thermal denaturations of modified and unmodified RNAs. The effect of the methylated uridines on the thermal stability of each ASL and TSL was analyzed by comparing their thermal denaturations to that of the corresponding unmodified RNAs. Thermal denaturations of the unmodified TSL and that with m^5m^6U at loop position 54 are shown in the top panel. Thermal denaturations of the unmodified ASL and those with m^6U and m^5m^6U at loop position 33 and m^5m^6U at stem position 40 are shown in the bottom panel.

kcal/mole increase in ΔG for both oligonucleotides closely matches the 1.0 kcal/mole thermodynamic contribution predicted for a UC loop closing[16].

Placement of m^6U at position 33 in the loop of the ASL destabilized the structure compared to that of the unmodified control (FIG.4). The high temperature and major transitions of the modified and unmodified ASLs were almost identical. However, a low temperature transition was now evident in the modified ASL. In the tRNA's crystal structure U_{33} stabilizes the loop backbone conformation into the now accepted U-turn

TABLE 2 Thermodynamic Parameters of TSL and ASL analogs

Sampe ID	Tm	+/-	ΔH	+/-	ΔS	+/-	ΔG	+/-
Unm TSL	54.5	0.3	-49	1	-149	4	-2.6	0.1
m5U54	56.7	0.4	-52	2	157	7	-3.1	0.1
m6U 54	49.0	0.3	-46	1	-142	1	-1.7	0.1
m5m6U 54	49.6	0.5	-44	2	-137	8	-1.7	0.1
Unm ASL	63.8	0.5	-54.4	2	-161	8	-4.3	0.1
m6U 33	61.1	0.8	-58.8	1	-176	2	-4.2	0.1
m5m6U 33	62.9	0.6	-53.3	2	-159	5	-4.1	0.1
m5m6U 40	29.0	0.9	-24.4	4	-80.7	12	-0.6	0.1

motif with three interactions[15], two hydrogen bonds (N3-H to the phosphate of A_{36} and 2'OH to the N7 of A_{35}) and stacking (between C_{32} and the phosphate of A_{35}). Probably, one or more of these interactions were disrupted when U_{33} was replaced with m^6U. What is less understood is the thermodynamic effect of a *syn* nucleotide in the loop region of a stem. While an increasing number of well ordered loop structures in the literature report a diversity of stable conformations, a quantitative and predictive understanding of possible loop structures is lacking. The loop inherently affords greater freedom for alternative structures. The variation between the ASL with $m^5m^6U_{33}$ and that with m^6U_{33} demonstrates this situation. While the ASL with m^6U_{33} has a low, as well as high temperature, transition during thermal denaturation, the ASL with $m^5m^6U_{33}$ has a simple bi-phasic denaturation (Fig.4). This result suggests that at least two different structural solutions exist to accommodate the modification and unusual nucleotide conformation. For example, the *syn* conformation disrupts base stacking important for the U-turn structure; however, the added hydrophobicity of the doubly methylated $m^5m^6U_{33}$ may actually rescue this situation by improving base stacking. We have discovered that this is the case when the stability of a hairpin with N1,N3 dimethylpseudouridine is compared to that with N3-methyluridine or N3-methylpseudouridine[18]. Further experimentation by NMR will be required for a more complete understanding of this result.

CONCLUSION

Automated chemical synthesis permits the site-selected incorporation of natural and non-natural modified nucleosides into nucleic acids. The introduction of non-natural

base modifications with known chemical or physical properties can be used to perturb the structures, and thus, possibly the functions of RNAs. The phosphoramidites of the 6-methylated uridines m^6U and m^5m^6U provide the investigator with pyrimidine substitutes that are *syn* conformers and therefore, can be used to probe specific relationships between RNA structure and function.

