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# Solution conformations of two naturally occurring RNA nucleosides: 3-Methyluridine and 3-methylpseudouridine

Jean-Paul Desaulniers, Helen M.-P. Chui and Christine S. Chow*

*Department of Chemistry, Wayne State University, Detroit, MI 48202, USA*

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**Abstract**—The conformations of 3-methyluridine and 3-methylpseudouridine are determined using a combination of sugar proton coupling constants from 1D NMR spectra and 1D NOE difference spectroscopy. Both $C_2'$-*endo* and $C_3'$-*endo* conformations are observed for 3-methyluridine (59:41, 37 °C, $D_2O$) and 3-methylpseudouridine (51:49, 37 °C, $D_2O$). 3-Methyluridine preferentially adopts an *anti* conformation in solution, whereas 3-methylpseudouridine is primarily in a *syn* conformation. *anti*/*syn*-Relationships are deduced by 1D NOE difference spectroscopy.


## 1. Introduction

Modified nucleosides are important in biological systems because they offer novel chemical structures that can alter function, stability, or structures of small oligonucleotides or large, naturally occurring RNAs. Nucleoside modifications range in complexity; however, the simplest and most common type of modification is base methylation. In DNA, methylation is most commonly observed at the 5-position of cytosines.[1] In contrast, methylation sites in RNAs are arrayed over a large number of sequences and are found in many of the major classes of RNA.[2]

Ribose methylation at the 2′-*O*-position is a prominent type of modification that is postulated to increase RNA stability. For example, thermophiles, organisms that thrive at high temperatures, contain a large number of 2′-*O*-methyl nucleosides compared to other bacterial species.[3] Other types of ribonucleoside methylation include base modification. Site-specific methylation of the purine and pyrimidine bases is observed in over 50 different ribonucleosides.[2b,4] These aromatic methylations lead to increased hydrophobicity and stacking ability of the nucleoside, and alter the hydrogen-bonding capacity and charge distribution within the purine or pyrimidine ring.[4]

In this study, the solution conformations of two naturally occurring nucleosides, 3-methyluridine ($m^3U$) and its isomer 3-methylpseudouridine ($m^3\Psi$) (Fig. 1), were determined. The first reported isolation of $m^3U$ was in 1963,[5] and it has been detected in 23S rRNA of archaea, 16S and 23S rRNA of eubacteria, and 18S, 25S, and 28S of eukaryotic ribosomal RNAs.[6] Its isomer, $m^3\Psi$, was detected much later in 23S rRNA of *Escherichia coli* (*E. coli*).[7] To date, the location of $m^3\Psi$ appears to be limited to the rRNAs of eubacteria and chloroplasts, and has been observed at position 1915 (*E. coli* numbering) of the large subunit rRNAs from *E. coli*, *Bacillus subtilis*, *Deinococcus radiodurans*, and *Zea mays* chloroplasts.[8] RluD is a specific pseudouridine synthase that isomerizes uridine to pseudouridine post-transcriptionally at position 1915 in 23S rRNA.[9] In contrast, the mechanism and enzyme responsible for methylation at N3 of Ψ 1915 remains unknown. The $m^3\Psi$ residue at

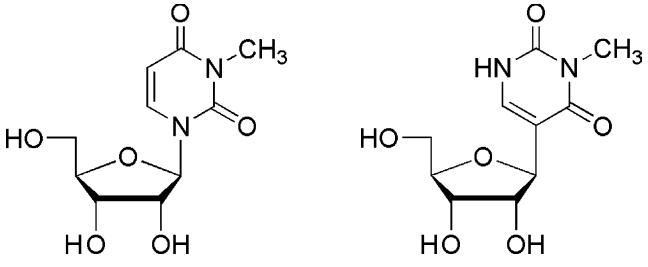


**Figure 1.** The structures of 3-methyluridine (left) and 3-methylpseudouridine (right) are shown.

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* Corresponding author. Tel.: +1 313 577 2594; fax: +1 313 577 8822; e-mail: csc@chem.wayne.edu

