



Synthesis of a 3-Methyluridine Phosphoramidite to Investigate the Role of Methylation in a Ribosomal RNA Hairpin

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Abstract—The synthesis of a 5'-O-BzH-2'-O-ACE-protected-3-methyluridine phosphoramidite is reported [BzH, benzhydryloxy-bis(trimethylsilyloxy)silyl; ACE, bis(2-acetoxyethoxy)methyl]. The phosphoramidite was employed in solid-phase RNA synthesis to generate a series of RNA hairpins containing single or multiple modifications, including the common nucleoside pseudouridine. Three 19-nucleotide hairpin RNAs that represent the 1920-loop region (G₁₉₀₆–C₁₉₂₄) of *Escherichia coli* 23S ribosomal RNA were generated. Modifications were present at positions 1911, 1915, and 1917. The stabilities and structures of the three RNAs were examined by using thermal melting, circular dichroism, and NMR spectroscopy © 2001 Elsevier Science Ltd. All rights reserved.

Introduction

The natural base modification 3-methylpseudouridine (m³Ψ) occurs in the 1920-loop region of *Escherichia coli* (*E. coli*) 23S ribosomal RNA (rRNA) at position 1915.¹ A pseudouridine residue (Ψ) at position 1915 appears to be conserved among all organisms examined to date, however, methylation of the pseudouridine at this position may be less common.² A number of recent cross-linking and crystallographic studies on the ribosome have implicated the 1920 loop of 23S rRNA (helix 69) in playing an important role in mediating subunit association and tRNA interactions.^{3–7} Many questions still remain, however, regarding the role of pseudouridine in this process and the influence of pseudouridine methylation on the overall structure and function of 23S rRNA.

The goal of the research described here was to probe the specific structural and stabilizing role of the methyl group at position 1915 in 23S rRNA by using the nucleotide analogue 3-methyluridine (m³U). In order to achieve this goal, the analogue was first converted into a phosphoramidite and incorporated site-specifically into short RNA oligonucleotides by using solid-phase chemistry. We reported recently on the synthesis of a

new pseudouridine phosphoramidite and examined the role of Ψ residues at positions 1911, 1915, and 1917 of *E. coli* 23S rRNA.⁸ The synthetic strategy involved the use of a 5'-O-silyl-2'-O-orthoester protection system^{9,10} to generate the new phosphoramidite. In this study, a similar protection system was employed to generate a 5'-O-BzH-2'-O-ACE-3-methyluridine-3'-phosphoramidite [BzH, benzhydryloxy-bis(trimethylsilyloxy)silyl; ACE, bis(2-acetoxyethoxy)methyl] that was compatible with solid-phase RNA synthesis.

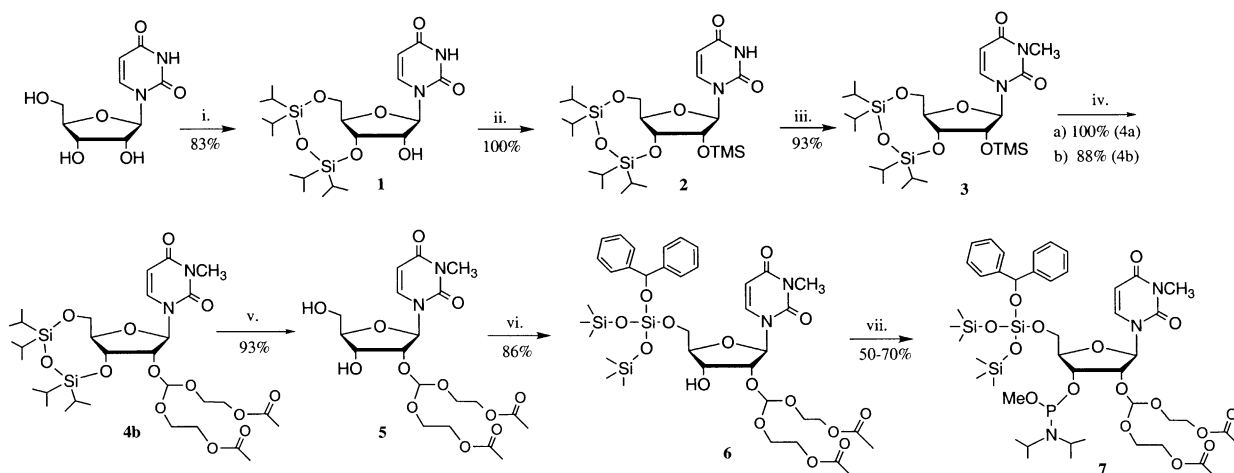
Three hairpin RNAs were synthesized in which their nucleotide sequences were derived from the 1920 region of *E. coli* 23S rRNA. These RNAs contained a combination of uridine, 3-methyluridine, and pseudouridine at positions 1911, 1915, and 1917. In order to understand more clearly the role of methylation at position 1915, the stabilities and structures of the modified and unmodified RNAs were examined through a variety of biophysical methods and compared to previous studies with RNAs containing as many as three pseudouridine residues.⁸

Results

Synthesis of a 3-methyluridine phosphoramidite

We first prepared 3',5'-O-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)uridine (**1**) (Scheme 1) according to the

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Scheme 1. (i) TIPDSCl₂, pyridine, 0 °C→rt; (ii) TMSCl, Et₃N, CH₂Cl₂, 0 °C→rt; (iii) *N,N*-dimethylformamide dimethylacetal (DMF-DMA), benzene, reflux; (iv) (a) pTSA-H₂O, Et₃N, THF; (b) tris(2-acetoxyethoxy)orthoformate, pyridinium *p*-toluenesulfonate, 4-(*tert*-butyldimethylsilyloxy)-3-penten-2-one, dioxane, rt for 48 h; (v) TMEDA-HF, CH₃CN, 0 °C; (vi) BzH-Cl, diisopropylamine, CH₂Cl₂, 0 °C; (vii) methyl tetraisopropyl phosphoradimidate, 4,5-dicyanoimidazole, CH₂Cl₂, rt.

