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A Practical Synthesis of the Modified RNA Nucleoside Pseudouridine

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Abstract: An alternative synthesis of the modified RNA nucleoside pseudouridine is reported. This procedure employs coupling of an iodinated pyrimidine and a suitably protected lactone. The resulting hemiacetal is reduced and deprotected to yield pseudouridine. © 1999 Elsevier Science Ltd. All rights reserved.

Pseudouridine is the most common RNA modification currently known in nature (reviewed in ref 1). This structural analogue of uridine comprises 2-3% of all the nucleotides in tRNA and up to 8% of the uridines found in the large subunit of rRNA.[2] The locations of pseudouridines in rRNA and tRNA have been linked to areas of functional importance, such as the peptidyl transferase center and the anticodon stem-loop domain.[3] In many cases, the positions of these pseudouridine residues are highly conserved throughout phylogeny ranging from bacteria to mammals.[1–3] Therefore, it is likely that these natural modifications play pivotal structural or functional roles in given RNAs.

To our knowledge, there have been only a few studies of the structural role of pseudouridine within the context of small model RNAs.[4–6] We believe that studies of the role of pseudouridine in more complex RNA structures have been limited by several factors. The nucleoside, which is typically isolated from natural sources, is very expensive (*e.g.*, $707/g from Sigma). Consequently, the corresponding phosphoramidite which can be used for solid-phase RNA synthesis is difficult to obtain. The goal of this work was to develop a practical synthesis of pseudouridine that is economical and involves few synthetic transformations. Once pseudouridine is readily available in sufficient quantities, novel and convenient routes to the phosphoramidite can be explored. The synthetic scheme that we have devised involves a minimal number of synthetic steps and uses commercially available and inexpensive starting materials.

The *C*-glycosidic bond of pseudouridine is unique to this naturally modified nucleoside and its structural analogues. Prior methods for the coupling of ribose sugars to pyrimidines and purines are generally applicable to the more abundant *N*-linked nucleosides.[7] Recently, however, Matulic-Adamic and coworkers reported a useful synthetic method for producing unnatural *C*-linked nucleosides.[8–10] Their scheme involved coupling of a suitably protected nucleoside base to the corresponding sugar lactone, and subsequent reduction of the hemiacetal to give the desired nucleoside. Although the overall yields of their syntheses ranged from 6 to 34%, multiple advantages of their approach are apparent. A low number of steps are required in the synthesis, the starting materials are readily available, and standard protective groups and routine synthetic transformations

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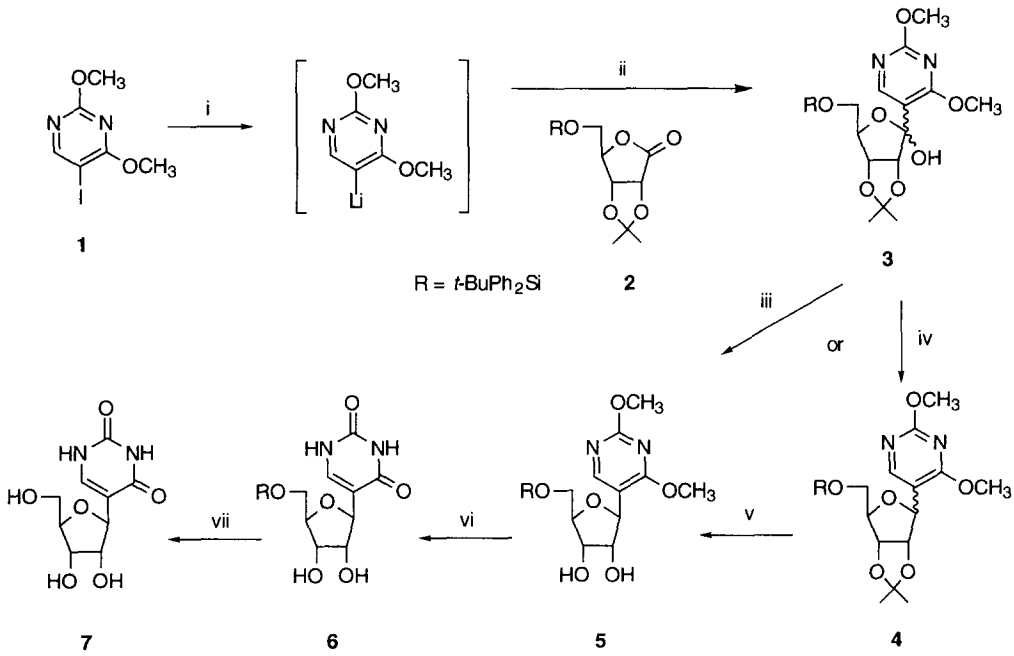
are employed. More importantly, their procedure provided sufficient quantities of *C*-nucleosides that could be subsequently converted to the phosphoramidites and used for solid-phase RNA synthesis.

