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Synthesis of ^{15}N-Enriched Pseudouridine Derivatives

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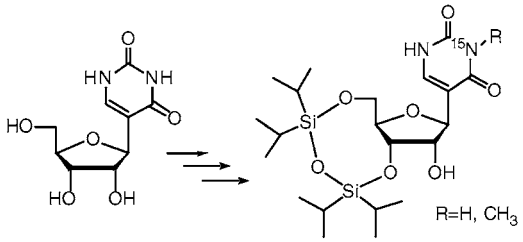
ABSTRACT



A procedure for the chemical synthesis of [3-^{15}N]-labeled pseudouridine and a methylated derivative was developed. A suitably protected pseudouridine precursor was nitrated at N3 followed by treatment with $^{15}NH_4Cl$ to afford the ^{15}N-labeled product in six steps with a 20% yield. This methodology will allow for the production of RNAs with [3-^{15}*N*]pseudouridine and [3-^{15}*N*-methyl]pseudouridine at specific locations.

The site-specific incorporation of ^{15}N labels is important for the structural analysis of RNA or the study of ligand interactions with nucleic acids.[1] ^{15}N labels allow overlapping ^{1}H NMR resonances to be resolved and can serve as local probes of hydrogen bonding or protonation because they display a wide range of chemical shifts that are sensitive to small changes in the local environment.[2] The synthesis of RNAs with ^{15}N labels at specific locations can be accomplished by using chemical or biological methods.[3] In the case of the modified nucleoside pseudouridine (Ψ), N1 or N3 labeling can be achieved using a biosynthetic approach.[4] Our main goals in using a purely chemical approach to label pseudouridine were to (1) use an inexpensive labeling source ($^{15}NH_4Cl$), (2) label the nucleoside at a specific location (N3), and (3) produce partially labeled samples (3-^{15}N-Ψ at specific residues).

A traditional method of incorporating ^{15}N into a desired biomolecule consists of growing a bacterial strain in ^{15}N-rich media and then extracting the ^{15}N-enriched material.[4] This biosynthetic method works well for the isolation of total RNAs, whereas the isolation of smaller RNA fragments is generally tedious and the quantity obtained may not be in sufficient quantities for NMR structural analysis. Nucleotides can be efficiently coupled using solid-phase chemistry to yield short polynucleotides in milligram quantities.[5] The four standard RNA nucleosides, as well as some modified bases, have been site-specifically enriched with ^{15}N through a wide variety of synthetic techniques.[6]

Natural RNAs are comprised of a large number of modified nucleosides such as the C-glycoside pseudouridine (Ψ).[7] Pseudouridine is the most common modified nucleotide and is universally conserved in several RNAs at regions of biological importance.[8,9] One region of considerable interest

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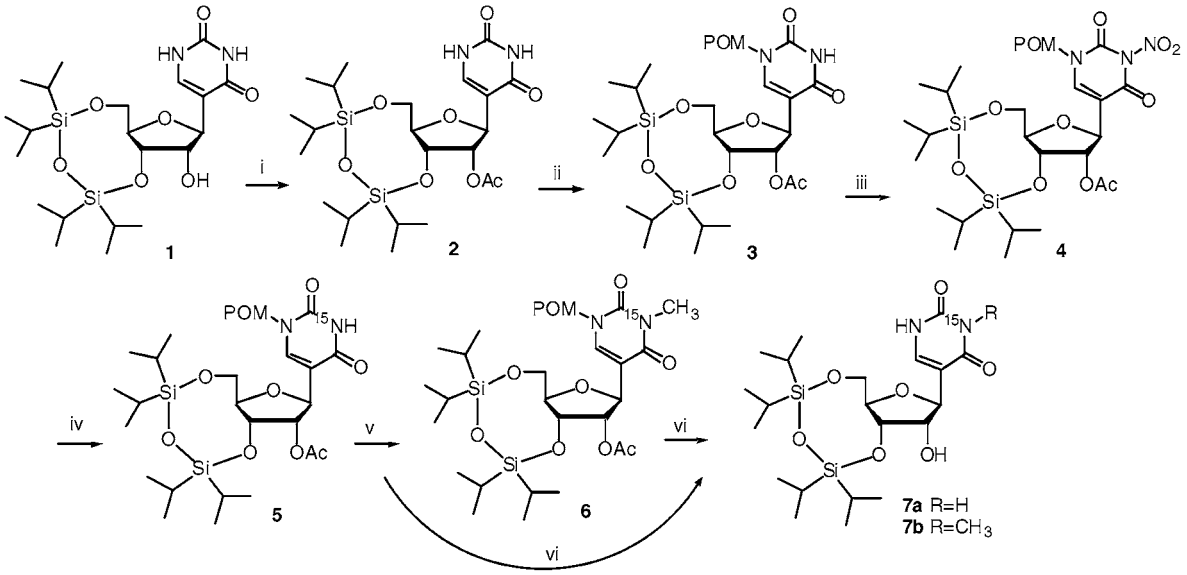
Scheme 1. POM Protection, N-Nitration, Ammoniolysis, and Deprotection Reactions[a]