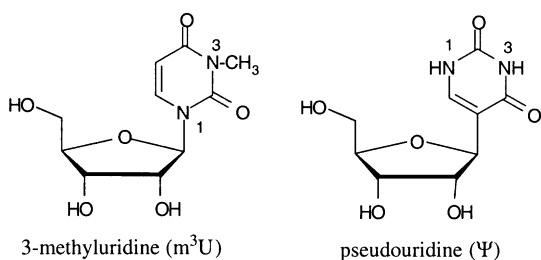
literature^{11–14} from commercially available uridine. Simultaneous protection of the 3'- and 5'-hydroxyl groups allows for the selective addition of the 2'-hydroxyl protective group. Furthermore, the disiloxanediyl ester can be cleanly and efficiently removed by exposure to TMEDA and hydrogen fluoride. We next prepared the 2'-*O*-trimethylsilyl derivative **2** in quantitative yield followed by methylation of the uridine moiety. Conventional methylation of base residues typically involves the use of methyl iodide or trimethyl

phosphate, but these procedures generally require long reaction times.¹⁵ In contrast, methylation of the base moiety with *N,N*-dimethylformamide dimethylacetal¹⁶ is a convenient and facile method that takes place under neutral conditions with shorter reaction times. This method was used to generate 3-methyl-5'-*O*-(1,1,3,3-tetraisopropyl)-1,3-disiloxanediyl)-2'-*O*-(trimethylsilyl)uridine (**3**) in 93% yield.

Compound **3** was treated with *p*-toluenesulfonic acid and triethylamine to expose the 2'-hydroxyl group which was then reacted with tris(2-acetoxyethoxy)-orthoformate and pyridinium-*p*-toluenesulfonate followed by 4-(*tert*-butyldimethylsilyloxy)-3-penten-2-one to give the 2'-*O*-ACE protected compound **4b** in 88% yield. Subsequent removal of the 3',5'-*O*-protective group, silylation of the 5'-OH with the BzH group, and 3'-*O*-phosphitylation to form the phosphoramidite were carried out as described previously.^{8–10} The final phosphoramidite product **7** was synthesized in seven steps with an overall 27–38% yield (Scheme 1). The RNA building block **7** containing the 5'-*O*-BzH ether, 2'-*O*-ACE orthoester protective group, and 3'-methyl-*N,N*-diisopropylphosphoramidite was fully characterized by ¹H, ¹³C, and ³¹P NMR spectroscopy, electrospray mass spectrometry, and elemental analysis. The remaining nucleotides (G, C, A, U, and Ψ) were converted to their corresponding phosphoramidites as described previously.^{8–10}

Synthesis and analysis of RNAs representing the sequence from the 1920 region of *E. coli* 23S ribosomal RNA

Three RNAs were synthesized in high yields (~4 mg per 1 μmol synthesis) from the building block **7** and the remaining G, C, A, U, and Ψ phosphoramidites. Figure 1 shows the sequences of the RNAs with the positions of modifications (m³U or Ψ). The natural *E. coli* 23S rRNA has pseudouridine residues at positions 1911 and



3-methyluridine (m³U)

pseudouridine (Ψ)

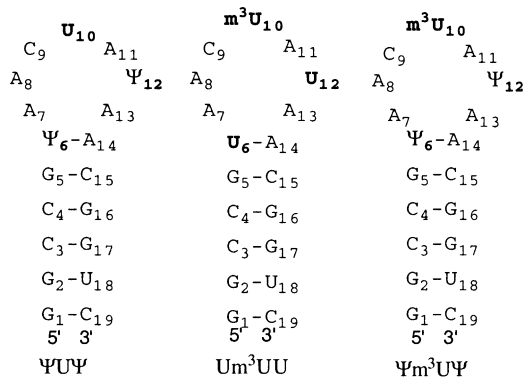


Figure 1. The structures of 3-methyluridine (m³U) and pseudouridine (Ψ) are shown. Secondary structure representations of the synthetic RNAs are based on the 1920-hairpin region (helix 69) of *E. coli* 23S rRNA (nucleotides G₁₉₀₆ to C₁₉₂₄). The nucleotide positions have been renumbered consecutively from G₁ to C₁₉. Positions 6 (1911), 10 (1915), and 12 (1917) are modified with m³U or Ψ.

1917² and a methylated derivative, m³Ψ, at position 1915.¹ In this study, uridines at positions 1911, 1915, and 1917 were replaced with combinations of pseudouridine and 3-methyluridine. All RNAs will be referred to by a three-nucleotide notation indicating the composition at positions 1911, 1915, and 1917, respectively (i.e., Ψm³UΨ, Um³UU, and ΨUΨ). The presence of the modified nucleosides was confirmed by P1 nuclease digestion and alkaline phosphatase treatment of each RNA, followed by HPLC analysis (data not shown). Three other RNAs, UUU, ΨΨΨ and UΨU, were used for comparison with the m³U-containing RNAs. These RNAs were synthesized and studied in previous work⁸ and refer to the unmodified RNA and oligoribonucleotides containing pseudouridines at all three positions (1911, 1915, and 1917) or one position (1915), respectively.

The effects of N3 methylation on the stability of the 1920-region RNAs

For the three new RNAs, Um³UU, Ψm³UΨ, and ΨUΨ, absorbance versus temperature profiles were obtained at pH 7.0 in low salt conditions (35 mM Na⁺) and analyzed in terms of melting temperature (T_m), ΔH° , ΔS° , and ΔG°_{37} .^{8,17,18} Each measurement was taken in duplicate and a range of RNA concentrations was examined (~5–200 μM). The normalized absorbance plots for single RNA concentrations are shown in Figure 2. The helix-to-coil transitions were independent of RNA concentration over the range examined, thus confirming the existence of hairpin structures for each RNA. The thermodynamic parameters for the three RNAs (Um³UU, Ψm³UΨ, and ΨUΨ) are given in Table 1.

The thermal melting data demonstrate that the presence of the m³U₁₉₁₅ methyl group has little effect on RNA hairpin stability (see Table 1; the ΔG°_{37} values for UUU

and Um³UU differ by 0.1 kcal/mol). In contrast, it was shown previously that the presence of Ψ₁₉₁₅ leads to a 0.7 kcal/mol destabilization relative to U₁₉₁₅ when two uridines are present at positions 1911 and 1917.⁸ A further comparison of the Ψ-containing RNAs (ΨUΨ, and Ψm³UΨ) reveals that U₁₉₁₅ and m³U₁₉₁₅ have similar effects on RNA stability. In contrast, previous work demonstrated that Ψ₁₉₁₅ was destabilizing by 0.5 kcal/mol if pseudouridines are present at positions 1911 and 1917.⁸ As summarized in Table 1, N3 methylation of U₁₉₁₅ does not appear to have any effect on the stability of the 1920-region RNA hairpin, regardless of whether the neighboring nucleotides in the loop region (position 1917) and closing base pair (position 1911) are uridine or pseudouridine.