We introduced the methylated uridine *syn* conformers at three locations in two tRNA fragments. At two of these locations the uridine *syn* conformer substituted for a naturally occurring methylated pyrimidine, m^5U_{54} in the TSL loop and m^5C_{40} in the ASL stem. At the third location, the uridine *syn* conformer substituted for the invariant U_{33}, important to the ubiquitous "U-turn" of tRNA anticodons. Purines in RNA structures have been found to take the *syn* conformation without modification, whereas *syn* pyrimidines are more unusual. A cytidine involved in a U-C base pair in the L3 loop of the hepatitis delta virus ribozyme is in a *syn* conformation[19]. Not unexpectedly, our substituting the uridine *syn* conformer for m^5C_{40} disrupted the ASL stem and drastically reduced stability of the RNA. However, substitution of the ASL loop U_{33} had little effect on the ASL's thermostability and increased ΔG by only 0.1-0.2 kcals/mol, if at all. Substitution of m^6U for U_{33} significantly enhanced a low temperature transition only slightly apparent in the unmodified ASL, but not the high temperature transition. Since the modification is in the anticodon loop and located at the invariant U_{33} position, the appearance of the low temperature transition must be attributed to a change in loop conformational dynamics. However, the low temperature transition was not observed when U_{33} was substituted with m^5m^6U. We have found that the stacking interaction of U_{33} is the most important of the nucleoside's physicochemical contributions to the U-turn [20]. Therefore, we speculate that the introduction of the 5-position methyl group restores stacking interactions, though the nucleoside remains in the *syn* conformation. Restoration of the stacking interactions that the 5'-side of the loop has with the 5'-side of the stem results in stability evidenced by lack of the low temperature transition. We have also substituted dihydrouridine, D, the only naturally occurring, non-aromatic, non-planar nucleoside for U_{33} and D completely disrupted the stability of the ASL [20]. Yet, substituting a *syn* for an *anti* conformer at position 33 had the same negative effect on the ASL's ability to bind the ribosome as did D [20].

In contrast to these results, substituting a *syn* uridine for the *anti* m^5U_{54} (T_{54}) had a

dramatic effect on stability, decreasing the Tm by 7-8 °C and increasing ΔG by 1.4 kcal/mol. The natural methylation of U_{54} in the TSL domains tRNAs has been reported to increase stability [21]. We found that introduction of the natural modification of m^5U_{54} to the TSL accounted for 2.2 °C increase in stability of the 6 °C increase reported for an entire tRNA. The structure of the m^5U_{54}-containing TSL is unusual in that the loop folds back upon the stem in a conformation unique among RNA structures and may be a pre-folding intermediate for tRNA [22]. This structure may not be possible with a *syn* conformer at position 54. The unmodified heptadecamer TSL is recognized by the *E. coli* m^5U_{54}-tRNA methyltransferase for production of m^5U_{54}.[23]. However, the m^6U_{54}-containing TSL is not a substrate of the enzyme (R. Sengupta and P. F. Agris, unpublished). It is predictable that substitution of a natural nucleoside by a uridine *syn* conformer within a stem will disrupt the base pairing and RNA stability. However, from our experience reported here, the effects of loop substitutions are not predictable and are probably sequence context dependent.

EXPERIMENTAL

Synthesis of 5'-*O*-4,4'-dimethoxytrityl derivatives of 6-methyluridine (3) and 5,6-dimethyluridine (4)

The methylated derivatives of uridine were obtained according to the previously described procedure by condensation of silylated 6-methyluracil and 5,6-dimethyluracil with 1-*O*-acetyl-2,3,5-*O*-tribenzoyl-D-ribofuranose in the presence of trimethylsilyl trifluoromethanesulfonate in acetonitrile[2]. The methylated uridines were derivatized with the dimethoxytrityl 5'OH protection by addition of 4,4'-dimethoxytrityl chloride (1.12 g; 3.3 mmol) to 3 mmol of the appropriate methylated uridine derivative in 22.5 mL of anhydrous pyridine. The reaction mixture was stirred in the dark at room temperature for 1-2 days. The tritylation reaction was periodically assessed by thin layer chromatography (TLC) of reactants and products. Analysis and purification of products was accomplished with TLC using fluorescent Merck F254 plates and by column chromatography with Merck 60 silica gel. When TLC analysis ($CHCl_3$/MeOH 9:1) showed no starting material, the reaction was quenched with 7 mL of ice water and then extracted with chloroform. The organic solution was dried over $MgSO_4$, filtered and evaporated to dryness. After co-evaporation with toluene to remove traces of pyridine,

the residue was purified by flash column chromatography using a 0-4% MeOH/$CHCl_3$ elution gradient to yield pure product in good yield.

