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Synthesis of Helix 69 of *Escherichia coli* 23S rRNA Containing Its Natural Modified Nucleosides, $m^3\Psi$ and Ψ

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The synthesis of 3-methylpseudouridine ($m^3\Psi$) phosphoramidite, 5′-*O*-[benzhydryloxybis(trimethylsilyloxy)silyl]-2′-*O*-[bis(2-acetoxyethoxy)methyl]-3-methylpseudouridine-3′-(methyl-*N*,*N*-diisopropyl)phosphoramidite, is reported. Selective pivaloyloxymethyl protection of the Ψ N1 followed by methylation at N3 was used to generate the naturally occurring pseudouridine analogue. The $m^3\Psi$ phosphoramidite was used in combination with pseudouridine (Ψ) and standard base phosphoramidites to synthesize a 19-nucleotide RNA representing helix 69 of *Escherichia coli* 23S ribosomal RNA (rRNA) (residues 1906−1924), containing a single $m^3\Psi$ at position 1915 and two Ψ's at positions 1911 and 1917. Our synthesis of the fully modified helix 69 RNA demonstrates the ability to make milligram quantities of RNA that can be used for further high-resolution structure studies. Site-selective introduction of the methyl group at the N3 position of pseudouridine at position 1915 causes a slight increase in the thermodynamic stability of the RNA hairpin relative to pseudouridine; RNAs containing either uridine or 3-methyluridine at position 1915 have similar stability. One-dimensional imino proton NMR and circular dichroism spectra of the modified RNAs reveal that the methyl group does not cause any substantial changes in the RNA hairpin structure.

Introduction

The site of protein synthesis in the ribosome is comprised of a set of specific structural motifs of the large and small subunit rRNAs, known as 23S and 16S rRNA in *Escherichia coli*. Interestingly, a large number of nucleotides in this region (from 23S rRNA, 16S rRNA, and tRNAs) are modified, including methylation and pseudouridylation.[1−3] Helix 69 of domain IV, or residues 1906−1924 in 23S rRNA, is centered at the heart of the ribosome machinery, and recent crystallographic studies revealed that this conserved, highly modified hairpin is in close proximity to the decoding region of 16S rRNA and tRNAs.[4] More specifically, the minor groove of helix 69 interacts with the minor groove of the D stem of the P-site tRNA, and the 1915 loop region interacts with the D stem of the A-site tRNA.[4] The loop portion of helix 69 was disordered in the *Haloarcula marismortui* 50S subunit structure,[5] which can be explained by the absence of direct stacking or packing interactions with the 30S subunit. In the *Thermus thermophilus* 70S ribosome structure, helix 69 is clearly observed contacting the decoding region near position 1408. Helix 69 of domain IV appears to be involved with almost all of the RNA−RNA contacts on the 50S subunit side of the 70S intersubunit interface; thus, it is likely to play a critical role in protein synthesis.[4] The exact biological role of helix 69 is still unclear, and its structural role is also not completely defined because of the dynamic nature of this region. Helix 69 likely undergoes conformational changes during protein synthesis because of its contacts with the mobile tRNAs.

The secondary structure of helix 69 is highly conserved throughout phylogeny, and three of its modified residues are pseudouridines or pseudouridine analogues. Therefore, structural information obtained on this hairpin may be relevant across a broad range of organisms. The disorder of helix 69 in the 50S subunit X-ray structure may be significant and suggests that interactions with the 30S subunit and tRNAs in the peptidyltransferase center may be coupled with RNA loop flexibility. Modified bases such as pseudouridine may play a role in controlling RNA flexibility or in making direct contacts with RNA motifs in its vicinity. Understanding the dynamic nature of RNA can be approached by carrying out solution NMR studies, which require milligram quantities of the RNAs with all of their natural modifications. Our approach is to synthesize the 19-nucleotide helix 69 RNA (Figure 1) by using RNA phosphoramidite chemistry[6] and then to carry out biophysical studies on the isolated hairpin RNA. The resulting synthetic RNA contains three natural modified nucleotides, Ψ_{1911}, $m^3\Psi_{1915}$, and Ψ_{1917}, in which Ψ is pseudouridine and $m^3\Psi$ is

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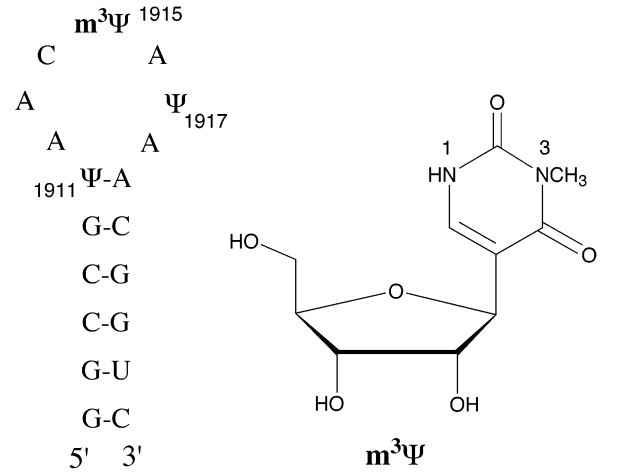

FIGURE 1. Secondary structure of the 19-nucleotide sequence of helix 69 (1920 loop) of *E. coli* 23S rRNA domain IV and the structure of modified nucleoside $m^3\Psi$, which occurs at position 1915.

3-methylpseudouridine. The advantage of the synthetic approach is that the effects of pseudouridine and the methyl substitution can be assessed individually, but within the context of the natural 23S rRNA helix 69 sequence.

Although Ψ and U are structural isomers, the presence of the additional NH group in pseudouridine can dramatically influence the overall structure of an RNA oligonucleotide.[7] The effects are more subtle in the helix 69 RNA,[8] and methylation of a uridine at position 1915 does not have much influence on the stability or secondary structure of the RNA hairpin.[9] This study focused on the synthesis of the natural nucleoside at position 1915 of *E. coli* 23S rRNA, 3-methylpseudouridine, and its conversion into the corresponding phosphoramidite and incorporation into a 19-nucleotide helix 69 RNA. This modified nucleoside has been synthesized previously, but not in quantities sufficient for phosphoramidite synthesis or high-resolution NMR structure studies.[3,10,11] Our synthetic approach involved the selective protection of N1 of the uracil base with pivaloyloxymethyl (POM), followed by N3 methylation. Helix 69 RNAs (also referred to as 1920 loop RNAs) containing one or all of the natural modifications were synthesized, and circular dichroism, UV melting, and NMR studies were carried out to determine the effects of $m^3\Psi$ on the RNA structure and stability.