[a] Reagents and conditions: (i) Ac_2O, DMAP, pyridine, rt, 6 h, 99%. (ii) POMCl (18 equiv), Et_3N, pyridine, rt, 5 days, 86%. (iii) NH_4NO_3, TFAA, CH_2Cl_2, 0 °C, 90 min, 73%. (iv) $^{15}NH_4Cl$, K_2CO_3, Et_3N, CH_3CN, H_2O, rt → 55 °C, 48 h, 53% (45% ^{15}N enrichment). (v) DMF−DMA, benzene, reflux, 3 h, 80%. (vi) Excess NH_3/CH_3OH, rt → 50 °C, 48 h, (**7a**, 76%; **7b**, 63%).

is helix 69 of *Escherichia coli* 23S rRNA. Crystallographic data show that this helix is situated proximally to the decoding region of 16S rRNA and tRNAs in the ribosome.[10] The crystal studies also suggest that this helix is dynamic and may play an intimate role in protein synthesis.[10,11] Thus, ^{15}N NMR spectroscopy may be useful to study the dynamics and interactions of this RNA segment with either the ribosome or isolated ligands.

In our previous work, we developed a strategy for the synthesis of a suitably protected 3-methylpseudouridine ($m^3\Psi$) phosphoramidite.[12] The protective groups used in that work can be employed in the synthesis of ^{15}N-enriched pseudouridine derivatives [3-^{15}*N*-methyl]-3′,5′-*O*-TIPDS-pseudouridine (TIPDS, tetraisopropyl disiloxane) and [3-^{15}*N*]-3′,5′-*O*-TIPDS-pseudouridine, which serve as precursors to the corresponding phosphoramidites.

Ariza et al. successfully synthesized [3-^{15}*N*]uridine and [1-^{15}*N*]inosine by first employing a mild nitrating reagent, nitronium trifluoroacetate (NO_2OCOCF_3), for generation of the *N*-nitro nucleosides.[3c] The presence of the strong electron-withdrawing group at the imino site rendered the neighboring carbonyl reactive toward ^{15}N-labeled ammonia ($^{15}NH_4Cl$).[3c] We used an analogous reaction with the appropriate pseudouridine derivative. Scheme 1 illustrates the synthetic steps used to generate two pseudouridine derivatives. Compound **1** was generated using standard procedures[13] and then acetylated with acetic anhydride and DMAP in pyridine to give **2** in 99% yield. A catalytic amount of DMAP was required for successful production of **2**. The N1 position was protected with pivaloyloxymethyl (POM) to yield **3** using previously developed strategies.[12] Compound **3** was nitrated using a mixture of TFAA and NH_4NO_3 for 90 min at 0 °C to provide **4** in a 73% yield. Minimal *N*1-POM deprotection was observed under these conditions.