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position 1915 is located at the top of the loop region of helix 69 (H69), which plays an important role in forming one of the intersubunit bridges of the ribosome on account of its close proximity to tRNAs and helix 44.[10]

Helix 69 is postulated to participate in a 'ratchet'-like motion during ribosomal protein synthesis.[10c,11] A recent three-dimensional cryo-electron microscopic map shows that specific amino acid residues of a ribosome recycling factor make specific contacts with $m^3\Psi$ at position 1915[12a] or the backbone of H69.[12b] These results imply that $m^3\Psi$ is important for RNA-protein contacts and may be necessary for proper recycling of deacetylated tRNAs.

To begin understanding the biological roles of $m^3\Psi$ in the ribosome, it is important to know its solution conformation. In this study, the two isomers, $m^3U$ and $m^3\Psi$, were examined and compared using a combination of circular dichroism and 1D $^1H$ NMR spectroscopy. The recent development of a convenient synthetic route to $m^3\Psi$ allows for the production of sufficient quantities of this modified nucleoside for structure studies.[13] Previous studies also provide an efficient synthetic route to $m^3U$.[14]

## 2. Results and discussion

NMR spectroscopy was used to determine the solution conformations of $m^3U$ and $m^3\Psi$. Nucleoside conformation is often characterized by a dominant sugar pucker and preferred glycosidic bond angle.[15] In 1D $^1H$ NMR experiments, NOEs are useful in the assignments of protons in close proximity and for conformer determination.[16] Since the magnitude of an NOE signal is inversely proportional to its distance ($d$) (magnitude of NOE = $1/d^6$), relative proton–proton distances can be deduced. The relative distances between purine H8 or pyrimidine H6 to sugar H1′, H2′, and H3′ protons can be used to approximate *syn* or *anti* conformer preference. It is known from the work of Rosemeyer et al. that when a *syn* conformer dominates, irradiation at H6/H8 will result in a strong NOE to H1′.[17] In contrast, when an *anti* conformer predominates, irradiation at H6/H8 will result in strong NOEs at both H2′ and H3′.[17] In an *anti* conformation, the aromatic H6/H8 lies in close proximity to both H2′ and H3′. Therefore, the overall magnitude of the combined sum of the NOEs of H2′ and H3′ compared to the NOE magnitude obtained at H1′ (from H6/H8 irradiation) is used to characterize *anti*/*syn* relationships.[17] Based on our observations, irradiation of H6 of 3-methyluridine results in stronger NOEs at H2′ and H3′ (8.2% combined) at 25 °C (Table 1). This observation is supported by a reverse experiment, in which irradiations of H2′ and H3′ both result in NOEs at H6 (5.1% and 2.2%, respectively). Furthermore, the NOE trend does not change significantly between 4 and 37 °C. This result demonstrates that $m^3U$ has a higher population of the *anti* conformation. In contrast, $m^3\Psi$ exhibits a dominant NOE at H1′ (6.1%) and much weaker NOEs at H2′ and H3′ (2.7% combined) when H6 is irradiated at 25 °C (Table 1). Similarly, this experiment is supported by a reverse experiment in which a strong NOE at H6 (5.0%) is observed when H1′ is irradiated. This observation reveals that $m^3\Psi$ exists predominantly in a *syn* conformation in solution. NOE studies of its precursor, pseudouridine, also show that the *syn* conformation predominates in solution.[18] NOE methods were employed to study the *anti*/*syn* relationship for $m^3U$ and $m^3\Psi$, and this process is standard for examining other nucleosides.[17,19] For example, when cytidine H6 is irradiated, a weak NOE at H1′ (3.6%) and stronger ones at H2′ and H3′ (8.5% combined) are observed.[17] This result is indicative of a predominantly *anti* conformation for cytidine.[17]