Results and Discussion

$m^3\Psi$ Phosphoramidite Synthesis. The first goal of this work was to generate the natural modified nucleoside 3-methylpseudouridine ($m^3\Psi$) and the corresponding 5′-*O*-BzH-2′-*O*-ACE-protected phosphoramidite (BzH, benzhydryloxybis(trimethylsilyloxy)silyl; ACE, bis(2-acetoxyethoxy)methyl). Previous studies have been carried out in our laboratories to synthesize pseudouridine[12] and its corresponding 5′-*O*-BzH-2′-*O*-ACE-protected phosphoramidite.[8] In nature, pseudouridines (Ψ) are synthesized through isomerization of uridines within the RNA motifs by one of many specific pseudouridine synthases, such as RluD.[13] Methylations of pseudouridines are presumed to occur through the action of specific RNA methyltransferases. Although the natural enzymes appear quite capable of specific methylation of N1 or N3 of Ψ, such specific modification is considerably more challenging to the organic chemist. Luyten et al. reported that pseudouridine N1 is only slightly more acidic ($pK_a = 9.3$) than N3 ($pK_a = 9.6$).[14] Thus, one can infer that the chemical reactivities of N1 and N3 in solution will be similar. To generate 1-methylpseudouridine or 3-methylpseudouridine, an efficient method to protect selectively either the N1 or N3 position is necessary. Several approaches to synthesize methylated pseudouridines, such as 1-methylpseudouridine and 1,3-dimethylpseudouridine, in high yields have been reported previously.[15−17] One group has produced 3-methylpseudouridine and the corresponding phosphoramidite; however, details of the syntheses and compound characterizations were not provided in their reports.[10,18] Other reports on 3-methylpseudouridine syntheses have lacked in complete characterization of the product(s), or the reactions proceeded with poor regioselectivity.[11,19] For example, Cohn showed that N-alkylation of pseudouridine in the presence of diazomethane formed multiple methylated products.[19] Matsuda et al. reported an 80% yield of 3-methylpseudouridine by using trimethylsilylated pseudouridine and generating the 1-acetyl derivative.[11] The 1-acetylpseudouridine product was methylated to give 3-methylpseudouridine; however, the assignment of the regiochemistry of the methyl group at N3 was ambiguous. The reported chemical shift of the methyl group in DMSO was shifted by ∼0.8 ppm (2.52 ppm) compared to those in other spectra of methylated pseudouridine taken in the same solvent (3.29 ppm).[11,20] The synthesis reported by Kowalak et al. was motivated by the desire to identify 3-methylpseudouridine in *E. coli* 23S rRNA by mass spectrometry.[3] For that reason, their synthesis was only carried out on the milligram scale, and the product was not characterized by NMR.

Here we report on a facile and selective method for production of 3-methylpseudouridine and preparation of the corresponding phosphoramidite. Our approach involved an N1 protection−deprotection strategy in which the N3 position can then be selectively methylated. The

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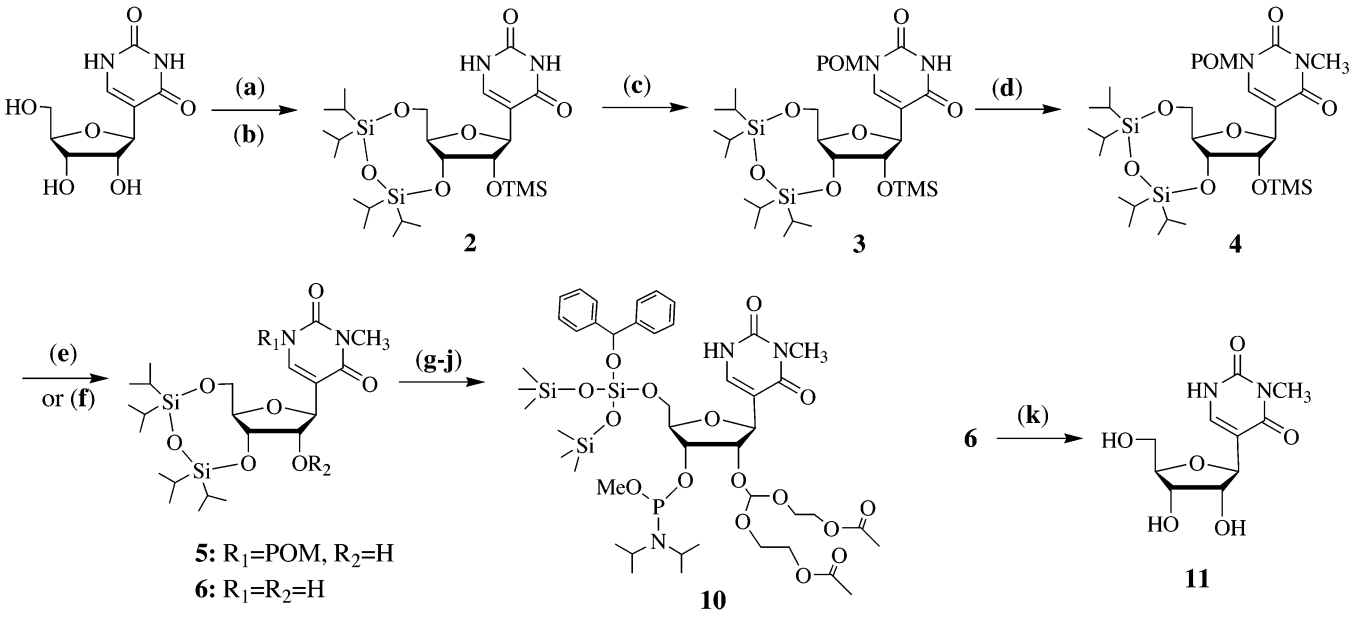
SCHEME 1. Synthesis of the m³Ψ Phosphoramidite[a]



[a] Reagents and conditions: (a) $TIPDSCl_2$, pyridine, 0 °C → rt, 94% yield of **1**; (b) TMSCl, Et_3N, CH_2Cl_2, 0 °C → rt, 99% yield of **2**; (c) POMCl (9 equiv), Et_3N, pyridine, rt, 91% yield of **3**; (d) DMF-DMA, benzene, reflux, 98% yield of **4**; (e) (i) pTSA·H_2O, Et_3N, THF, 95% yield of **5**; (ii) NH_3/CH_3OH, rt, 94% yield of **6**; (f) excess NH_3/CH_3OH, rt, 92% yield of **6**; (g) tris(2-acetoxyethoxy)orthoformate, pyridinium *p*-toluenesulfonate, 4-(*tert*-butyldimethylsilyloxy)-3-penten-2-one, dioxane, rt → 40 °C, 69−80% yield of **7**; (h) TMEDA/HF, CH_3CN, 0 °C, 68% yield of **8** from **6**; (i) BzHCl, diisopropylamine, CH_2Cl_2, 0 °C, 68% yield of **9**; (j) methyl tetraisopropyl phosphorodiamidite, 1*H*-tetrazole, CH_2Cl_2, rt, 77% yield of **10**; (k) TMEDA/HF, CH_3CN, 0 °C, 85% yield of **11**.

synthetic route is illustrated in Scheme 1. The first step involved simultaneous protection of the 3′- and 5′-hydroxyl groups of pseudouridine using dichlorotetraisopropyldisiloxane in the presence of pyridine at 0 °C to give compound **1** (94% yield). Compound **1** was treated with trimethylsilyl chloride in the presence of triethylamine to give compound **2** in 99% yield. The final phosphoramidite will contain 2′-*O*-ACE and 5′-*O*-BzH protective groups; however, the ACE group was found to be unstable under the conditions of the N1 protection reaction. For this reason, the TMS group was employed for 2′-hydroxyl protection. The TMS group can also be removed simultaneously or selectively in the presence of the N1 protective group POM under mild conditions. Following protection of the ribose hydroxyl groups, the N1 position was selectively protected by reaction with triethylamine/pyridine (6:1) and excess (>9 equiv) pivaloylmethyl chloride (POMCl) for 5 days to give compound **3** in 91% yield. The addition of a single portion of POMCl (9 equiv) gave a lower yield of **3** (71%), and we found that it was necessary to add the reagent in smaller aliquots (∼3 equiv) in 24 h intervals.