Ariza et al. employed KOH in their ^{15}N incorporation step.[3c] The stronger base will readily remove the *N*1-POM group that is necessary for selective methylation at N3 in a subsequent step. When the ammoniolysis reaction was carried out with K_2CO_3 for 5 days at room temperature, **5** was obtained in 10−15% yields. ^1H NMR spectroscopy revealed a doublet at 8.4 ppm with a J_{NH} value of 91 Hz, which is consistent with that reported for 3-^{15}*N*-uridine.[3c] The ^1H NMR spectrum of **5** also revealed a singlet between the split $^{15}N-H$ doublet, corresponding to the 3-^{14}N proton and accounting for half of the naturally occurring isotope. Integration of the 3-^{15}N proton signal versus 3-^{14}N proton signal revealed a 1:1 ratio of ^{15}N to ^{14}N. The level of ^{15}N-enrichment was 50%, suggesting that the NO_2 group was not completely stable to the conditions of the ammoniolysis reaction. Our immediate concern, however, was to improve the overall yield of the ammoniolysis reaction. Adding more equivalents of $^{15}NH_4Cl$ or K_2CO_3 did not improve the yield. The use of higher temperature (55 °C) or decreased reaction time (24 h) gave a modest increase in the overall isolated yield of **5** (28−38%); however, ^1H NMR data revealed that the ratio of ^{15}N:

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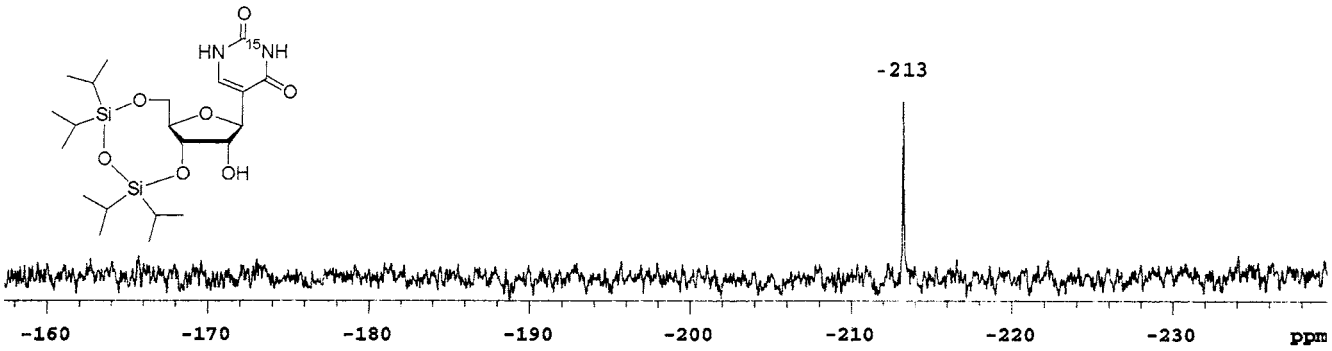

Figure 1. ^{15}N NMR spectrum of **7a**. A total of 350 scans were acquired on a Varian Unity 500 MHz spectrometer with a 25 mM solution in 700 μL $CDCl_3$.

^{14}N decreased from 1:1 to 1:2. When the reaction was stirred vigorously at room temperature for 24 h, followed by an increase in the temperature to 55 °C for another 24 h, the isolated yield of **5** increased to 53%, and the relative ratio of ^{15}N:^{14}N was 0.9:1. ^{15}N NMR analysis confirmed the isotopic enrichment of ^{15}N by a sharp singlet at −215 ppm. A proton-coupled ^{15}N spectrum revealed a doublet with a J_{NH} value of 91 Hz, consistent with the 3-^{15}N−H couplings observed in the ^{1}H NMR spectrum.