The effects of N3 methylation on the structure of the 1920-region RNAs. The circular dichroism (CD) spectra of the 1920-region hairpin RNAs were obtained in order to compare the effects of uridine methylation on their structures. As shown in Figure 3, the spectra of all RNAs in this study have maxima centered around 265 nm and minima near 240 nm, which is typical for A-form RNAs. The CD spectra for UUU and Um³UU RNAs (Figure 3, open circles and closed circles, respectively) are nearly identical, suggesting that when uridines are present at residues 1911 and 1917 there is little influence from uridine methylation at position 1915 on the overall folded RNA structure. Similarly, comparison of the CD spectra for ΨUΨ and Ψm³UΨ (Figure 3, open triangles and closed triangles, respectively) reveals no differences. In contrast, comparison of the Ψ₁₉₁₁- and Ψ₁₉₁₇-containing RNAs (Figure 3, open and closed triangles) with U₁₉₁₁- and U₁₉₁₇-containing RNAs (Figure 3, open and closed circles) indicates differences in the peak shapes and cross-over points (242 nm vs 250 nm for the Ψ- and U-containing RNAs, respectively). These results are consistent with previous studies in which pseudouridine modification had an impact on the folded 1920-region RNA structure.⁸ In both cases, however, N3 methylation of U₁₉₁₅ appears to have a negligible influence on the global RNA fold.

NMR spectroscopy was used to examine the hydrogen-bonding interactions in the 1920-region RNAs containing methylations and pseudouridines. The ¹H NMR spectra (imino proton region) of the RNAs are shown in

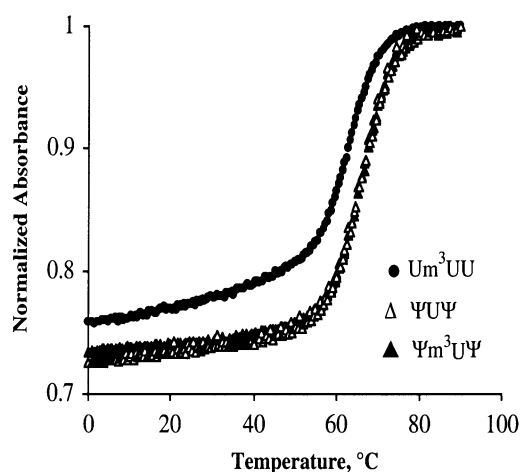


Figure 2. Representative normalized UV melting curves for the modified RNAs taken in 15 mM NaCl, 20 mM sodium cacodylate, 0.5 mM EDTA, pH 7.0 are shown. The melting curves were obtained using an Aviv 14DS UV-vis spectrometer. The curves for singly modified RNA (Um³UU), pseudouridine-modified RNA (ΨUΨ), and triply modified RNA (Ψm³UΨ) are represented by closed circles, open triangles, and closed triangles, respectively. The melting curves were normalized at 95°C.

Table 1. Thermodynamics of 1920-loop RNAs, 5'-G₁₉₀₆GCCGXAACYAZAACGGUC_{1924-3'}, with pseudouridine (Ψ) and 3-methyluridine (m³U) modifications at positions X₁₉₁₁, Y₁₉₁₅, and Z₁₉₁₇

XYZ	ΔG°_{37} (kcal/mol) ^a	ΔH° ^a (kcal/mol)	ΔS° ^a (e.u.)	T_m (°C)
UUU ^b	-4.9	-62.2	-184.8	63.7
Um ³ UU	-5.0	-63.6	-188.9	63.5
ΨUΨ	-5.3	-61.3	-180.3	66.5
Ψm ³ UΨ	-5.3	-60.9	-179.5	66.4

^aConservative estimates of standard errors for ΔG°_{37} , ΔH° , and ΔS° are 5%, 7%, and 8%, respectively.^{8,17,18} Best fits were obtained by assuming hairpin formation (the melting profiles were concentration independent). The buffer conditions were 15 mM NaCl, 20 mM sodium cacodylate, and 0.5 mM Na₂EDTA, pH 7.0.

^bData were obtained from ref 8.

Figures 4 and 5. The spectrum of the unmodified RNA (UUU) (Fig. 4A) shows five imino proton resonances that were assigned previously by using 1D NOE difference spectroscopy.⁸ The formation of three G-C base pairs and one G-U mismatch are evident from the spectrum. The loop closing U₆-A₁₄ pair (the numbering system 1–19 has been used for NMR analysis) was not observed.⁸ The ¹H NMR spectrum for the newly synthesized Um³UU RNA (Fig. 4B) is very similar to the UUU RNA spectrum, except for one additional weak resonance at 10.2 ppm (data not shown) that could not be assigned based on the 1D NOE difference spectra. The ¹H NMR spectrum of UΨU was also assigned previously and is shown here for comparison (Fig. 4C).⁸ These data suggest that N3 methylation at position 1915 is not having much influence on the overall RNA secondary structure, in contrast to pseudouridine which had subtle effects on the hairpin loop structure. These results are consistent with the CD data in which m³U₁₉₁₅ had little influence on the global RNA structures when uridines were present at positions 1911 and 1917, whereas the presence of Ψ₁₉₁₅ led to modest changes in the CD spectrum.⁸

In contrast, the ΨUΨ RNA spectrum (Fig. 5A) exhibits seven imino proton resonances. These resonances are associated with five base pairs, including a Ψ₆-A₁₄ pair. The Ψ₆-A₁₄ assignment is based on the additional resonance at 13.1 ppm (Ψ₆N3H). The Ψ₆N1H assignment at 10.2 ppm is based on a strong NOE from Ψ₆N1H to Ψ₆H6 (data not shown). The previously assigned ΨΨΨ RNA spectrum⁸ is shown in Figure 5C for comparison. The Ψm³UΨ RNA spectrum (Fig. 5B) exhibits eight