We observed that the selectivity of the POM reaction with N1 or N3 was solvent dependent and for the reaction to be driven to completion a large excess of POMCl (∼9−15 equiv) was required. These reaction conditions were based on an earlier report by Pieles et al. in which the POM group was employed to protect both N1 and N3 of pseudouridine.[21] Some of the results for solvent effects are summarized in Table 1. Solvent polarity was based on dielectric constant and dipole moment characteristics.[22] In polar solvents (DMSO, CH_3CN, and DMF), the formation of *N*1-POM-protected pseudouridine (**3**) occurred in a relatively short time in low yield with competing formation of the *N*1,*N*3-bis-POM product. Reactions with stoichiometric amounts of POMCl (1.1 equiv) and K_2CO_3 in DMF at room temperature gave near equimolar mixtures of *N*1-POM- and *N*1,*N*3-bis-POM-protected pseudouridines along with ∼40% starting material (**2**). In nonpolar solvents (Et_3N, $CHCl_3$, and CCl_4), the formation of *N*1-POM required longer reaction times with little production of *N*1,*N*3-bis-POM-protected pseudouridine. The use of THF as a solvent led to production of *N*1-POM-protected pseudouridine in low yield, even with long reaction times. As more POMCl was added (in THF), the formation of *N*1,*N*3-bis-POM product increased and the amount of *N*1-POM-protected pseudouridine remained the same. When THF/K_2CO_3 or DMF/NaH was used, only the formation of *N*1-POM-protected pseudouridine (**3**) in ∼30% and 70% yields, respectively, was observed after 24−72 h at room temperature. Thus, the *N*1-POM-protected pseudouridine appears to be the thermodynamically favored product, and the selectivity of POM for N1 or N3 is dependent on the solvent polarity. The complete details of the solvent conditions, temperature, and bases employed are currently under further investigation.

Following successful protection of N1, compound **3** was methylated under neutral conditions by refluxing in *N*,*N*-dimethylformamide dimethyl acetal (DMF-DMA) in benzene for 6 h to obtain a single product, **4**, in 98% yield. Stepwise removal of 2′-*O*-TMS and *N*1-POM protective groups can be achieved by treatment with *p*-toluene-

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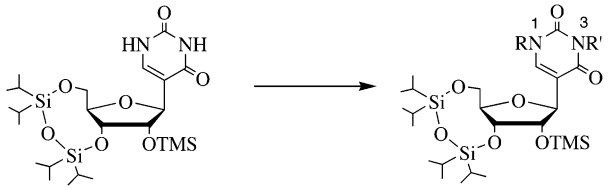
TABLE 1. Reaction of 2 with POMCl



2

solvent	base	amt of POMCl (equiv)	time (h)	product distribution (%) R = POM, R′ = H	R = H, R′ = POM	R = R′ = POM	**2**
DMSO	K_2CO_3	1.1	12	~30[a]	~30[a]	~30[a]	~10[a]
CH_3CN	K_2CO_3	1.1	12	~10[a]	0[a]	~80[a]	~10[a]
DMF	K_2CO_3	1.1	12	31[b]	0	25[b]	44[b]
THF	K_2CO_3	1.1	24	0	0	0	100[b]
THF	K_2CO_3	1.1	60	29[b]	0	0	58[b]
THF	K_2CO_3	5.0	60	~33[a]	0[a]	~33[a]	~33[a]
Et_3N/py	none	1.1	48	45[b]	0	0	35[b]
Et_3N/py	none	9.0	96	91[b]	0	<5[a]	4[b]
$CHCl_3$	K_2CO_3	1.1	48	~70[a]	0	~15[a]	~15[a]
CCl_4	NaH	1.1	48	~70[a]	0	~15[a]	~15[a]

[a] Reaction yields are estimated by TLC analysis (2:1 hexane/EtOAc). [b] Isolated yields. All reactions were carried out at room temperature.

sulfonic acid (pTSA) followed by methanolic ammonia to give **5** and then **6** (89% for two steps), or compound **6** can be obtained in one step by reacting **4** with excess methanolic ammonia (92%). The 3′,5′-*O*-TIPDS-protected (TIPDS = 1,1,3,3-tetraisopropyldisiloxanediyl) 3-methylpseudouridine can be obtained in an overall 76% yield from pseudouridine on a gram scale. The products were fully characterized by NMR spectroscopy and mass spectrometry. The ^{1}H NMR spectrum of **4** in $CDCl_3$ exhibited a methyl signal at δ 3.34 ppm. The unprotected product 3-methylpseudouridine (**11**) was obtained in 85% yield by treatment of **6** with TMEDA/HF and examined by NOE spectroscopy to confirm the identity and location of the methyl group. No NOEs between base protons were observed upon irradiation of either the *N*3-CH_3 or H6 peaks. The regioisomer 1-methyl-3-pivaloyloxymethyl-3′,5′-*O*-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)-2′-*O*-(trimethylsilyl)pseudouridine was also synthesized and characterized by NMR. In this case, the ^{1}H NMR spectrum taken in $CDCl_3$ exhibited a methyl signal at δ 3.37 ppm, and a strong NOE between H6 and *N*1-CH_3 was observed (Supporting Information). We have also observed that upon methylation of the Ψ NH position, the two methylene protons of the POM group on compound **4** or its regioisomer exhibit major differences in their *J* coupling patterns in the proton NMR spectra. The methylene group of POM showed different spin-coupling patterns depending on its location on Ψ. For **4**, the spin coupling between the two geminal protons (δ 5.46 (d) and 5.76 (d) ppm) exhibited a typical first-order AX pattern (where $\Delta\nu/J = 12.5$). In contrast, the spin coupling between the two methylene protons (δ 5.92 (d) and 5.95 (d) ppm) on the regioisomer exhibited an AB splitting pattern (where $\Delta\nu/J = 1.2$) (Supporting Information).

