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

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Supporting Information (JO026364m)

NMR Spectra, Mass Spec Data, and Melting Curves

- **S4.** ¹H NMR spectrum for **1-Pivaloyloxymethyl-3',5'-O-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)-2'-O-(trimethylsilyl)pseudouridine (3)**. (CDCl₃)
- **S5.** ¹H NMR spectrum for **3-Methyl-1-pivaloyloxymethyl-3',5'-O-(1,1,3,3-tetraisopropyl-1,3-disiloxane-diyl)-2'-O-(trimethylsilyl)pseudouridine (4)**. (CDCl₃)

Note the AX splitting pattern for the N1-CH₂- from the POM methylene protons at 5.46 (d) and 5.76 (d) ppm and the N3-CH₃ at 3.34 ppm.

- **S6.** ¹H NMR spectrum for **3-Methyl-1-pivaloyloxymethyl-3',5'-O-(1,1,3,3-tetraisopropyl-1,3-disiloxane-diyl)pseudouridine (5)**. (CDCl₃)
- **S7.** ¹H NMR spectrum for **3-Methyl-3',5'-O-(1,1,3,3-tetraisopropyl-1,3-disiloxane-diyl)pseudouridine (6)**. (CDCl₃)
- **S8.** ¹H NMR spectrum for **2'-O-[Bis(2-acetoxyethoxy)methyl]-3-methylpseudouridine (8)**. (CDCl₃)
- **S9.** ¹H NMR spectrum for **5'-O-[Benzhydryloxy-bis(trimethylsilyloxy)-2'-O-[bis-(2-acetoxyethoxy)methyl]-3-methylpseudouridine (9)**. (CDCl₃)

- **S10.** ^1H NMR spectrum for **5'-O-[Benzhydryloxy-bis(trimethylsilyloxy)silyl]-2'-O-[bis(2-acetoxyethoxy)methyl]-3-methylpseudouridine-3'-(methyl-*N,N*-diisopropyl)phosphoramidite (10).** (CDCl_3)
- **S11.** ^{31}P NMR spectrum for **5'-O-[Benzhydryloxy-bis(trimethylsilyloxy)silyl]-2'-O-[bis(2-acetoxyethoxy)methyl]-3-methylpseudouridine-3'-(methyl-*N,N*-diisopropyl)phosphoramidite (10).** (CDCl_3)
- **S12.** ESI-MS data for **5'-O-[Benzhydryloxy-bis(trimethylsilyloxy)silyl]-2'-O-[bis(2-acetoxyethoxy)methyl]-3-methylpseudouridine-3'-(methyl-*N,N*-diisopropyl)phosphoramidite (10).**
- **S13.** ^1H NMR spectrum for **3-methylpseudouridine (11).** (D_2O)
- **S14.** ^{13}C NMR and DEPT spectra for **3-methylpseudouridine (11).** (D_2O)
- **S15.** ESI-MS data for **3-methylpseudouridine (11).**
- **S16.** 1D NOE difference spectra for **3-methylpseudouridine (11).** (500 MHz, D_2O)

The top panel shows that there are no NOE signals upon irradiation of N3-CH_3 . The lower panel shows NOEs to H1' , H2' , and H3' upon irradiation of H6 , but no NOE signal at N3-CH_3 .

- **S17.** 1D NOE difference spectra for **1-methyl-3-pivaloyloxymethyl-3',5'-O-(1,1,3,3-tetraisopropyl-1,3-disiloxane-diyl)-2'-O-(trimethylsilyl)pseudouridine** (400 MHz, CDCl_3)

Note the AB splitting pattern for the N3-CH_2 from the POM methylene protons at 5.92 (d) and 5.95 (d) ppm and the N1-CH_3 at 3.37 ppm.

The top panel shows a 7.44% NOE signal at H6 upon irradiation of N1-CH_3 . The middle panel shows a 10.57% NOE signal at N1-CH_3 upon irradiation of H6 .

- **S18.** Representative normalized UV melting curves for the modified RNAs $\Psi m^3 \Psi \Psi$ (closed circles) and $U m^3 \Psi U$ (open circles) taken in 15 mM NaCl, 20 mM sodium cacodylate, 0.5 mM EDTA, pH 7.0. The melting curves were obtained on an Aviv 14DS UV-vis spectrometer and normalized at 95 °C.
- **S19.** ESI-MS data (a) and **S20.** Mass transform (b) of the acquired ESI-MS data for the RNA product $\Psi m^3 \Psi \Psi$. The spectrum on top is the computer generated isotopic distribution for molecular formula $C_{182}H_{228}N_{73}O_{130}P_{18}$. MW calcd: 6075.75 (average mass) or 6072.87 (monoisotopic mass); found: 6076.0 (6072.9 for monoisotopic mass).
- **S21.** The 1D 1H gradient NMR spectrum of $\Psi m^3 \Psi \Psi$ RNA taken in 100% D_2O , 30 mM NaCl, 10 mM sodium phosphate, 0.2 mM Na_2EDTA , pH 6.5, at 37 °C (500 MHz, 500 ms mixing time) is shown. The N3-methyl peak from residue m^3_{1915} is clearly visible at 3.04 ppm.

Mercury 400 spectrometer

Pulse Sequence: s2pul

Mercury 400 spectrometer

Pulse Sequence: s2pul

Mercury 400 spectrometer

Pulse Sequence: s2pul

Unity 500 spectrometer

Pulse Sequence: s2pul

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Mercury 400 spectrometer

Pulse Sequence: s2pul

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Unity 500 spectrometer