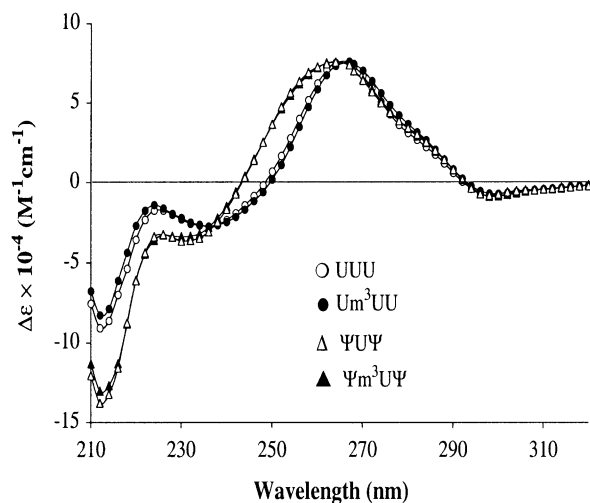


Figure 3. CD spectra of the unmodified and modified RNAs taken on a Jasco J600 spectropolarimeter from 210 to 320 nm at ambient temperature in 15 mM NaCl, 20 mM sodium cacodylate, 0.5 mM EDTA, pH 7.0 are shown. The molar ellipticities were normalized to RNA concentration (4.9, 7.4, 3.2, and 3.6 × 10⁻⁶ M in molecules of RNA for UUU, Um³UU, ΨUΨ, and Ψm³UΨ, respectively) based on an extinction coefficient of 188,860 cm⁻¹ M⁻¹ for all RNAs and A₂₆₀ values at 90 °C. The spectra for unmodified (UUU), singly modified (Um³UU), pseudouridine-modified (ΨUΨ), and triply modified (Ψm³UΨ) RNAs are represented by open circles, closed circles, open triangles, and closed triangles, respectively. Each spectrum is the average of four scans. The spectrum for UUU was obtained previously⁸ and is shown here to allow for direct comparison to Um³UU.

imino resonances. One additional peak is observed at 13.3 ppm (*), which also could not be assigned based on the 1D NOE difference spectra. Overall, these results are consistent with the CD studies in which the Ψm³UΨ

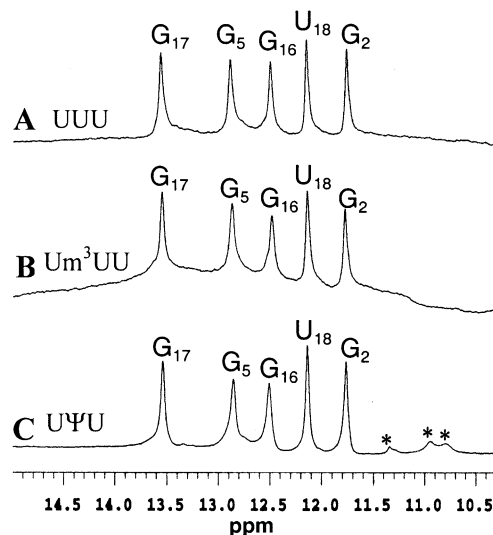


Figure 4. The 1D imino proton (guanine N1H/uridine N3H/pseudouridine N1H and N3H) NMR spectra obtained at 3 °C on a Varian UNITY 500MHz spectrometer in 30 mM NaCl, 10 mM sodium phosphate, 0.5 mM Na₂EDTA, pH 6.5 (90% H₂O/10% D₂O) are shown. The RNA concentrations for UUU (A), Um³UU (B), and UΨU (C) were 0.8 to 1 mM. Nucleotide assignments based on the 1D-NOE difference spectroscopy (data not shown) are indicated above each resonance with the nucleotides numbered consecutively from G₁ to C₁₉. The spectra in panels A and C were assigned previously⁸ and are shown here to allow for direct comparison to Um³UU.

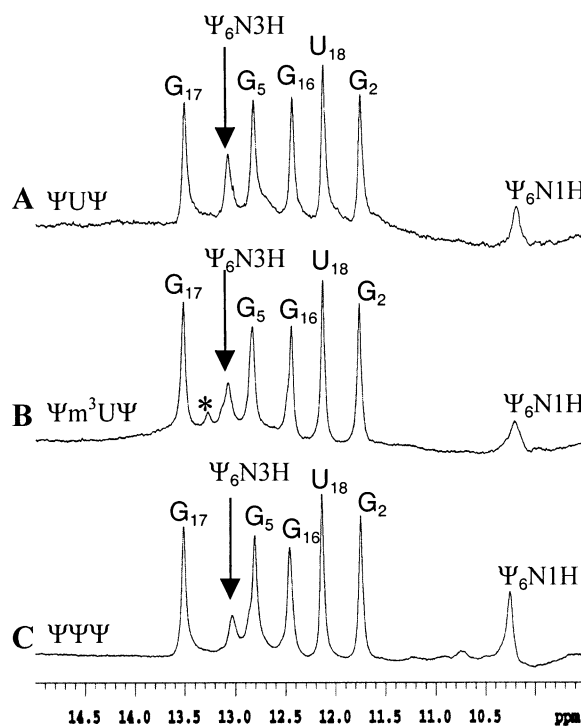


Figure 5. The 1D imino proton (guanine N1H/uridine N3H/pseudouridine N1H and N3H) NMR spectra for RNAs ΨUΨ (A), Ψm³UΨ (B) and ΨΨΨ (C) are shown. The experimental conditions were the same as those described in Figure 4. The spectrum in panel C was assigned previously⁸ and is shown here for comparison.

and $\Psi\text{U}\Psi$ RNAs had essentially the same global A-form structures. In both of these RNAs, the presence of Ψ_{1911} (Ψ_6 by the NMR numbering scheme) appears to be important for maintaining the closing base pair (Ψ_6 – A_{14}), which may further influence the loop structure. The presence Ψ_{1917} of may also influence the loop structure or closing base pair stability. Such effects have been observed previously in other Ψ -containing RNAs.^{8,19,20}

Discussion

The properties of the natural modification $\text{m}^3\Psi$ at position 1915 that contribute to the *E. coli* 23S rRNA structure and function are still not well understood. One possible function of $\text{m}^3\Psi_{1915}$ in the ribosome might be to maintain a specific folded conformation of the 23S rRNA, perhaps by stabilizing the 1920-loop structure through its N1H or N3CH₃ groups. Alternatively, this modified nucleoside might be involved in long-range contacts with other regions of the ribosome such as 16S rRNA and tRNAs.⁷ In order to understand the contribution of the N3 methyl group to the local structure of the 1920 region, we synthesized several model RNAs containing a 3-methyluridine derivative at position 1915. The m^3U residue is isosteric with $\text{m}^3\Psi$ on the major groove side, such that the influence of the methyl group alone can be assessed. The structures of the modified RNAs were compared to an unmodified RNA through a variety of techniques, including CD spectroscopy, thermal melting, and 1D imino proton NMR spectroscopy.

Modifications at positions 1911, 1915, and 1917 of the 1920 region of 23S rRNA were shown previously to modulate the stability and local secondary structure of the RNA.⁸ For example, pseudouridines either stabilize or destabilize the structure, depending on which positions they occupy. A singly modified Ψ_{1915} variant ($\text{U}\Psi\text{U}$) was 0.7 kcal/mol less stable than the corresponding unmodified RNA (UUU), and a U_{1911} – A_{1919} loop-closing base pair (U_6 – A_{14} in the NMR numbering system) was not observed in either case.⁸ In contrast, only negligible effects on stability are observed in this study for the singly modified Um^3UU RNA relative to the unmodified RNA, and the closing U_{1911} – A_{1919} pair is not observed.