The basis of our pseudouridine synthesis, shown in Figure 1, involves a lithium-halogen exchange with 5-iodo-2,4-dimethoxypyrimidine (**1**) and *tert*-butyllithium, followed by coupling to 5-*O*-*tert*-butyldiphenylsilyl-2,3-*O*-isopropylidene-D-ribono-1,4-lactone (**2**). To our knowledge, this particular combination of protective groups has not been used previously in pseudouridine syntheses. Other reported syntheses of pseudouridine typically used starting materials that are more difficult to obtain, such as protected aldehydo sugars[11,12] or a bicyclic enone,[13] or used protective groups that are potentially labile or difficult to remove.[14,15] Following coupling, the resulting lactol **3** can be subsequently reduced to **4**. Alternatively, novel reduction conditions can be employed that reduce the lactol and remove the acetonide group in a single step to generate a pseudouridine precursor **5** which can be conveniently deprotected in two steps to provide pseudouridine (**7**) in ~20% overall yield from **2**.

Figure 1. The overall synthetic scheme for pseudouridine synthesis is shown.



Reagents and Conditions: i) *t*-BuLi (2 equiv) /THF/-78 °C (30 min); ii) **2** (0.91 equiv)/THF/-78 °C (1.5 h); iii) $BF_3{\cdot}Et_2O$ (1.1 equiv)/Et_3SiH (40 equiv)/CH_2Cl_2/4 °C (1 h) followed by warming to 20 °C (overnight) and further addition of $BF_3{\cdot}Et_2O$ (4 equiv); iv) $BF_3{\cdot}Et_2O$ (1.1 equiv)/Et_3SiH (6 equiv)/CH_2Cl_2/4 °C, 21 h; v) 70% CH_3COOH (reflux, 60 min); vi) NaI/CH_3COOH (glacial) (reflux, 25 min); vii) 3% HCl in MeOH (4 h).

The syntheses of the starting materials **1** and **2** were done essentially as described previously.[16,17] In order to avoid bis-silylation in the preparation of **2**, the isopropylidene 2,3-*O*-group was added first under

controlled temperature and reaction time (4 °C over 4 h). The primary hydroxyl was then protected with *tert*-butylchlorodiphenylsilane to generate **2**. The coupling reaction was carried out by first treating **1** with 2 equiv of *tert*-butyllithium at -78 °C, followed by addition of a -78 °C solution of 0.91 equiv of **2**. The resulting mixture of anomers (1:8 of α:β) **3** was obtained in 60% yield after silica-gel purification.[18] Since the stereoselectivity of the coupling reaction is lost during the reduction step, the mixture of anomers **3** was subjected to $BF_3{\cdot}Et_2O$ in the presence of triethylsilane. By carefully controlling the reducing conditions, it was possible to isolate the fully protected, coupled product as a mixture of anomers **4** in 51% yield (1:1 α:β) following silica-gel chromatography.[19] Alternatively, we were able to conveniently obtain the naturally occurring β anomer **5** without the isopropylidene group as a single isomer after chromatography in 20–35% yield by using higher triethylsilane ratios (typically 40 equiv) and an excess of $BF_3{\cdot}Et_2O$.[20] The advantage of the latter approach is that the desired β anomer **5** is easier to isolate and separate from the α anomer by silica-gel chromatography when the 2',3'-*O*-protective group is absent. The stereochemistries of **4** and **5** were verified as described previously.[21]

The ability to selectively deprotect intermediates **4** or **5** is critical because either may serve as a novel entry point into the desired phosphoramidite synthesis of this modified uridine. Previous methods that were reported for the pseudouridine amidite synthesis generally employed multiple protection steps, or did not use any protection of the base nitrogens.[4,22,23] Deprotection of **5** was accomplished by treatment with NaI in refluxing acetic acid for 25 min followed by an appropriate work-up to generate compound **6**. Finally, the *tert*-butyldiphenylsilyl group was removed by stirring at room temperature in 3% methanolic HCl. The organic impurities were removed by extracting the product into water; the HCl was removed as described previously[15] to yield pure **7** in overall 10-20% yield from **2**. The yield for both deprotection steps was >95% and provided pseudouridine that is identical to an authentic sample (Sigma) as determined by ^1H and ^{13}C NMR, mass spectrometry (FAB+), and UV-visible spectroscopy.

