Synthesis of Oligoribonucleotides Containing 2-Thiouridine: Incorporation of 2-Thiouridine Phosphoramidite without Base Protection

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Received July 31, 1995

Transfer RNAs are unique among the naturally occurring RNAs in that a very high percentage of the nucleosides are chemically modified.[1,2] With few exceptions, the functional role of these diverse nucleosides is unknown. The naturally occurring nucleoside modifications comprise a structurally diverse group, and many simple classes of chemical modifications that one could envisage, particularly with respect to the bases, are represented in nature. One simple base modification among uridine nucleotides that has dramatic effects on nucleoside conformation is the replacement of oxygen at the C-2 position of the base with sulfur. For 2-thiouridine, it has been shown that sulfur substantially stabilizes the 3′-endo sugar conformation at the nucleoside and dinucleotide level,[3,4] but due to the synthetic difficulties this property has not been confirmed for oligonucleotides.

Because of the chemical reactivity introduced by sulfur substitution, thiouridines are incompatible with the normal conditions used on commercial automated oligonucleotide synthesizers. Specifically, the sulfur is readily oxidized by the aqueous iodine reagent used to oxidize the phosphoramidite during each coupling cycle.[5] Incorporation of 4-thiouridine poses the additional problem of hydrolysis during ammonia deprotection; this has been overcome by introducing the sulfur after oxidation is completed and also by modifying the deprotection strategy.[6] A recent report described the use of a new protection strategy for the incorporation of 2′-deoxy-2-thiouridine and 2′-deoxy-2-thiothymidine into oligodeoxyribonucleotides.[7] Unprotected 2′-deoxy-2-thiothymidine has also been incorporated into oligodeoxyribonucleotides using the conventional solid-phase phosphoramidite method, but the yield was quite low, making this approach unacceptable for synthesizing longer oligonucleotides.[8] Desulfurization leading to the 4-pyrimidone was a major side reaction when the aqueous iodine reagent was used in the oxidation step of the solid-phase method.[9] The base-protecting strategy appears to be a viable approach for oligodeoxynucleotides, but there has been no report of the solid phase synthesis of oligoribonucleotides containing 2-thiouridine.

Given the additional difficulty of synthesizing RNA compared to DNA, we were interested in a general strategy for incorporating 2-thiouridine with a minimum of protection/deprotection steps. Furthermore, there are a number of naturally occurring modified uridines that have sulfur at the 2 position along with further modifications at other ring positions;[3] protection as described by Kuimelis and Nambiar[7] could be problematic for these nucleosides. Oxidation of phosphoramidites with *tert*-butylhydroperoxide has been described previously,[10,11] and we determined in a preliminary experiment monitored by thin-layer chromatography that the 2-thiouridine nucleoside was stable to treatment with *tert*-butyl hydroperoxide for 24 h under the conditions described below. We found that 2-thiouridine phosphoramidite could be incorporated into RNA oligonucleotides without base protection by using *tert*-butyl hydroperoxide for the oxidation step. The stepwise and overall yields were comparable to those obtained with unmodified RNA using iodine/water oxidation. This alternative oxidation method allows for the introduction of 2-thiolated uridines into any sequence context, expanding the repertoire of potential oligonucleotide stabilizing modifications.

The synthesis of 2-thiouridine was carried out using the procedure of Vorbruggen.[12] The 5′-(dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-2-thiouridine 3′-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite) was synthesized according to well-established literature procedures as shown in Scheme 1 in the supporting information.[13] The protected phosphoramidite was characterized by ^{1}H and ^{31}P NMR and high-resolution FAB mass spectrometry. RNA oligonucleotide synthesis was done on an Applied Biosystems 394 synthesizer using standard RNA protocols for the 10 μmol scale except that the oxidations were done twice for 6 min each with an acetonitrile wash between oxidations. The oxidation reagent was a 10% solution of *tert*-butyl hydroperoxide (Aldrich) in acetonitrile.

As part of a project to investigate the influence of RNA modification on codon–anticodon interactions, we synthesized three oligonucleotide pentamers using the *tert*-butyl hydroperoxide oxidation protocol. The sequences GAAAC, GUUUC, and Gs^2UUUC were synthesized on a 10 μmol scale using 0.05 M solutions of Perseptive Biosearch Expedite phosphoramidites and a 0.1 M solution of the 2-thiouridine phosphoramidite. Trityl release assays indicated that the couplings were greater than 95%, and C18 HPLC analysis indicated overall yields consistent with 98% couplings for each pentamer. An initial synthesis using a 0.05 M solution of the 2-thiouridine phosphoramidite resulted in a coupling yield of 75% at the modified site, but in subsequent preparations the coupling at the thiouridine step was identical within experimental error to that of the unmodified phosphoramidites. The oligonucleotides were each deprotected using published RNA protocols[14,15] and the oligonucleotides purified by PRP-1 HPLC.[16] We have extended the methodology to longer oligonucleotides; an 18-mer with four consecutive s^2U residues at positions 8, 9, 10, and 11 has been synthesized as well as a 17-mer corresponding to the anticodon of $tRNA^{Lys}$ with a single s^2U at the