5'-*O*-4,4'-dimethoxytrityl-6-methyluridine (3): yield 92.9%; TLC: Rf=0.41 ($CHCl_3$/ MeOH 9:1); ^{1}H NMR (CD_3Cl) δ (ppm): 2.31(s, 3H, 6-**CH_3**), 3.69 (s, 6H, 2 × O-C**H_3**) 3.35-3.41 (m, 2H, **H5', H5"**), 3.70-3.80 (m, 1H, **H4'**), 4.51 (pt, 1H, $J_{3',2'}$=$J_{3',4'}$=6.5Hz, **H3'**), 4.98 (dd, 1H, $J_{2',1'}$=2.25Hz, $J_{2',3'}$=4.00Hz, **H2'**), 5.52 (s, 1H, **H5**), 5.77 (d, 1H, $J_{1',2'}$=2.25Hz, **H1'**), 6.76-7.43 (m, 13H, **Ph_3**CO-5').

5'-*O*-4,4'-dimethoxytrityl-5,6-dimethyluridine (4): yield 76.7%; TLC: Rf=0.47 ($CHCl_3$/MeOH; 9:1); ^{1}H NMR (CD_3Cl) δ (ppm): 1.93 (s, 3H, 5-**CH_3**), 2.31 (s, 3H, 6-**CH_3**), 3.35-3.49 (m, 2H, **H5',H5"**), 3.69 (s, 6H, 2 × O-C**H_3**), 3.91-3.94 (m, 1H, **H4'**), 4.51 (pt, 1H, $J_{3',2'}$=6.25, $J_{3',4'}$=5.00Hz, **H3'**), 4.71 (dd, 1H, $J_{2',1'}$=2.25Hz, $J_{2',3'}$=6.25Hz, **H2'**), 5.55 (d, 1H, $J_{1',2'}$=2.25Hz, **H1'**), 6.76-7.40 (m, 13H, **Ph_3**CO-5').

Synthesis of 5'-*O*-4,4'-dimethoxytrityl-2'-*O*-*tert*-butyldimethylsilyl derivatives of 6-methyluridine (5) and 5,6-dimethyluridine (6)

The 5'-*O*-4,4'-dimethoxytrityl of methylated uridine derivative (2 mmol) was dissolved in 20 mL of anhydrous tetrahydrofuran (THF). While the solution was stirred, $AgNO_3$ (440 mg; 2.6 mmol), dry pyridine (0.66 mL; 8 mmol) and *tert*-butyldimethylsilyl chloride (400 mg; 2.6 mmol) were added. Stirring was continued overnight at room temperature. When TLC analysis of the reaction mixture ($CHCl_3$/MeOH 95:5 or benzene/ethyl acetate 3:1) showed that the starting material was still present in the solution, more $AgNO_3$ (100 mg, 0.6 mmol) and *tert*-butyldimethylsilyl chloride (90 mg, 0.6 mmol) were added. The reaction was allowed to proceed for another 6 hours. After this time, the solution was filtered into 5% $NaHCO_3$ (~30 mL). The aqueous phase was extracted with CH_2Cl_2 and the combined organic solutions were dried over $MgSO_4$, filtered and evaporated to yield white foam. This material was purified by column chromatography on silica gel with a gradient elution of CH_2Cl_2/acetone (gradient 100:0 to 90:10) to give trace amounts of the 2',3'-*O*-di-*tert*-butyldimethylsilyl derivative, the 3'-*O*-silyl derivative and the desired 2'-*O* -silyl isomer.

5'-*O*-4,4'-dimethoxytrityl-2'-*O*-*tert*-butyldimethylsilyl-6-methyluridine (5): yield 37%, Rf=0.46, $CHCl_3$/MeOH (95:5); 0.44 benzene/ethyl acetate (3:1); ^{1}H NMR (CD_3Cl) δ (ppm): 0.93 (s, 9H, C(**CH_3**)$_3$), 2.34 (s, 3H, 6-**CH_3**), 3.42-3.33 (m, 2H, **H5',**

H5"), 3.77 (s, 6H, 2×OC**H**$_3$), 3.97-3.91 (m, 1H, **H4'**), 4.24 (dd, 1H, $J_{3',2'}$=6.25, $J_{3',4'}$=5.75Hz, **H3'**), 4.94 (dd, 1H, $J_{2',1'}$=3.75Hz, $J_{2',3'}$=6.25Hz, **H2'**), 5.45 (d, 1H, $J_{1',2'}$=3.75Hz, **H1'**), 5.60 (s, 1H, **H5**), 6.80 (m, 13H, **Ph**$_3$CO-5').