This work and previous studies⁸ reveal that a pseudouridine at position 1911 is important for stabilizing the Ψ_{1911} – A_{1919} closing base pair of the hairpin loop. The presence of Ψ_{1911} in a singly modified RNA (ΨUU) led to a 1.0 kcal/mol stabilization and appearance of additional resonances in the 1D imino proton spectrum in comparison to the UUU RNA.⁸ Related studies have shown that pseudouridines at loop-closing base pairs stabilize RNA structure, possibly due to water-mediated hydrogen-bonding interactions between the RNA-phosphate backbone and Ψ -N1H.^{19,21} The presence of closing Ψ -A pairs may also influence the conformation or stability of the adjacent hairpin loops.^{8,19,20} In the 1920-region $\Psi\text{m}^3\text{U}\Psi$ RNA, the Ψ_{1911} – A_{1919} pair and/or

residue Ψ_{1917} have an influence on the hairpin loop structure as shown by differences in the shapes and crossover points of the CD spectra (compare closed circles and closed triangles in Fig. 3) and presence of additional resonances in the NMR spectra relative to the Um^3UU RNA (compare Figs 4B and 5B).

In summary, the 1920-region RNA with sequence $\text{U}_{1911}\text{m}^3\text{U}_{1915}\text{U}_{1917}$ lacks the formation of a U_{1911} – A_{1919} pair as was observed previously with $\text{U}\Psi\text{U}$ and UUU RNAs.⁸ In contrast, RNAs with the sequences $\Psi_{1911}\text{U}_{1915}\Psi_{1917}$ and $\Psi_{1911}\text{m}^3\text{U}_{1915}\Psi_{1917}$ contain a Ψ_{1911} – A_{1919} closing base pair that serves to stabilize the hairpin structure. The same effect was observed with $\Psi\Psi\Psi$ and ΨUU RNAs.⁸ For both sequence contexts, U and m^3U at position 1915 are iso-energetic, whereas Ψ was destabilizing by ~ 0.5 kcal/mol.⁸ In the present study, the fact that methylation at the N3 position of U_{1915} , which is isosteric with Ψ -N3, has no effect on the RNA stability or secondary structure suggests that the N3H of Ψ_{1915} does not participate in hydrogen-bonding interactions.

Taken together, these results suggest important roles for both Ψ_{1911} and $\text{m}^3\text{U}_{1915}$ in modulating the RNA structure and stability. The more hydrophilic nucleotide Ψ_{1911} is stabilizing relative to U, presumably due to additional hydrogen bonding through its N1H.⁸ On the other hand, the presence of the N1H (on Ψ) at position 1915 is destabilizing,⁸ whereas N3 methylation (on m^3U) has no effect on the RNA stability. These results are in contrast to other work in which a m^3U residue is destabilizing relative to U when the modification is located at a closing base pair and the U-N3H is involved in hydrogen-bonding interactions.²¹ Results similar to those obtained in this work were observed, however, when a m^3U residue replaced a uridine in a U-turn motif from the anticodon loop of tRNA.²⁰ Within the tRNA anticodon sequence context, m^3U was *iso*-energetic with uridine. Ashraf and coworkers suggested that modifications at this site in the RNA were more likely to influence long-range interactions with other RNAs rather than playing a role in the local secondary folding of the anticodon loop.²⁰ Similarly, Rife and coworkers demonstrated that an N^2 -methylguanine (m^2G) modification is *iso*-energetic with guanine in the context of several common secondary-structure motifs.²² These authors suggested that the methylated nucleoside might be introduced into natural RNAs in order to stabilize RNA–RNA or RNA–protein interactions rather than local secondary structures. The creation of a hydrophobic patch on the RNA through methylation could play a role in specific long-range tertiary interactions.

In the 1920-region RNA, the lack of secondary structure stabilization or alteration in conformation from N3 methylation suggests that this modification is also more likely to play a role in long-range tertiary interactions in the ribosome. This idea is supported by cross-linking and X-ray crystal studies on the ribosome in which the 1920 helix was shown to be involved with close contacts with the 30S subunit and 16S rRNA or tRNA.^{3–5,7} A

recent X-ray structure of the free 50S subunit revealed that the 1920 hairpin is solvent exposed,⁶ and chemical probing experiments in our laboratory have also shown that the 1915 residue in this hairpin is solvent exposed (data not shown). Thus, it can be concluded that the modified base $m^3\psi_{1915}$ in *E. coli* 23S rRNA is more likely to be involved in long-range interactions than in the maintenance of local RNA conformations, although the effects of methylation on the electronics of ψ could not be assessed here. Such long-range interactions could be important for ribosomal subunit association. The specific role of the $m^3\psi_{1915}$ in place of the unnatural analogue m^3U_{1915} in the 1920-region RNA is the subject of current investigations in our laboratories. The efficient chemical synthesis of specifically N3-methylated pseudouridine and the corresponding phosphoramidite still remains a challenge and limitation for structure studies on this naturally occurring modification.

Experimental

General

All reactions were carried out at room temperature under an inert atmosphere and anhydrous condition unless other noted. Tris(acetoxyethoxy)orthoformate, benzhydryloxy-bis(trimethylsilyloxy)silyl chloride, and 4-(*tert*-butyldimethylsilyloxy)-3-penten-2-one were prepared as described elsewhere.^{9,10} All other reagents were obtained from Aldrich (Milwaukee, WI). All solvents for RNA purification and HPLC analysis were spectra grade. ¹H, ¹³C and ³¹P NMR spectra were obtained in deuterated solvents on either 400 or 500 MHz spectrometers (Varian UNITY-400 and Unity-500, respectively). The chemical shifts of the imino protons of the 1920 hairpin RNAs were reported relative to 3-(trimethylsilyl)propionate (Wilmad, Buena, NJ) at 0.0 ppm. Microanalysis (elemental analysis) for all compounds was performed by Midwest Microlab (Indianapolis, IN). Nuclease digestions and HPLC analyses for the RNAs were performed as described previously.⁸ The RNA sample preparation and experimental conditions for the thermal melting studies, circular dichroism (CD) spectroscopy, and NMR studies were as reported previously.⁸