To complete the synthesis of the 3-methylpseudouridine phosphoramidite, compound **6** was reacted with tris(2-acetoxyethoxy)orthoformate in the presence of pyridinium *p*-toluenesulfonate and 4-(*tert*-butyldimethylsilyloxy)-3-penten-2-one (TBDMS-acac) to achieve 2′-*O*-ACE protection. The addition of TBDMS-acac was necessary to drive the reaction to completion; we presume that this reagent works by reacting with the primary alcohol that is formed upon ionization of the tris(2-acetoxyethoxy)orthoformate. In our previous studies, the ACE protection reaction was carried out at 55 °C or room temperature.[6,8,9] For the 3-methylpseudouridine analogue, ACE protection was carried out at 40 °C for 48 h to obtain **7** in 69−80% yield (reactions at 55 °C were not successful). After 2′-*O*-ACE protection, the 3′,5′-*O*-TIPDS group was removed under mild and neutral conditions (TMEDA/HF at 0 °C; 68% yield). The free 5′-hydroxyl was then protected with BzH by using BzHCl and diisopropylamine at 0 °C to afford a 68% yield of **9**. Finally, compound **9** was reacted with freshly prepared methyl tetraisopropyl phosphorodiamidite and 1*H*-tetrazole to generate the target 3-methylpseudouridine phosphoramidite **10** in 77% yield (27% yield in nine or ten steps from pseudouridine).

RNA Synthesis. The modified helix 69 RNA (1920 loop) shown in Figure 1 was synthesized using building block **10** and the G, C, A, U, and Ψ phosphoramidites. The modified base $m^3\Psi$ was incorporated at position 1915, along with two Ψ's at positions 1911 and 1917 (this RNA is referred to as $\Psi m^3\Psi\Psi$). A second RNA containing only $m^3\Psi$ modification at position 1915 and uridines at position 1911 and 1917 was also synthesized (referred to as $Um^3\Psi U$). The two RNAs were synthesized in high yields (~4 mg/μmol synthesis or 1 mg/0.2 μmol synthesis), deprotected, and purified by 20% polyacrylamide gel electrophoresis. The isolated RNAs were desalted and used for further biophysical studies. Electrospray mass spectrometry was used to confirm the identity of the modified RNAs (Supporting Information). The presence of the modified nucleosides was also confirmed by P1 nuclease digestion and alkaline phosphatase treatment of each RNA, followed by HPLC analysis (data not shown). In addition, the methyl group of $m^3\Psi$ is clearly visible in the 1D ^{1}H NMR spectrum of the $\Psi m^3\Psi\Psi$ RNA in D_2O at δ 3.04 ppm (Supporting Information).

Effects of $m^3\Psi$ on Helix 69 Stability and Structure. Previous studies have shown that Ψ at position

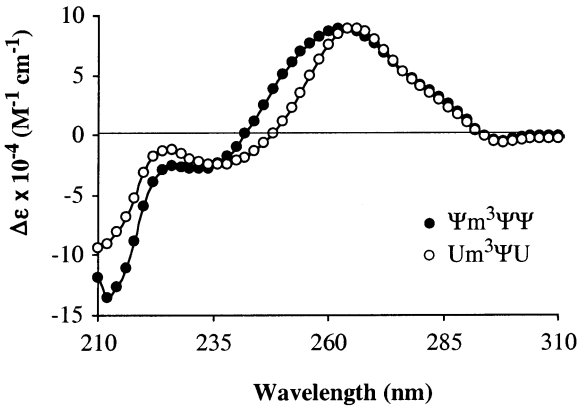


FIGURE 2. CD spectra of the 3-methylpseudouridine-modified RNAs taken in 15 mM NaCl, 20 mM sodium cacodylate, 0.2 mM Na_2EDTA, pH 7.0. The molar ellipticities are normalized to RNA concentrations (6.4×10^{-6} and 4.9×10^{-6} M in molecules of RNA for $\Psi m^3\Psi\Psi$ (closed circles) and $Um^3\Psi U$ (open circles), respectively). Each spectrum is the average of four scans.

1915 slightly destabilizes helix 69 by 0.5 kcal/mol compared to uridine, whereas m^3U has no effect.[9] For the newly synthesized RNA $\Psi m^3\Psi\Psi$, methylation of pseudouridine at position 1915 leads to a 0.5 kcal/mol stabilization relative to pseudouridine; RNAs with uridine and 3-methylpseudouridine at position 1915 have similar stability. The $\Delta G°_{37}$ values are −5.3,[9] −5.3,[9] −4.8,[8] and −5.2 kcal/mol for ΨUΨ, $\Psi m^3U\Psi$, ΨΨΨ, and $\Psi m^3\Psi\Psi$, respectively, in which the three letters for the RNAs designate the nucleosides at positions 1911, 1915, and 1917. Similar results were observed with the $Um^3\Psi U$ RNA ($\Delta G°_{37}$ values are −4.9,[8] −5.0,[9] −4.2,[8] and −4.7 for UUU, Um^3UU, UΨU, and $Um^3\Psi U$, respectively) in which $m^3\Psi$ at position 1915 has little effect on helix 69 RNA stability relative to uridine or 3-methyluridine, but stabilizes the RNA by ∼0.5 kcal/mol relative to pseudouridine. The CD spectrum of $Um^3\Psi U$ in Figure 2 is essentially identical to the previously reported spectra for UUU and Um^3UU RNAs.[8,9] Similarly, the CD spectrum of $\Psi m^3\Psi\Psi$ overlaps completely with the spectra for ΨUΨ and $\Psi m^3U\Psi$ RNAs. Thus, the observed differences between the $Um^3\Psi U$ and $\Psi m^3\Psi\Psi$ spectra appear to arise from the presence of Ψ modifications at positions 1911 and 1917. The imino proton NMR spectrum of $\Psi m^3\Psi\Psi$ shown in Figure 3 indicates that the effect of $m^3\Psi$ on the RNA structure is also very subtle. Seven major imino proton peaks are observed (G_{1922}, Ψ_{1911} N3H, G_{1910}, G_{1921}, U_{1923}, G_{1907}, and Ψ_{1911} N1H at 13.5, 13.0, 12.8, 12.4, 12.1, 11.7, and 10.3, respectively), which are similar to those seen previously in 1D imino proton NMR spectra of ΨΨΨ, ΨUΨ, and $\Psi m^3U\Psi$ RNAs.[8,9] An additional peak is observed at 11.2 ppm corresponding to G_{1906} (closing base pair). The Ψ_{1911} N3H and N1H imino resonances observed at 13.0 and 10.3 ppm, respectively, were confirmed by 1D NOE difference and 2D NOESY spectroscopy and indicate base pairing with A_{1919}. Three unassigned peaks occur at 12.6, 10.7, and 9.7 ppm, and the G_{1910} and Ψ_{1911} N1H peaks both have shoulders, suggesting possible conformational exchange in the region containing the modified nucleotides. These peaks do not occur in the corresponding ΨΨΨ spectrum; therefore, the conformational exchange in this region likely results from