The ribose moieties of RNA typically adopt either a $C_3'$-*endo* (south) or $C_3'$-*endo* (north) conformation. Altona and Sundaralingam defined the major sugar puckers based on $J_{H1'\text{-}H2'}$ and $J_{H3'\text{-}H4'}$ coupling constants.[20] The percentages of $C_3'$-*endo* versus $C_2'$-*endo* are summarized from the equations: % $C_2'$-*endo* = $100 \times J_{1',2'}/J_{1'2'} + J_{3',4'}$) and % $C_3'$-*endo* = 100–% $C_2'$-*endo*[20] Tables 2 and 3 list these parameters for $m^3U$ and $m^3\Psi$. For $m^3U$, a slight preference for the $C_3'$-*endo* (55–60%) conformation is observed at 4, 25, and 37 °C. In contrast, $m^3\Psi$ exists in a near equal distribution of $C_2'$- and $C_3'$-*endo* conformations (50–54 % and 46–50%, respectively), which may indicate a slightly lower rotational energy barrier between the conversion of the two sugar puckers. Figure 2 illustrates the proposed conformations of the isomers based on the aforementioned NMR studies.

**Table 1.** Irradiation and NOE data for 3-methyluridine and 3-methylpseudouridine at 4, 25, and 37 °C

| Compound | Irradiated proton | NOEs | | |
|---|---|---|---|---|
| | | 4 °C | 25 °C | 37 °C |
| $m^3U$ | H6 | H1′ (4.6) | H1′ (5.0) | H1′ (3.5) |
| | | H2′ + H3′ (10.7) | H2′ + H3′ (8.2) | H2′ + H3′ (6.1) |
| | H1′ | H6 (7.0) | H6 (4.9) | H6 (3.5) |
| | H2′ | H6 (7.2) | H6 (5.1) | H6 (4.6) |
| | H3′ | H6 (3.4) | H6 (2.9) | H6 (2.9) |
| $m^3\Psi$ | H6 | H1′ (5.8) | H1′ (6.1) | H1′ (4.7) |
| | | H2′ + H3′ (3.6) | H2′ + H3′ (2.7) | H2′ + H3′ (2.1) |
| | H1′ | H6 (5.8) | H6 (5.0) | H6 (4.2) |
| | H2′ | H6 (2.7) | H6 (2.0) | H6 (1.6) |
| | H3′ | H6 (1.3) | H6 (1.1) | H6 (0.9) |

**Table 2.** $^{1}H$–$^{1}H$ coupling constants for 3-methyluridine and 3-methylpseudouridine at 4, 25, and 37 °C

| Temp | $J_{1',2'}$ | $J_{2',3'}$ | $J_{3',4'}$ | $J_{4',5'}$ | $J_{4',5''}$ |
|---|---|---|---|---|---|
| *$^{1}H$–$^{1}H$ coupling constants for $m^3U$* | | | | | |
| 4 °C | 4.0 | 5.0 | 6.0 | 3.0 | 4.2 |
| 25 °C | 4.5 | 5.0 | 5.5 | 3.0 | 4.2 |
| 37 °C | 4.0 | 4.5 | 5.8 | 3.2 | 4.5 |
| *$^{1}H$–$^{1}H$ coupling constants for $m^3\Psi$* | | | | | |
| 4 °C | 5.0 | 5.2 | 5.8 | 2.5 | 4.8 |
| 25 °C | 5.5 | 5.5 | 5.5 | 3.2 | 4.8 |
| 37 °C | 5.5 | 5.2 | 5.8 | 3.0 | 5.0 |

**Table 3.** Percentages of $C_3'$-*endo* (*N*) versus $C_2'$-*endo* (*S*) conformers and equilibrium constants (*N*/*S*) for 3-methyluridine and 3-methylpseudouridine at 4, 25, and 37 °C

| Compound | Temperature (°C) | % $C_3'$-*endo* (*N*) | % $C_2'$-*endo* (*S*) | $K_{eq}$ (*N*/*S*) |
|---|---|---|---|---|
| $m^3U$ | 4 | 60 | 40 | 1.5 |
| | 25 | 55 | 45 | 1.2 |
| | 37 | 59 | 41 | 1.4 |
| $m^3\Psi$ | 4 | 54 | 46 | 1.2 |
| | 25 | 50 | 50 | 1.0 |
| | 37 | 51 | 49 | 1.0 |