Preparation of the 5'-O-BzH-2'-O-ACE-3-methyluridine-3'-phosphoramidite

3',5'-O-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)-uridine (1). Uridine (1.30 g, 5.35 mmol) was dried by azeotropic removal of water with benzene in vacuo for 2 h and then dissolved in 10 mL pyridine to give a clear solution. 1,3-Dichloro-1,1,3,3-tetraisopropylidisiloxane (TIPDSCI₂) (1.53 mL, 4.86 mmol) was added dropwise to the cooled solution (0 °C) and stirred for 6 h. The solvent was evaporated and the remaining residue was taken up in ethyl acetate, washed twice with saturated NH₄Cl, pH 8, and washed once with water. The organic layers were combined and washed with aqueous brine, dried over Na₂SO₄, filtered, and concentrated. The crude product was purified by flash chromatography on silica gel using

a 20–60% ethyl acetate in hexane gradient to give a white solid (1.96 g, 83%). ¹H NMR (CDCl₃, 400 MHz) δ (ppm): 1.00–1.10 (28H, m), 3.17 (1H, d, J =1.6 Hz), 4.00 (1H, dd, J =2.4, 13.2 Hz), 4.11 (1H, dt, J =2.4, 9.2 Hz), 4.17–4.22 (2H, m), 4.36 (1H, dd, J =4.8, 8.8 Hz), 5.69 (1H, dd, J =1.6, 8.4 Hz), 5.73 (1H, s), 7.70 (1H, d, J =8.0 Hz), 8.87 (1H, s); ¹³C NMR (CDCl₃, 400 MHz) δ (ppm): 12.5, 12.9 (d), 13.3, 16.8, 16.89, 16.94, 17.0, 17.21, 17.27, 17.35, 17.4, 60.3, 69.0, 75.1, 81.9, 90.9, 101.9, 140.0, 149.8, 163.1.

3', 5'-O-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)-2'-O-(trimethylsilyl)uridine (2). A solution of **1** (1.96 g, 4.03 mmol) in CH₂Cl₂ (45 mL) was cooled to 0 °C. To this mixture were added slowly via syringe triethylamine (2.80 mL, 20.2 mmol) and trimethylsilyl chloride (TMSCl) (1.54 mL, 12.1 mmol). The solution was stirred for 2 h at 0 °C then for 1.5 h at room temperature. The resulting red solution was poured over 100 mL of cold 1.0 M aqueous NaHCO₃ and extracted twice with CH₂Cl₂. The organic layers were combined, washed with brine, dried over Na₂SO₄, and concentrated in vacuo to afford a light yellow foam (2.30 g, 100%) which was used for the next step without further purification: ¹H NMR (CDCl₃, 400 MHz) δ (ppm): 0.19 (9H, s), 0.99–1.09 (28H, m), 3.96 (1H, dd, J =2.4, 13.6 Hz), 4.05 (1H, dd, J =4.4, 9.4 Hz), 4.12 (1H, d, J =4.4 Hz), 4.16 (1H, d, J =9.6 Hz), 4.25 (1H, d, J =13.6 Hz), 5.58 (1H, s), 5.66 (1H, dd, J =1.6, 8.0 Hz), 7.97 (1H, d, J =8.0 Hz), 9.36 (1H, s, NH); ¹³C NMR (CDCl₃, 400 MHz) δ (ppm): 0.5, 13.0, 13.1, 13.5, 13.6, 17.0, 17.1, 17.2, 17.3, 17.4, 17.6, 17.7, 17.8, 59.7, 68.3, 76.6, 81.7, 91.4, 101.4, 140.0, 150.2, 164.1. Exact mass calculated for C₂₄H₄₆N₂O₇Si₃: 558.6; found by LC-MS (ES⁺): [M + Na]⁺ 581.2.

3-Methyl-3',5'-O-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)-2'-O-(trimethylsilyl)uridine (3). To a solution of **2** (2.30 g, 4.12 mmol) in 25 mL benzene was added dropwise *N,N*-dimethylformamide dimethyl acetal (DMF-DMA) (1.64 mL, 12.3 mmol). The reaction refluxed for 7 h and the solvent was evaporated. The crude product was purified by flash chromatography on silica gel using a 25–60% ethyl acetate in hexane gradient to afford a white solid (2.20 g, 93%). ¹H NMR (CDCl₃, 400 MHz) δ (ppm): 0.2 (9H, s), 0.98–1.09 (28H, m), 3.33 (3H, s), 3.96 (1H, dd, J =2.4, 14.0 Hz), 4.05–4.11 (2H, m), 4.16 (1H, dd, J =1.6, 8.8 Hz), 4.25 (1H, d, J =13.6 Hz), 5.59 (1H, s), 5.70 (1H, d, J =8.0 Hz), 7.93 (1H, d, J =8.4 Hz); ¹³C NMR (CDCl₃, 400 MHz) δ (ppm): 0.3, 12.7, 12.8, 13.2, 13.4, 16.8, 16.9, 16.96, 17.0, 17.2, 17.3, 17.4, 17.5, 27.4, 59.5, 68.1, 76.5, 81.4, 91.5, 100.6, 137.3, 150.7, 163.2. Exact mass calculated for C₂₅H₄₈N₂O₇Si₃: 572.6; found by LC-MS (ES⁺): [M + Na]⁺ 595.1, [2M + Na]⁺ 1167.4.

3-Methyl-3',5'-O-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)uridine (4a). To a clear solution of **3** (2.12 g, 3.70 mmol) in THF (53 mL) was added *p*-toluenesulfonic acid monohydrate (pTSA-H₂O) (1.06 g, 5.55 mmol). After stirring the reaction solution for 1.5 h, triethylamine (1.1 mL, 8.0 mmol) was added dropwise and stirred for another 15 min. The reaction was quenched with

5% aqueous NaHCO_3 and extracted with ethyl acetate (2×100 mL). The organic layers were combined, washed with brine, dried over Na_2SO_4 , and concentrated to give a light yellow oil which was purified by flash chromatography on silica gel with 25–50% ethyl acetate in hexane gradient to give a colorless thick oil. Repeated co-evaporation with CH_2Cl_2 gave a white foam (1.90 g, 100%). ^1H NMR (CDCl_3 , 400 MHz) δ (ppm): 1.00–1.09 (28 H, m), 2.84 (1H, d, $J=1.2$ Hz), 3.33 (3H, s), 4.00 (1H, dd, $J=2.4$, 12.9 Hz), 4.09 (1H, ddd, $J=2.4$, 8.7 Hz), 4.14 (1H, d, $J=5.1$ Hz), 4.21 (1H, dd, $J=1.8$, 12.9 Hz), 4.37 (1H, dd, $J=5.1$, 9.1 Hz), 5.74 (1H, d, $J=8.1$ Hz), 5.76 (1H, s), 7.66 (1H, d, $J=8.4$ Hz); ^{13}C NMR (CDCl_3 , 400 MHz) δ (ppm): 12.5, 12.8, 12.9, 13.3, 16.8, 16.87, 16.94, 17.0, 17.2, 17.24, 17.3, 17.4, 27.5, 60.2, 69.0, 75.3, 81.8, 91.2, 101.2, 137.5, 150.7, 162.9. Exact mass calculated for $\text{C}_{22}\text{H}_{40}\text{N}_2\text{O}_7\text{Si}_2$: 500; found by LC-MS (ES^+): $[\text{M} + \text{H}]^+$ 501.