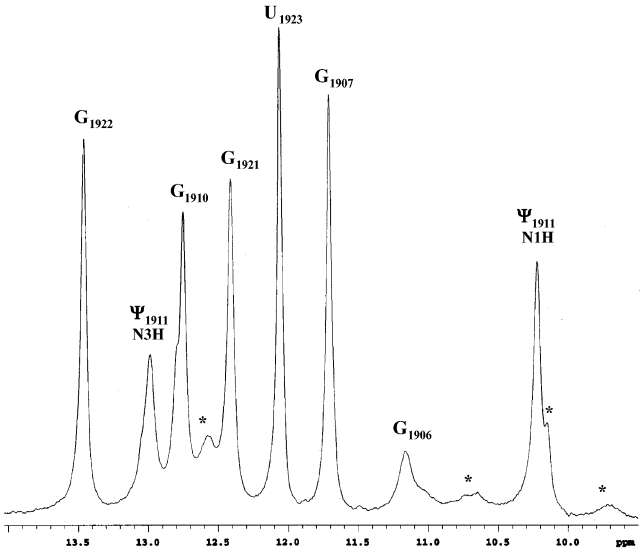


FIGURE 3. 1D imino proton (guanine N1H/uridine N3H/pseudouridine N1H and N3H) NMR spectrum of $\Psi m^3\Psi\Psi$ RNA taken in 90% H_2O/10% D_2O, 30 mM NaCl, 10 mM sodium phosphate, 0.2 mM Na_2EDTA, pH 6.5, at 3 °C. The nucleotide assignments are based on the 1D NOE difference spectra and 2D NOESY spectrum.

changes in the loop region due to the presence of $m^3\Psi$. Our biophysical studies are still preliminary; however, the thermodynamic and 1D NMR data are both consistent with the methyl group playing a minor structural role when present on pseudouridine. This result is in contrast to previous studies in which methylation of uridine had negligible effects on structure or stability. Even slight changes in RNA structure resulting from the presence of a methyl group may have important biological implications such as correct positioning of the 1920 loop in the ribosome. Further high-resolution structure studies on the fully modified helix 69 RNA are necessary to understand these effects more clearly.

In conclusion, we have reported on the synthesis of 3-methylpseudouridine and its corresponding phosphoramidite. Our approach involved an N1 protection−deprotection strategy in which the N3 position of pseudouridine can be efficiently modified by methylation. A similar strategy should also be possible for selective N3 alkylation, aminoylation, ^{15}N isotopic enrichment, or fluorescent labeling of pseudouridine. Furthermore, the protection−deprotection strategy presented will also allow for specific combinations of modifications at the N1, N3, and 2′-O positions of pseudouridine. Thus, large quantities of pseudouridine derivatives can be synthesized for applications in RNA structure−function studies or as novel nucleoside inhibitors.

Experimental Section

General Procedures. All reactions were carried out at room temperature under an inert atmosphere and anhydrous conditions unless otherwise noted. Tris(acetoxyethoxy)orthoformate, benzhydryloxybis(trimethylsilyloxy)silyl chloride, methyl tetraisopropyl phosphorodiamidite, and 4-(*tert*-butyldimethylsilyloxy)-3-penten-2-one were prepared as described elsewhere.[6] All other reagents were obtained from commercial sources. All solvents for RNA purification and HPLC analysis were spectra grade. ^1H, ^{13}C, and ^{31}P NMR spectra were

obtained in deuterated solvents on either 400 or 500 MHz spectrometers. The chemical shifts of the imino protons of the 1920 hairpin RNAs were reported relative to that of 3-(trimethylsilyl)propionate at 0.0 ppm. Nuclease digestions and HPLC analyses for the modified RNAs were performed as described previously.[8] The RNA sample preparation and experimental conditions for the thermal melting studies, CD spectroscopy, and NMR studies were done as reported previously.[8]

3′,5′-*O*-(1,1,3,3-Tetraisopropyl-1,3-disiloxanediyl)-pseudouridine (1). Pseudouridine (0.95 g, 3.89 mmol) was dried by azeotropic removal of water with benzene in vacuo overnight and then dissolved in 50 mL of freshly distilled pyridine. The white suspension was stirred for 1 h to give a clear solution and then cooled to 0 °C. 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane (TIPDSCl$_2$) (1.37 mL, 4.28 mmol) was added dropwise to the cooled solution and stirred for 1 h at 0 °C and then overnight at rt. The solvent was evaporated, and the white residue was taken up in CH_2Cl_2, washed with 5% $NaHCO_3$, and then washed with water. The organic layers were combined and washed with aqueous brine, dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by flash column chromatography on silica gel using a 0−5% EtOH in CH_2Cl_2 gradient to give compound **1** as a white solid (1.78 g, 94%): ^{1}H NMR ($CDCl_3$, 500 MHz) δ (ppm) 1.02−1.09 (m, 28H), 3.07 (s, 1H), 3.94−4.00 (m, 2H), 4.08 (dd, 1H, J = 12.5, 3, 3.5 Hz), 4.12 (dd, 1H, J = 5.5, 2.0 Hz), 4.31 (dd, 1H, J = 8.0, 5.5 Hz), 4.75 (s, 1H), 7.54 (s, 1H), 9.86 (s, 1H), 10.08 (s, 1H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ (ppm) 12.57, 12.69, 13.05, 13.38, 16.93, 17.01, 17.03, 17.14, 17.28, 17.33, 17.41, 61.28, 70.85, 74.57, 79.99, 80.89, 112.83, 138.83, 152.51, 163.04; ESI-MS (ES$^+$) m/z calcd for $C_{21}H_{38}N_2O_7Si_2$ 486.2, found 487.0 (MH$^+$), 509.0 (M + Na), 995.1 (2M + Na).

3′,5′-*O*-(1,1,3,3-Tetraisopropyl-1,3-disiloxanediyl)-2′-*O*-(trimethylsilyl)pseudouridine (2). A solution of **1** (1.78 g, 3.65 mmol) in 60 mL of CH_2Cl_2 was cooled to 0 °C. To the stirring solution was added triethylamine dropwise (2.47 mL, 17.75 mmol). The clear solution was stirred for 30 min at 0 °C followed by the addition of TMSCl (1.35 mL, 10.65 mmol). The solution was stirred for 1 h at 0 °C and then overnight at rt. The resulting red solution was poured over 100 mL of cold 5% aqueous $NaHCO_3$ and extracted twice with CH_2Cl_2. The organic layers were combined, washed with brine, dried over Na_2SO_4, and concentrated in vacuo to afford a light yellow foam that was further purified by flash chromatography on silica gel using a 0−5% EtOH in CH_2Cl_2 gradient to afford compound **2** as a white solid (2.02 g, 99%). A 25−50% gradient of EtOAc in hexanes was also successful and gave a comparable yield of product: ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) 0.18 (s, 9H), 0.90−1.06 (m, 28H), 3.91 (dd, 1H, J = 13.2, 1.6 Hz), 4.00−4.07 (m, 2H), 4.16 (d, 1H, J = 4 Hz), 4.14 (d, 1H, J = 5.6 Hz), 4.70 (s, 1H), 7.61 (dd, 1H, J = 5.6, 1.6 Hz), 10.04 (s, 1H), 10.21 (d, 1H, J = 5.2 Hz); ^{13}C NMR ($CDCl_3$, 400 MHz) δ (ppm) 0.32, 12.68, 12.91, 12.97, 13.43, 16.93, 17.06, 17.14, 17.27, 17.30, 17.36, 17.41, 60.11, 69.29, 75.74, 79.65, 80.90, 113.91, 138.25, 152.84, 162.89; ESI-MS (ES$^+$) m/z calcd for $C_{24}H_{46}N_2O_7Si_3$ 558.3, found 559.0 (MH$^+$), 581.0 (M + Na), 1139.2 (2M + Na).