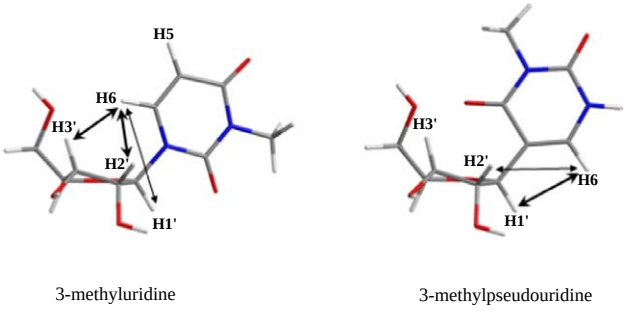


**Figure 2.** The preferred conformations of 3-methyluridine (*anti*) and 3-methylpseudouridine (*syn*) are determined by 1D NOE experiments. Thick arrows represent strong NOEs whereas smaller ones represent weak NOEs. At 37 °C the percent $C_3'$-*endo* is 59% for 3-methyluridine and 51% for 3-methylpseudouridine.

Circular dichroism (CD) spectroscopy determines the differential absorption of a left or right circularly polarized light by a molecule.[21] The CD spectrum of $m^3U$ exhibits a minimum at 234 nm and a maximum at 284 nm (Fig. 3). In contrast, $m^3\Psi$ exhibits a very different CD spectrum, which is almost a mirror image of the $m^3U$ spectrum (Fig. 3). Specifically, a minimum at 282 nm and a maximum at 240 nm are observed in the $m^3\Psi$ spectrum. For $m^3\Psi$, the long-wavelength Cotton effect is negative, in agreement with earlier studies on Ap$\Psi$[22] The observable optically active groups, the pyrimidines, differ in orientation and their electronic dipole moments for $m^3U$ and $m^3\Psi$ because of their glycosidic bonds (C–N vs C–C) to the ribose. Therefore, the contrasting CD spectra suggest that the two isomeric compounds, $m^3U$ and $m^3\Psi$, exhibit very different transition moments due

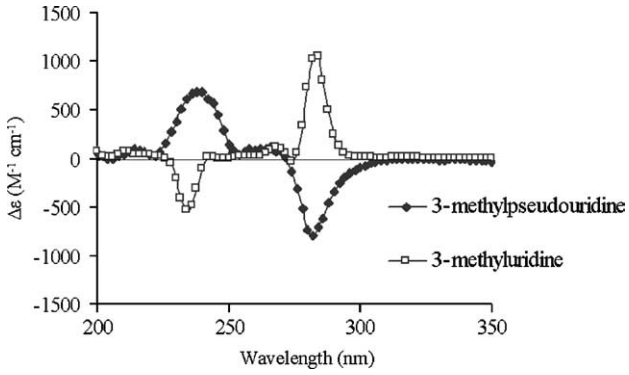


**Figure 3.** CD spectra of 3-methyluridine and 3-methylpseudouridine in $ddH_2O$ at room temperature are shown. The molar ellipticities were normalized from concentrations of 1.1 and 0.6 mM for $m^3U$ and $m^3\Psi$, based on an extinction coefficient of $1.0 \times 10^4$ $cm^{-1}$ $M^{-1}$ and $8.1 \times 10^3$ $cm^{-1}$ $M^{-1}$, respectively. Each curve represents the average of four scans.

to differences in position of the uracil residue, relative to the ribose moiety. The C–C nucleosides typically favor a higher population of $C_2'$-*endo* conformers than C–N nucleosides due to a decreased anomeric effect.[23] As such, C–C nucleosides have enhanced rotational freedom about the C–C glycosidic bond.[23] Although the electronic and anomeric effects are modest, the conformations of $m^3U$ and $m^3\Psi$ are in agreement with the conformational data observed for other C–C and C–N nucleosides.