5'-*O*-4,4'-dimethoxytrityl-2'-*O*-*tert*-butyldimethylsilyl-5,6-dimethyluridine (6): yield 35%, Rf=0.52, $CHCl_3$/MeOH 95:5; ^{1}H NMR (CD_3Cl) δ (ppm): 0.91 (s, 9H, C(C**H**$_3$)$_3$), 2.01 (s, 3H, 5-C**H**$_3$), 2.36 (s, 3H, 6-C**H**$_3$), 3.33-3.44 (m, 2H, **H5', H5"),** 3.80 (s, 6H, 2×OC**H**$_3$), 3.88-3.94 (m, 1H, **H4'**), 4.26 (dd, 1H, $J_{3',2'}$=6.25Hz, $J_{3',4'}$=6.00Hz, **H3'**), 4.93 (dd, 1H, $J_{2',1'}$=3.25Hz, $J_{2',3'}$=6.25Hz, **H2'**), 5.52 (d, 1H, $J_{1',2'}$=3.50Hz, **H1'**), 6.79-7.49 (m, 13H, **Ph**$_3$CO-5').

Synthesis of phosphoramidite of 5'-*O*-4,4'-dimethoxytrityl-2'-*O*-*tert*-butyldimethylsilyl- 6-methyluridine (7) and 5,6-dimethyluridine (8)

5'-*O*-Dimethoxytrityl-2'-*O*-*tert*-butyldimethylsilyl-ribonucleoside (1 mmol) was dried by two co-evaporations with anhydrous toluene (2 mL) and methylene chloride (3 mL). The residue was dissolved in anhydrous methylene chloride (5 mL) under an argon atmosphere. N,N-diisopropylethylamine (0.68 mL; 4 mmol) and 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (0.51 mL; 2 mmol) were added dropwise through a rubber septum and the reaction mixture was stirred at room temperature. Starting materials had been completely consumed after 3 hr as confirmed by TLC (benzene/ethyl acetate/triethylamine, 7:2:1). The reaction mixture was added to methylene chloride (50 mL), washed with aqueous 5% $NaHCO_3$, dried over $MgSO_4$, filtered and evaporated to dryness. The residue was co-evaporated with toluene and purified on a column of silica gel with CH_2Cl_2 /triethylamine (99:1) to give pure product as a white foam.

5'-*O*-4,4'-dimethoxytrityl-2'-*O*-*tert*-butyldimethylsilyl-6-methyluridine-3'-*O*-(2-cyanoethyl)-N,N-diisopropylphosphoramidite (7): yield 75%, Rf=0.57, benzene/ethyl acetate/triethylamine 7:2:1. ^{31}P NMR (C_6D_6) δ (ppm): 149.5; 151.2; FAB MS, -VE: 873 (M-H).

5'-*O*-4,4'-dimethoxytrityl-2'-*O*-*tert*-butyldimethylsilyl-5,6-dimethyluridine 3'-*O*-(2-cyanoethyl)-N,N-diisopropylphosphoramidite (8): yield 80%, Rf=0.69 benzene/ethyl acetate/triethylamine 7:2:1. ^{31}P NMR (C_6D_6) δ (ppm): 149.9; 150.9; FAB MS, -VE: 887 (M-H).

Characterization of uridine phosphoramidites: NMR spectroscopy and MS

^{1}H NMR spectra of the methylated uridine phosphoramidites and the intermediates in their syntheses were recorded on a Bruker DPX-250 spectrometer with tetramethylsilane as an internal standard. ^{31}P NMR spectra were obtained as downfield shifts relative to 85% phosphoric acid as an external standard. Fast atom bombardment (FAB) mass spectra were collected on Finnigan MAT 95 spectrometer working in negative ion mode.

Oligoribonucleotide synthesis.