2'-O-[bis(2-acetoxyethoxy)methyl]-3-methyl-3',5'-O-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)uridine (4b). To a 100 mL round bottom flask, compound **4a** (1.85 g, 3.70 mmol in 10 mL CH_2Cl_2), tris(2-acetoxyethoxy)-orthoformate (2.74 g, 8.51 mmol) and pyridinium *p*-toluenesulfonate (1.11 g, 4.40 mmol) were added to give a clear solution. The reaction was stirred for 2 h, then 4-(*tert*-butyldimethylsilyloxy)-3-penten-2-one (1.6 mL, 6.7 mmol) was added and stirred for 48 h. The reaction progress was monitored by TLC with 25% acetone in hexane containing 0.5% *N,N,N',N'*-tetramethylethylenediamine (TMEDA). Upon completion, the reaction was quenched by the addition of TMEDA (0.2 mL, 1.8 mmol) and stirred for 15 min. The resulting brown reaction mixture was diluted with 3 mL of hexanes/ CH_2Cl_2 (5:1) and purified by flash chromatography on silica gel using 0.5% TMEDA in hexane/acetone (4:1) to afford a colorless oil (2.32 g, 88%). ^1H NMR (CDCl_3 , 400 MHz) δ (ppm): 0.99–1.09 (28H, m), 2.05 (3H, s), 2.07 (3H, s), 3.31 (3H, s), 3.82–4.0 (7H, m), 4.16–4.30 (6H, m), 5.72 (2H, d, $J=8.4$ Hz), 5.77 (1H, d, $J=7.2$ Hz), 7.84 (1H, d, $J=8.4$ Hz); ^{13}C NMR (CDCl_3 , 400 MHz) δ (ppm): 12.5, 12.8, 13.0, 13.3, 16.7, 16.9, 17.0, 17.1, 17.2, 17.3, 17.4, 20.8, 20.9, 27.4, 59.2, 60.7, 62.0, 63.1, 63.4, 67.8, 77.2, 81.7, 89.4, 101.0, 111.6, 136.8, 150.5, 162.8, 170.8, 170.9. Exact mass calculated for $\text{C}_{31}\text{H}_{54}\text{N}_2\text{O}_{13}\text{Si}_2$: 718.5; found by LC-MS (ES^+): $[\text{M} + \text{Na}]^+$ 741.3.

2'-O-[bis(2-acetoxyethoxy)methyl]-3-methyluridine (5). CH_3CN (6.5 mL), TMEDA (2.42 mL, 16.1 mmol) and HF (48% aq stock solution, 0.36 mL, 11.3 mmol) were added to a 50 mL round bottom flask at 0°C dropwise via syringe. The HF/TMEDA mixture was stirred at 0°C for 10 min, then transferred to a light yellow solution of **4b** (2.32 g, 3.23 mmol in 6.5 mL CH_3CN) at 0°C dropwise over 5 min by cannula. The resulting solution was stirred at 0°C for 10 min then at room temperature until no more of the starting material was apparent by TLC analysis in 10% CH_3OH in CH_2Cl_2 . Upon completion of the reaction, the solvent was evaporated and the residue was taken up in a mixture of hexane/ CH_2Cl_2 5:1 (5 mL) containing 0.2% TMEDA. The crude product was purified by flash chromatography on silica gel

with 0.5% TMEDA in the eluents (33% ethyl acetate in hexane, then 0–5% CH_3OH in ethyl acetate) to give a pale yellow oil (1.43 g, 93%). ^1H NMR (CDCl_3 , 500 MHz) δ (ppm): 2.07 (6H, s), 2.97 (1H, bs), 3.04 (1H, bs), 3.31 (3H, s), 3.70–3.75 (1H, m), 3.77–3.85 (4H, m), 3.97 (1H, dd, $J=2.0$, 12.5 Hz), 4.12–4.14 (1H, m), 4.17–4.26 (4H, m), 4.34 (1H, t, $J=5.0$ Hz), 4.66 (1H, t, $J=5.0$ Hz), 5.45 (1H, s), 5.71 (1H, d, $J=4.5$ Hz), 5.78 (1H, d, $J=8.5$ Hz), 7.59 (1H, d, $J=8.0$ Hz); ^{13}C NMR (CDCl_3 , 400 MHz) δ (ppm): 20.78, 20.81, 27.6, 61.9, 62.7, 62.8, 62.9, 63.2, 69.8, 75.7, 85.2, 92.0, 101.9, 112.5, 139.8, 151.1, 162.7, 170.86, 170.89. Exact mass calculated for $\text{C}_{19}\text{H}_{28}\text{N}_2\text{O}_{12}$: 476.1; found by LC-MS (ES^+): $[\text{M} + \text{Na}]^+$ 499.1.

5'-O-[benzhydryloxy-bis(trimethylsilyloxy)silyl]-2'-O-[bis(2-acetoxyethoxy)methyl]-3-methyluridine (6). Solution A: to a solution of **5** (0.221 g, 0.46 mmol in 2.5 mL CH_2Cl_2) was added diisopropylamine (65 μL , 0.46 mmol) and cooled to 0°C . Solution B: diisopropylamine (156 μL , 1.1 mmol) was added dropwise to benzhydryloxy-bis(trimethylsilyloxy)silyl chloride (BzH-Cl) (395 mg, 0.93 mmol) in CH_2Cl_2 (0.8 mL) at 0°C . Aliquots of solution B (0.5 equiv and 0.16 equiv $\times 5$ aliquots) were added to solution A at 0°C dropwise over 2 min and stirred for 10 min after each addition. The reaction progress was monitored by TLC (hexane/ethyl acetate 1:1). Upon completion, the reaction was quenched with 5% NaHCO_3 and extracted with CH_2Cl_2 (2 100 mL). The combined organic layers were washed with brine, dried over Na_2SO_4 , concentrated, and purified by flash chromatography on silica gel with 0.5% triethylamine (TEA) in the eluents (hexane/acetone 4:1 followed by hexane/ethyl acetate/acetone 3:1:1) to afford a colorless oil (343 mg, 86%). ^1H NMR (CDCl_3 , 400 MHz) δ (ppm): 0.02 (9H, s), 0.03 (9H, s), 2.00 (3H, s), 2.02 (3H, s), 2.98 (1H, d, $J=6.0$ Hz), 3.26 (3H, s, NCH_3), 3.73–3.82 (5H, m), 3.93–3.98 (2H, m), 7.14–7.31 (10H, m), 7.75 (1H, d, $J=8.0$ Hz); ^{13}C NMR (CDCl_3 , 500 MHz) δ (ppm): 1.28, 1.29, 20.57, 20.59, 27.3, 61.2, 62.6, 62.7, 62.8, 68.3, 76.7, 77.3, 83.7, 88.0, 101.3, 112.2, 126.0, 126.1, 127.1, 128.1, 137.3, 143.5, 143.6, 150.8, 162.5, 170.5, 170.6. Exact mass calculated for $\text{C}_{38}\text{H}_{56}\text{N}_2\text{O}_{15}\text{Si}_3$: 864.6; found by LC-MS (ES^+): $[\text{M} + \text{Na}]^+$ 887.2.