1-Pivaloyloxymethyl-3′,5′-*O*-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)-2′-*O* -(trimethylsilyl)pseudouridine (3). To a clear solution of **2** (31.6 mg, 0.057 mmol) in 1.2 mL of freshly distilled triethylamine and 0.2 mL of dry pyridine was added POMCl (25 μL, 0.173 mmol). The reaction was stirred for 70 h at rt. An additional 25 μL of POMCl was added and the reaction stirred for 24 h. This process was repeated with a third aliquot of 25 μL of POMCl. The reaction was quenched with 5% $NaHCO_3$ and extracted with EtOAc. The organic layer was washed with aqueous brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel using a 20−34% gradient of EtOAc in hexanes to yield a colorless oil (34.8 mg, 91%) which coevaporated with hexanes to give a white crystalline solid: ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) 0.19 (s, 9H), 0.92 (m, 28H), 1.20 (s, 9H), 3.94 (dd, 1H, J = 12.8, 1.6 Hz), 4.00−4.07 (m, 2H), 4.14 (d, 1H, J = 3.2 Hz), 4.16 (d, 1H, J = 13.6 Hz), 4.70 (s, 1H), 5.45 (d, 1H, J = 10.4 Hz), 5.75 (d, 1H, J = 9.6 Hz), 7.60 (d, 1H, J = 1.6 Hz), 9.14 (s, 1H); ^{13}C NMR ($CDCl_3$, 400 MHz) δ (ppm) 0.29, 12.75, 12.85, 12.95, 13.37, 16.93, 17.04, 17.08, 17.19, 17.32, 17.34, 17.44, 17.47, 26.86, 38.82, 60.18, 69.64, 70.96, 75.80, 79.66, 80.82, 114.55, 140.89, 150.29, 162.16, 177.62; ESI-MS (ES$^+$) m/z calcd for $C_{30}H_{56}N_2O_9Si_3$ 672.3, found 673.2 (MH$^+$), 695.1 (M + Na), 1367.2 (2M + Na).

3-Methyl-1-pivaloyloxymethyl-3′,5′-*O*-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)-2′-*O*-(trimethylsilyl)pseudouridine (4). To a solution of **3** (1.69 g, 2.51 mmol) in 30 mL of benzene was added dropwise DMF-DMA (1.00 mL, 7.53 mmol). The light yellow solution was refluxed for 6 h, and the solvent was evaporated. The deep yellow crude product was purified by flash chromatography on silica gel using a 10−25% EtOAc in hexanes gradient to afford compound **4** as a thick colorless oil in 98% yield (1.69 g): ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) 0.18 (s, 9H), 0.90−1.07 (m, 28H), 1.18 (s, 9H), 3.34 (s, 3H), 3.93 (dd, 1H, J = 12.8, 2.4 Hz), 3.99−4.07 (m, 2H), 4.09 (d, 1H, J = 3.2 Hz), 4.15 (d, 1H, J = 12.8 Hz), 4.70 (s, 1H), 5.46 (d, 1H, J = 9.6 Hz), 5.76 (d, 1H, J = 9.6 Hz), 7.57 (s, 1H); ^{13}C NMR ($CDCl_3$, 400 MHz) δ (ppm) 0.36, 12.76, 12.85, 12.95, 13.40, 16.93, 17.05, 17.09, 17.19, 17.32, 17.35, 17.44, 17.47, 26.87, 27.75, 38.81, 60.18, 69.61, 71.93, 75.95, 79.60, 81.24, 113.63, 138.74, 150.85, 161.66, 177.56; ESI-MS (ES$^+$) m/z calcd for $C_{31}H_{58}N_2O_9Si_3$ 686.3, found 709.2 (M + Na).

3-Methyl-1-pivaloyloxymethyl-3′,5′-*O*-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)pseudouridine (5). To a clear solution of **4** (190 mg, 0.28 mmol) in THF (8 mL) was added *p*-toluenesulfonic acid monohydrate (pTSA·H_2O) (79 mg, 0.42 mmol). After the solution was stirred for 3 h, triethylamine (84 μL, 0.6 mmol) was added. The reaction was quenched with 5% aqueous $NaHCO_3$ and extracted with EtOAc. The organic layers were combined, washed with brine, dried over Na_2SO_4, concentrated, and purified by flash chromatography on silica gel with a 10−33% EtOAc in hexanes gradient to give a colorless thick oil. Drying in vacuo gave compound **5** as a white foam (161 mg, 95%): ^{1}H NMR ($CDCl_3$, 500 MHz) δ (ppm) 1.01−1.08 (m, 28H), 1.19 (s, 9H), 2.90 (s, 1H), 3.33 (s, 3H), 3.9−3.94 (m, 1H), 4.00 (dd, 1H, J = 13.0, 3.0 Hz), 4.06 (dd, 1H, J = 13.0, 4.0, 4.5 Hz), 4.17 (dd, 1H, J = 5.5, 2.5 Hz), 4.35 (dd, 1H, J = 8.0, 5.8 Hz), 4.70 (dd, 1H, J = 2.3, 1.5 Hz), 5.58 (d, 1H, J = 10.0 Hz), 5.60 (d, 1H, J = 10.0 Hz), 7.53 (d, 1H, J = 1.0 Hz); ^{13}C NMR ($CDCl_3$, 500 MHz) δ (ppm) 12.53, 12.63, 13.02, 13.31, 16.92, 16.99, 17.01, 17.14, 17.26, 17.30, 17.35, 17.40, 26.86, 27.77, 38.82, 61.48, 71.27, 71.34, 74.42, 80.82, 80.99, 112.45, 139.50, 150.88, 161.60, 177.99; ESI-MS (ES$^+$) m/z calcd for $C_{28}H_{50}N_2O_9Si_2$ 614.3, found 615.2 (MH$^+$), 637.2 (M + Na), 1251.5 (2M + Na).

3-Methyl-3′,5′-*O*-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)pseudouridine (6). Method A. The POM protective group can be removed following 2′-*O*-TMS removal (from **5**). Compound **5** (1.19 g, 1.93 mmol) was dissolved in 35 mL of methanolic ammonia (2.0 M in methanol) and stirred overnight. The solvent was evaporated and the product isolated by flash chromatography on silica gel using a 15−20% EtOAc in hexanes gradient to afford **6** (0.91 g, 94%) as a white foam: ^{1}H NMR ($CDCl_3$, 500 MHz) δ (ppm) 1.00−1.09 (m, 28H), 2.94 (d, 1H, J = 1.5 Hz), 3.31 (s, 3H), 3.95−4.00 (m, 2H), 4.07−4.18 (m, 2H), 4.28 (dd, 1H, J = 8.5, 5.0 Hz), 4.79 (dd, 1H, J = 1.5 Hz), 7.47 (dd, 1H, J = 6.0, 1.0 Hz), 10.20 (d, 1H, J = 5.5 Hz); ^{13}C NMR ($CDCl_3$, 500 MHz) δ (ppm) 12.53, 12.67, 13.00, 13.38, 16.89, 16.98, 17.02, 17.10, 17.26, 17.28, 17.34, 17.40, 27.08, 60.93, 70.40, 74.71, 80.25, 80.56, 112.45, 135.88, 152.91, 162.24; ESI-MS (ES$^+$) m/z calcd for $C_{22}H_{40}N_2O_7Si_2$ 500.2, found 501.0 (MH$^+$), 523.0 (M + Na), 1023.2 (2M + Na).