The ASL and TSL oligoribonucleotides were synthesized using a Perkin-Elmer Applied Biosystems Model 394 synthesizer. The major nucleotide phosphoramidites were purchased from Glen Research (Sterling, Virginia USA). The standard 1 μM RNA synthesis protocol was followed[3,9,10]. Oligonucleotides were cleaved from the beads by incubating the sample for 18 hours at 55° in ethanolic ammonia. After cleavage the samples were dried using a Savant Speed Vac and the dried samples were stored at -20° C until the time of purification.

Oligonucleotide Purification

At the time of purification the 2'OH protection was removed by incubating the RNA overnight in 1.0 M *tert*-butylammonium fluoride (TBAF) in THF (Aldrich)[3,9]. Purification included gradient anion exchange HPLC using a Nucleogen 60 column (10 x 250 mm)[3]. Slight modifications to the gradient are made to optimize separation for each sequence. Fractions of the separation were collected and those containing the oligomer of interest were pooled. After lyophilization, the samples were desalted by solid phase chromatography using Sep Pak cartridges (Waters). Purity of each oligomer was analyzed by subjecting an aliquot to denaturing polyacrylamide gel electrophoresis (PAGE). The nucleoside composition was determined by HPLC.

Gel electrophoresis of ASLs and TSLs

In order to assess the purity of the oligonucleotide synthesis products, ASLs and TSLs were subjected to polyacrylamide (20%) gel electrophoresis under denaturing conditions (7 M urea). The RNAs within the gels were visualized with ethidium bromide staining.

Nucleoside composition of ASLs and TSLs

The nucleoside composition was determined for those ASLs and TSLs that contained modified uridines in order to confirm incorporation of the modified nucleosides.

Hydrolysis of the ASLs and TSLs and analysis of the constituent nucleosides were accomplished according to methods developed by Agris[24] and Gehrke[25]. Nucleosides were separated using a Waters 600 solvent delivery system and detection was accomplished with a Waters 990 photo-diode array detector monitoring 200 to 400 nm. Nucleoside standards of common nucleosides were obtained from Sigma. Retention times and spectral profiles of the nucleosides were matched to the standards of common and modified nucleosides.

MALDI-TOF mass spectrometry of ASLs and TSLs

MALDI-TOF mass spectra were recorded on Voyager-Elite (PerSeptive Biosystems, Framingham, MA) spectrometer equipped with UV nitrogen laser emitting at 337 nm. Oligomers (approximatly 0.02 OD) were mixed with 3-hydroxypicolinic acid / ammonium citrate as the matrix. Spectra were collected in negative ion mode by addition of 100 to 200 laser shots. Mass spectrometer was externally calibrated on T_8/T_{16} mixture.

NMR spectroscopy of ASLs and TSLs

NOESY and TOCSY spectra of oligoribonucleotides were collected on a Bruker DRX-500 spectrometer. Spectra were obtained at 25 °C with the RNA in 10 mM phosphate, pH 6, and 0.1 mM EDTA according to standard protocols for RNA[26,27]. Overlapped cross-peaks were fit as gaussian peaks using the fitting procedure available within the FELIX 97.0 package. For analysis of sugar pucker, conventional pulse sequences that are especially augmented for nucleic acid studies, such as DQFCOSY with and without ^{31}P broadband or selective coupling during acquisition, assist in the measuring of ^{1}H-^{1}H coupling constants[28]. Definition of the sugar pucker conformation that was hampered by the lack of coupling constants was approached through the patterns of intra- and internucleotide NOEs that infer whether the sugar is in 2'-endo or 3'-endo conformation.

UV-Monitored thermal denaturations

All thermal denaturations and renaturations of the ASLs and TSLs were monitored with a CARY 3 UV spectrophotometer. The spectrophotometer is equipped with a thermal control module. UV absorbance at 260 nm was monitored over a range of 5 to 90° C with a ramp rate of 1° per minute. Solution conditions were 100 mM NaCl, 10 mM phosphate buffer, pH 7.0. Data was collected for both denaturation and renaturation to be certain the process was reversible. To assess error, triplicate denaturations were made for each

sample. Tm was concentration independent for each sample in concentration range of 2 to 50 μM.

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