Methylation of **5** at 3-^{15}N was accomplished by using *N*,*N*-dimethylformamide dimethylacetal[14] in refluxing benzene for 2 h to produce **6** with an isolated overall yield of 80%. The ^{15}N NMR spectrum revealed a single peak at −213 ppm corresponding to 3-^{15}N. The advantage of using 2′-*O*Ac and *N*1-POM protective groups is that they can be removed in one step. Compounds **5** and **6** were treated with 2.0 M NH_3 in MeOH and heated to 50 °C to give **7a** in 76% yield and **7b** in 63% yield, respectively. The ^{15}N NMR spectra reveal peaks at −213 and −214 ppm for 3-^{15}N of **7a** and **7b**, respectively (Figures 1 and 2).

In summary, we have synthesized two pseudouridine derivatives: [3-^{15}*N*]-3′,5′-*O*-TIPDS-pseudouridine and [3-^{15}*N*-methyl]-3′,5′-*O*-TIPDS-pseudouridine with 45% ^{15}N enrichment. Recent NMR studies have demonstrated that DNA oligonucleotide resonances can be assigned by site-specific ^{15}N,^{13}C-enrichment at levels as low as 1%;[15] therefore, the level of enrichment for pseudouridine should be sufficient for further NMR studies. From **7a** and **7b**, it will be possible to synthesize the corresponding phosphoramidites in four steps by employing 5′-*O*-silyl-2′-*O*-orthoester-type chemistry[12,16] or by using other standard phosphoramidite techniques.[5] The resulting phosphoramidites will then be used for the synthesis of short RNA oligonucleotides containing these modifications. We synthesized the N3-methylated pseudouridine derivative because pseudouridine 1915 of *E. coli* 23S rRNA is naturally methylated at the N3 position. These labeled nucleosides will be important for probing the natural, biological roles of pseudouridine and 3-methylpseudouridine. Studies to date have revealed a dynamic nature of the regions containing these modifications.[10,11] The ^{15}N-labeled pseudouridine will have other practical applications; its incorporation into oligonucleotides will aid in complete RNA structure determination by NMR spectroscopy, probing the pK_a of the NH amines within the context of short RNAs, examining hydrogen-bonding interactions in

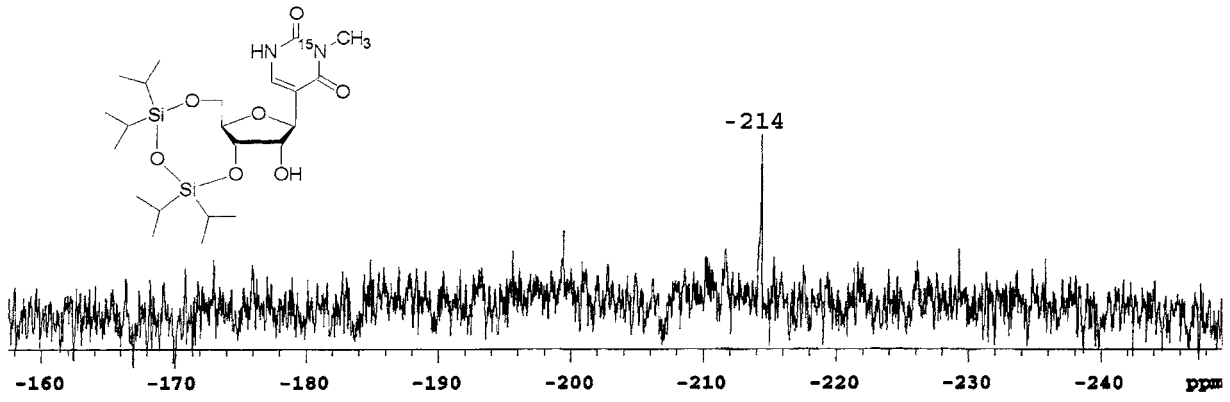

Figure 2. ^{15}N NMR spectrum (500 MHz) of **7b**. A total of 1120 scans were acquired on a 50 mM solution in 350 μL $CDCl_3$.

RNA duplexes, and screening for small-molecule−RNA or RNA−RNA interactions.

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Supporting Information Available: Complete experimental procedures, ^{1}H, ^{13}C, and ^{15}N NMR spectra, and ESI-MS spectra for compounds **2**−**7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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