Method B. The POM and TMS groups can be removed simultaneously from **4**. Compound **4** (211 mg, 0.31 mmol) was dissolved in 15 mL of methanolic ammonia (2.0 M in methanol)

at 0 °C to give a clear solution. The solution was gradually warmed to rt and stirred overnight. The resulting light blue solution was concentrated, and the product was purified by flash chromatography using a 0−5% EtOH gradient in hexanes/EtOAc (2:1 mixture) to afford **6** (142 mg, 92%) as a white foam.

2′-*O*-[Bis(2-acetoxyethoxy)methyl]-3-methyl-3′,5′-*O*-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)pseudouridine (7). In a 100 mL round-bottom flask, compound **6** (0.91 g, 1.81 mmol) was dissolved in 11 mL of 1,4-dioxane to give a clear solution. Tris(2-acetoxyethoxy)orthoformate (1.63 g, 5.07 mmol) and pyridinium *p*-toluenesulfonate (182 mg, 0.72 mmol) were then added to give a clear solution. The reaction was stirred for 1 h, and then 4-(*tert*-butyldimethylsilyloxy)-3-penten-2-one (0.78 mL, 3.26 mmol) was added. The solution was stirred for 12 h at rt and then at 40 °C for 24 h. The reaction progress was monitored by TLC using a hexanes/EtOAc (1:2) solution. Upon completion, the reaction was cooled to rt, and the cloudy mixture was quenched by the addition of TMEDA (0.15 mL, 1.0 mmol) and stirred for 15 min. The resulting clear brown reaction solution was first purified by flash column chromatography on silica gel using a 16−67% EtOAc in hexanes gradient to give a partially purified compound **7** (80%) that was further purified using a hexanes/EtOAc (1:7) mixture to afford a white solid (898 mg, 69%): ^{1}H NMR ($CDCl_3$, 500 MHz) δ (ppm) 0.83−0.88 (m, 4H), 0.91−1.00 (m, 24H), 1.97 (s, 3H), 1.98 (s, 3H), 3.20 (s, 3H), 3.74−3.86 (m, 5H), 3.89−3.95 (m, 2H), 4.04 (d, 1H, J = 4.0 Hz), 4.07−4.12 (m, 3H), 4.21 (t, 2H, J = 5 Hz), 4.80 (s, 1H), 5.70 (s, 1H), 7.45 (d, 1H, J = 5.5 Hz), 10.23 (d, 1H, J = 5.5 Hz); ^{13}C NMR ($CDCl_3$, 500 MHz) δ (ppm) 12.43, 12.70, 13.18, 16.65, 16.78, 16.84, 16.96, 17.04, 17.07, 17.14, 17.19, 20.59, 20.67, 26.74, 59.43, 60.17, 63.11, 63.45, 65.56, 68.83, 76.97, 78.40, 79.61, 111.39, 112.21, 135.73, 152.47, 161.88, 170.70, 170.80.

2′-*O*-[Bis(2-acetoxyethoxy)methyl]-3-methylpseudouridine (8). CH_3CN (1.0 mL), TMEDA (0.22 mL, 6.12 mmol), and HF (48% aq stock solution, 0.15 mL, 4.08 mmol) were added dropwise via syringe to a 50 mL round-bottom flask at 0 °C. The HF/TMEDA mixture was stirred at 0 °C for 10 min and then transferred to a clear solution of **7** (729 mg, 1.02 mmol, in 7 mL of CH_3CN) at 0 °C dropwise over 5 min by cannula. The resulting light yellow solution was stirred at 0 °C for 30 min and then at rt for 5 h. The reaction progress was monitored by TLC analysis in a hexanes/EtOAc (1:2) mixture. Upon completion of the reaction, the solvent was evaporated, and the residue was taken up in a mixture of hexanes/EtOAc (1:2) (5 mL). The crude product was purified by flash chromatography on silica gel with 0.5% TMEDA in the eluents (33−88% EtOAc in hexanes and then 0−5% CH_3OH in EtOAc) to give a pale yellow oil (585 mg, 68% yield from compound **6**): ^{1}H NMR ($CDCl_3$, 500 MHz) δ (ppm) 2.05 (s, 3H), 2.07 (s, 3H), 3.09 (s, 1H), 3.29 (s, 3H), 3.66−3.82 (m, 6H), 3.85 (dd, 1H, J = 12.0, 2.0 Hz), 4.07 (m, 1H), 4.16−4.28 (m, 5H), 4.55 (dd, 1H, J = 6.5, 4.5 Hz), 4.60 (d, 1H, J = 6.5 Hz), 5.39 (s, 1H), 7.41 (s, 1H), 10.17 (br s, 1H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ (ppm) 20.74, 20.79, 27.25, 62.12, 62.72, 62.76, 63.03, 63.16, 71.59, 76.85, 79.39, 85.04, 109.48, 112.89, 138.97, 152.16, 162.91, 171.02 (2 carbonyls); ESI-MS (ES^+) m/z calcd for $C_{29}H_{28}N_2O_{12}$ 476.2, found 499.2 (M + Na), 515.2 (M + K).

5′-*O*-[Benzhydryloxybis(trimethylsilyloxy]-2′-*O*-[bis(2-acetoxyethoxy)methyl]-3-methylpseudouridine (9). Solution A was prepared by adding diisopropylamine (172 μL, 1.23 mmol) dropwise to a solution of **8** (0.59 g, 1.23 mmol, in 7 mL of CH_2Cl_2) and cooling the resulting solution to 0 °C. Solution B was prepared by adding diisopropylamine (172 μL, 1.23 mmol) dropwise to benzhydryloxybis(trimethylsiloxy)silyl chloride (BzHCl; 1.3 g, 3.08 mmol) in 3 mL of CH_2Cl_2 at 0 °C. Aliquots of solution B (0.5 equiv and 0.33 equiv × 3) were added to solution A at 0 °C dropwise over 2 min, and the resulting solutions were stirred for 20 min after each addition. The reaction progress was monitored by TLC (hexanes/EtOAc, 1:2). The light yellow solution was stirred for 2 h at 0 °C. Upon completion, the reaction was quenched with 5% $NaHCO_3$ and extracted with CH_2Cl_2. The combined organic layers were washed with brine, dried over Na_2SO_4, concentrated, and purified by flash column chromatography on silica gel with 33−50% EtOAc in hexanes and then 0−5% CH_3OH in EtOAc to afford compound **9** as a thick clear oil in 68% yield: ^{1}H NMR ($CDCl_3$, 500 MHz) δ (ppm) 0.03 (s, 9H), 0.04 (s, 9H), 2.02 (s, 6H), 2.03 (s, 6H), 2.87 (d, 1H, J = 8.5 Hz), 3.21 (s, 3H), 3.78−3.85 (m, 4H), 3.87 (d, 1H, J = 4.5 Hz), 3.88 (d, 1H, J = 5.0 Hz), 3.96−4.01 (m, 2H), 4.12 (dd, 1H, J = 5.0, 1.5 Hz), 4.18−4.27 (m, 4H), 4.87 (s, 1H), 5.63 (s, 1H), 5.94 (s, 1H), 7.15−7.20 (m, 2H), 7.23−7.27 (m, 4H), 7.32−7.34 (m, 4H), 7.38 (d, 1H, J = 5.0 Hz), 9.16 (d, 1H, J = 5.0 Hz); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 1.26, 20.61, 20.64, 26.78, 61.32, 62.20, 62.39, 62.93, 63.02, 68.69, 76.56, 78.12, 78.55, 81.74, 111.53, 112.53, 126.14, 126.27, 127.12, 128.08 (2C), 136.03, 143.76, 143.85, 152.03, 162.16, 170.72, 170.76; ESI-MS (ES^+) m/z calcd for $C_{38}H_{56}N_2O_{15}Si_3$ 864.3, found 887.3 (M + Na).