5'-O-[benzhydryloxy-bis(trimethylsilyloxy)silyl]-2'-O-[bis(2-acetoxyethoxy)methyl]-3-methyluridine-3'-(methyl-*N,N*-diisopropyl)phosphoramidite (7). To a solution of **6** (1.00 g, 1.16 mmol) in CH_2Cl_2 (13 mL), methyl tetra-isopropyl phosphoradiamidite (0.93 mL, 3.25 mmol), and 4,5-dicyanoimidazole (137 mg, 1.20 mmol) were added. The resulting cloudy solution was stirred overnight, then quenched with 5% aqueous NaHCO_3 and extracted with CH_2Cl_2 . The organic layers were combined, dried over Na_2SO_4 , and concentrated. The crude product was purified two times by flash chromatography with 0.5% TEA in the eluents (20% acetone in hexane followed by 5–10% CH_2Cl_2 in hexane) to afford a colorless oil (0.60 g, 50%). Yields can be improved to 70–75% if the methyl tetra-isopropyl phosphoramidite reagent is freshly prepared.²³ ^1H NMR (CDCl_3 , 500 MHz) δ (ppm) (mixture of diastereomers): 0.02–0.06 (36H, four s, $\text{Si}(\text{CH}_3)_3$), 1.15–1.20 (24H, four s,

CH(CH₃)₃, 2.04 (6H, d, J = 1.5 Hz, COCH₃), 2.05 (6H, s, COCH₃), 3.295 and 3.298 (6H, two s, NCH₃), 3.31 and 3.37 (6H, two d, J = 12.5, 13.0 Hz, OCH₃), 3.53–3.63 (4H, m, CH(CH₃)₂), 3.72–4.36 (26H, m, OCH₂CH₂, H2', 3', 4', 5', 5''), 5.43 and 5.49 (2H, two d, J = 8.0 Hz, H5), 5.49 and 5.63 (2H, two s, OCH(Ph)₂), 5.92 and 5.93 (2H, two d, J = 3.0, 4.0 Hz, H1'), 7.18–7.34 (20H, m, Ph), 7.77–7.80 (2H, two d, J = 8.0 Hz, H6); ³¹P NMR (CDCl₃, 500 MHz) δ (ppm) (mixture of diastereomers): 151.89 and 151.92. Exact mass calculated for C₄₃H₇₂N₃O₁₆Si₃P: 1025.4; found by LC-MS (ES⁺) (CH₃OH): [M + H]⁺ 1026.3; [M + Na]⁺ 1048.3. Elemental anal. calcd for C₄₃H₇₂N₃O₁₆Si₃P: C, 52.66; H, 7.08; N, 4.10; found: C, 52.82; H, 6.97; N, 3.89.

Synthesis, deprotection, and purification of modified and unmodified RNA oligonucleotides. Two RNAs containing a 3-methyluridine (m³U) modification at position 1915 and one pseudouridine-modified RNA were synthesized chemically on a 1.0 μ mol scale using polystyrene supports as described previously.^{8–10} The sequences of the three RNAs are as follows with the numbering based on the full length *E. coli* 23S rRNA: 5'-G₁₉₀₆GCCGU₁₉₁₁AACm³U₁₉₁₅AU₁₉₁₇AACGGUC_{1924-3'} (referred to as Um³UU; the names of the RNAs correspond to the nucleotides at positions 1911, 1915, and 1917, respectively), 5'-G₁₉₀₆GCCG- Ψ ₁₉₁₁AACm³U₁₉₁₅A Ψ ₁₉₁₇AACGGUC_{1924-3'} (Ψ m³U Ψ ; where Ψ = pseudouridine), and 5'-G₁₉₀₆GCCG- Ψ ₁₉₁₁AACU₁₉₁₅A Ψ ₁₉₁₇AACGGUC_{1924-3'} (Ψ U Ψ). Each crude RNA was divided into four microcentrifuge tubes and deprotected by adding 400 μ L of 100 mM NaOAc buffer (pH 3.8) to each tube and heating at 60 °C for 30 min. The deprotected RNA oligonucleotides were then purified by gel electrophoresis on 20% denaturing polyacrylamide gels followed by electroelution with an Amicon centriluterTM and centricon 3's (Millipore Corporation, Bedford, MA). The gel-purified RNA oligonucleotides were desalted by dialysis against 3 $\times$ 4 L of RNase-free deionized water for two days. The dialyzed RNAs were then lyophilized to dryness and further purified over C-18 Sep-PakTM (Waters, Milford, MA) columns. Each Sep-PakTM column was prepared by addition of 10 mL CH₃CN followed by 10 mL RNase-free deionized water. Approximately 10 OD units (~300 μ g) of RNA oligonucleotide was loaded onto each column which was then washed with 5 mL deionized water. The RNA eluted from the column in three fractions with 60% CH₃OH in water. The final purified and desalted RNA oligonucleotides were dried under reduced pressure in a Speed-Vac concentrator to give a white solid. The single-stranded extinction coefficient (ϵ) for each RNA (188,860 cm⁻¹ M⁻¹) was calculated as described by Richards.²⁴ The extinction coefficient for uridine (1.0 $\times$ 10⁴ cm⁻¹ M⁻¹ at pH 7.0) was used for pseudouridine and 3-methyluridine because the nearest-neighbor extinction coefficients for those modified bases are unknown, thus some error in the RNA concentrations could be present.

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