In summary, we have demonstrated that the modified nucleoside pseudouridine can be synthesized in fewer than 7 steps from readily obtainable starting materials. The *C*-linked nucleoside synthesis devised by Matulic-Adamic *et al.*[8-9] for phenyl and pyridyl derivatives was easily adapted to accommodate a pyrimidine ring, and gave comparable yields of coupled and reduced products. The reactions described here have also been performed on a preparative scale to produce sufficient quantities of pseudouridine. We envision that this scheme could be expanded further to produce pseudouridine derivatives, such as the naturally occurring 1-, and 3-methylpseudouridines, or novel structural analogues. Such nucleosides could be incorporated into RNA for structure studies, or considered as potential antiviral agents. Currently, work is underway to synthesize the pseudouridine phosphoramidite from the intermediates shown in Figure 1 as different entry points. The resulting phosphoramidite will ultimately be used in solid-phase RNA synthesis to produce model RNA hairpins for structure studies.

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18. Characterization of **3**: (α/β 1:8 mixture) ^{1}H NMR ($CDCl_3$) δ (ppm): α anomer 1.00 (s, tBu), 1.38 (s, 3H, Me), 1.64 (s, 3H, Me), 3.73–3.92 (m, 2H, H-5'/H-5''), 3.98 (s, 3H, OMe), 4.02 (s, 3H, OMe), 4.69 (s, 1H, H-4'), 4.73 (m, 2H, H-2', H-3'), 7.24–7.69 (m, Ph), 8.56 (s, 1H, H-6); β anomer 1.08 (s, tBu), 1.21 (s, 3H, Me), 1.24 (s, 3H, Me), 3.98 (s, 3H, OMe), 4.02 (s, 3H, OMe), 4.22 (s, 1H, OH-1'), 4.35 (dt,, 1H, H-4'), 4.84 (dd, 1H, H-3'), 4.86 (d, 1H, H-2'), 7.24–7.69 (m, Ph), 8.56 (s, 1H, H-6); MS (+FAB): calc. 567.2, found 567.2.
19. Characterization of **4**: (α/β 1:1 mixture) ^{1}H NMR ($CDCl_3$) δ (ppm): α anomer 1.07 (s, tBu), 1.28 (s, 3H, Me), 1.38 (s, 3H, Me), 3.74–3.90 (m, 2H, H-5', H-5''), 3.94 (s, 3H, OMe), 3.98 (s, 3H, OMe), 4.22 (s, 1H, H-4'), 4.83–4.96 (m, 2H, H-2', H-3'), 5.46 (d, J=3.5, 1H, H-1'), 7.32–7.67 (m, Ph), 8.35 (s, 1H, H-6); β anomer 1.04 (s, tBu), 1.33 (s, 3H, Me), 1.58 (s, 3H, Me), 3.74-3.90 (m, 2H, H-5', H-5''), 3.92 (s, 3H, OMe), 3.96 (s, 3H, OMe), 4.11 (m, 1H, H-4'), 4.63 (m, 1H, H-2'), 4.69 (m, 1H, H-3'), 4.96 (d, J=4, 1H, H-1'), 7.32–7.37 (m, Ph), 8.28 (s, 1H, H-6); MS (+FAB): calc. 551.2, found 551.2.
20. Characterization of **5**: β anomer ^{1}H NMR ($CDCl_3$) δ (ppm): 1.03 (s, tBu), 3.0 (bs, 1H, OH-2'), 3.2 (bs, 1H, OH-3'), 3.86–3.93 (dd, 2H, H-5', H-5''), 3.96 (s, 3H, OMe), 3.96 (s, 3H, OMe), 4.03 (q, 1H, H-4'), 4.12 (t, J=5.5, 1H, H-2'), 4.24 (t, 1H, H-3'), 4.91 (d, J=5.5, 1H, H-1'), 7.35–7.68 (m, Ph), 8.34 (s, 1H, H-6); MS (+FAB): calc. 510.2, found 510.8.
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