5′-*O*-[Benzhydryloxybis(trimethylsilyloxy)silyl]-2′-*O*-[bis(2-acetoxyethoxy)methyl]-3-methylpseudouridine-3′-(methyl-*N*,*N*-diisopropyl)phosphoramidite (10). To a solution of **9** (452 mg, 0.52 mmol) in CH_2Cl_2 (3 mL) were added methyl tetraisopropyl phosphorodiamidite (0.42 mL, 1.46 mmol) and 1*H*-tetrazole (90 mg, 0.52 mmol). The resulting white suspension turned clear after being stirred for 1 h and was then stirred for an additional 20 h. The reaction was 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 by flash chromatography with 5−10% TEA in the eluents (15% CH_2Cl_2 in hexanes followed by 20−33% acetone in hexanes) to afford compound **10** as a colorless oil (413 mg, 77%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) (mixture of diastereomers) 0.02−0.06 (3s, 18H), 1.16, 1.17, 1.18, 1.19 (4s, 12H), 2.05, 2.060, 2.064, 2.07 (4s, 6H), 3.23 (s, 3H), 3.33, 3.37 (2s, 3H), 3.54−3.62 (m, 2H), 3.82−4.00 (m, 5H), 4.05−4.34 (m, 8H), 4.96 (s, 1H), 5.72, 5.79 (2s, 1H), 5.98 (1s, 1H), 7.24−7.38 (m, 10H), 7.42, 7.44 (2s, 1H); ^{31}P NMR ($CDCl_3$, 400 MHz) δ 150.45, 152.03; ESI-MS (ES^+) m/z calcd for $C_{45}H_{72}N_3O_{16}PSi_3$ 1025.4, found 1026.2 (MH^+), 1048.1 (M + Na).

3-Methylpseudouridine (11). Removal of the TIPDS protective group from compound **6** (70 mg, 0.14 mmol) in the presence of a HF/TMEDA (3:1) mixture in CH_3CN (as described for compound **8**) gave **11** as a white crystalline solid (31 mg, 85%): ^{1}H NMR (D_2O, 500 MHz) δ (ppm) 3.23 (s, 3H), 3.68 (dd, 1H, J = 12.0, 4.8, 4.8 Hz), 3.82 (dd, 1H, J = 12.4, 3.2, 3.2 Hz), 3.97 (m, 1H), 4.10 (t, 1H, J = 5.6, 5.6 Hz), 4.23 (t, 1H, J = 5.4, 6.0 Hz), 4.67 (d, 1H, J = 5.6, 5.6 Hz), 7.61 (s, 1H); ^{13}C NMR (D_2O, 500 MHz) δ (ppm) 27.20 ($N3$-CH_3), 61.51, 70.80, 73.46, 79.81, 83.16, 110.06, 139.34, 153.16, 164.75; ESI-MS (ES^+) m/z calcd for $C_{10}H_{14}N_2O_6$ 258.1, found 259 (MH^+), 281 (M + Na)$^+$, 539 (2M + Na).

Synthesis, Deprotection, and Purification of Modified RNA Oligonucleotides. Two RNAs containing 3-methylpseudouridine ($m^3\Psi$) at position 1915 and pseudouridines (Ψ) or uridines at positions 1911 and 1917 were synthesized chemically on 0.2 and 1.0 μmol scales using polystyrene supports as described previously.[6] The sequences of the two RNAs are as follows, with the numbering based on the full-length *E. coli* 23S rRNA: 5′-$G_{1906}GCCGU_{1911}AAC m^3\Psi_{1915}AU_{1917}AACGGUC_{1924}$-3′ (referred to as Um$^3\Psi$U; the names of the RNAs correspond to the nucleotides at positions 1911, 1915, and 1917, respectively) and 5′-$G_{1906}GCCG\Psi_{1911}AAC m^3\Psi_{1915}A\Psi_{1917}AACGGUC_{1924}$-3′ ($\Psi m^3\Psi\Psi$). The crude RNAs were divided into 0.20−0.25 μmol per microcentrifuge tube and deprotected by adding 400 μL of 100 mM NaOAc buffer (pH 3.8) and heating the solution at 60 °C for 30 min. The deprotected RNAs were then purified by gel electrophoresis on 20% denaturing polyacrylamide gels followed by electroelution. The gel-purified RNA oligonucleotides were desalted by dialysis against RNase-free deionized water (1 × 4 L), salt solution (1 × 4 L of 50 mM NaCl, 0.1 mM EDTA), and RNase-

free deionized water (1 × 4 L) for 12 h each. The dialyzed RNAs were lyophilized to dryness, resuspended in RNase-free water, and further purified over C-18 columns. Each column was prepared by addition of 10 mL of CH_3CN followed by 10 mL of RNase-free deionized water. Approximately 10 OD units (∼300 μg) of RNA was loaded onto each column and washed with 5 mL of deionized water. The RNA eluted from the column in three fractions with 60% CH_3OH 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) was calculated to be 188860 cm^{-1} M^{-1} for each RNA. The extinction coefficient for uridine (1.0×10^4 cm^{-1} M^{-1} at pH 7.0)[23] was used for pseudouridine and 3-methylpseudouridine because the nearest-neighbor extinction coefficients for those modified bases are unknown.

(23) Richards, E. G. Use of Tables in Calculation of Absorption, Optical Rotary Dispersion, and Circular Dichroism of Polyribonucleotides. In *Handbook of Biochemistry and Molecular Biology: Nucleic Acids*; Fasman, G. D., Ed.; CRC Press: Cleveland, OH, 1975; pp 596−599.

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Supporting Information Available: NMR data for compounds **1**−**11** and RNA and mass spectral data for compounds **10** and **11** and RNA. This material is available free of charge via the Internet at http://pubs.acs.org.

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