Although pseudouridine as a free nucleoside preferentially adopts a *syn* conformation, pseudouridine has been observed only in the *anti* conformation within the confines of an oligonucleotide.[24] In both single- and double-stranded RNAs, NMR[24b,c,25], X-ray crystal structures[26], and molecular dynamics simulations[27] have shown that a water molecule fits appropriately between the N1-H of pseudouridine and the phosphate backbone. Water-mediated hydrogen bonding conformationally constrains the pseudouridine in RNA such that increases in RNA stability occur when compared to uridine.[28] However, a previous work has shown that single-stranded loop pseudouridines of H69 ($\Psi$ 1915 and $\Psi$ 1917) are slightly destabilizing.[24b] Although we do not completely understand the structural significance of these destabilizing pseudouridines, one possibility is that different conformations are present. Recent NMR data from our laboratory suggest that $\Psi$ 1915N1, $\Psi$ 1915N3, and $\Psi$ 1917N3 imino protons contribute differently to the loop structure of H69 compared to uridine imino protons (unpublished results). Therefore, it is important to determine what conformation(s) $m^3\Psi$ at position 1915 will adopt within the H69 RNA hairpin.

Nature uses methylation as the simplest and most common type of derivatization for the major classes of RNA. Non-methylated nucleotides in 16S rRNA of reconstituted ribosomes reduce ribosomal efficiency to about 50%.[29] Therefore, methylation appears to be important for optimizing the overall efficiency of ribosomal protein synthesis. In tRNAs, methylation has been shown to be important for tRNA stabilization and

efficient tRNA-ribosome contacts, which may be important for proper gene readout.[30] More recently, ribosomal recycling factor has been shown to make contacts with $m^3\Psi$ at position 1915 of *E. coli*.[12] In this system, methylation may play a role in directing RNA-protein complex formation. Therefore, perturbation by methylation in RNA can alter its chemical properties, and hence adjust the biological role of the overall macromolecule.

## 3. Conclusions

In conclusion, we have determined the solution conformations of the modified isomeric nucleosides, $m^3U$ and $m^3\Psi$. The uridine analogue, $m^3U$, exhibits a slightly higher proportion of $C_3'$-*endo* sugar pucker and its base is positioned in the *anti* conformation. In contrast, its C-glycosidic isomer, $m^3\Psi$, exhibits near equal populations of both $C_2'$- and $C_3'$-*endo* conformations and its base is oriented in a *syn* conformation. It remains to be determined how these modified nucleosides behave within the context of an oligonucleotide. We are currently assessing the conformational impact of $m^3\Psi$ and other pseudouridine derivatives within the context of ~20-nucleotide model rRNAs.

## 4. Experimental section

The modified nucleosides, $m^3U$ and $m^3\Psi$, were synthesized as previously described.[13,14c] $^1H$ NMR data were acquired on a unity 500 MHz NMR spectrometer. NOE experiments were performed in 99.99% $D_2O$. Each proton was irradiated for 1D NOE differences at 4, 25, and 37 °C, and the intensity of irradiated signal for nearby protons was measured. CD spectra were acquired in $ddH_2O$ between 200 and 350 nm on a Jasco J600 spectropolarimeter at ambient temperature. The concentrations of the nucleotides were calculated from the extinction coefficient for uridine ($1.0 \times 10^3\ cm^{-1}\ M^{-1}$ at pH 7.0) and pseudouridine ($8.1 \times 10^3\ cm^{-1}\ M^{-1}$ at pH 7.0).[31] With these concentrations, the measured CD spectra were converted to molar ellipticity ($\Delta\varepsilon$).[32]

## 5. Supporting information

NMR spectra for 3-methyluridine and 3-methylpseudouridine are available.

## Acknowledgments

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.07.061.

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