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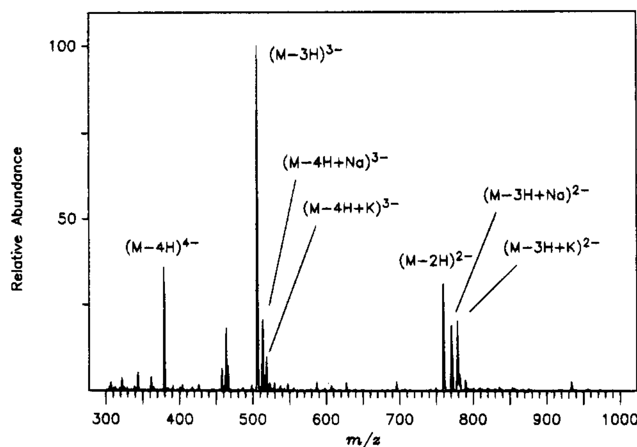

Figure 1. Negative ion electrospray mass spectrum of G s²UUUC. The molecular weight measured from the spectrum shown is 1522 ± 0.3, and the molecular weight calculated for Gs²UUUC is 1522.19. M designates the neutral oligonucleotide. Measured mass values: $(M - 4H)^{4-}$, 379.50; $(M - 3H)^{3-}$, 506.50; $(M - 2H)^{2-}$, 760.00.

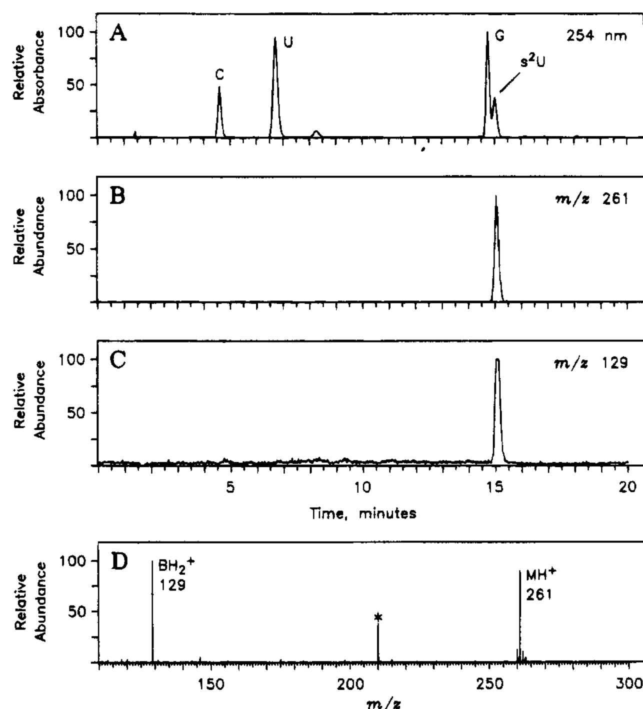

Figure 2. LC/MS analysis of nucleosides from an enzymatic digest of Gs²UUUC. Chromatography and mass spectrometry procedures are as described.[18] (A) Reversed-phase HPLC separation with UV detection at 254 nm. Assignments shown were determined from corresponding mass spectra and relative HPLC retention times.[18] (B) Reconstructed ion chromatogram for MH^+ ion of s^2U. (C) Reconstructed ion chromatogram for BH_2^+ ion of s^2U. (D) Mass spectrum acquired at 15.2 min (see panel A). Peak marked with an asterisk is due to background.

8 position. Overall yields were greater than 50% at the 10 μmol level with no sulfur oxidation.

The molecular weight of each pentamer was verified using electrospray mass spectrometry;[17] Figure 1 shows the electrospray mass spectrum of Gs²UUUC. Gs²UUUC and GUUUC were also analyzed for nucleoside composition by enzymatically digesting the oligonucleotides to

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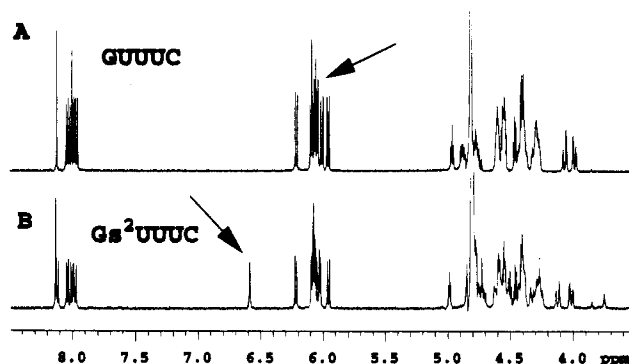

Figure 3. 500 MHz ¹H NMR spectrum at 35 °C of 2.5 mM (A) GUUUC and (B) Gs²UUUC oligonucleotides in 0.5 mL of 25 mM phosphate buffer, pH 7.0 containing 100 mM NaCl. The samples were lyophilized and then dissolved in 0.5 mM of 99.996% deuterium oxide. The arrows indicate the positions of the H1′ protons for the second U or s2U; the U H1′ resonance is not resolved in the 1D spectrum. The doublet at 6.59 ppm for the s²U residue is shifted downfield due to sulfur substitution, with a $^3J_{1'-2'}$ of 2.7 Hz compared to the analogous uridine in GUUUC with a $^3J_{1'-2'}$ of 5.8 Hz measured from the 2D PECOSY spectrum (not shown).

their component nucleosides and subsequent analysis by liquid chromatography/mass spectrometry (LC/MS).[18] Figure 2 shows the LC/MS data for Gs²UUUC which provides unambiguous verification as to the identity of the modified nucleoside and confirms that the nucleosides are present in the expected ratios. The ¹H NMR spectrum of Gs²UUUC in Figure 3 shows that the H1′ proton is shifted downfield by sulfur substitution and that the H1′-H2′ scalar coupling is reduced compared to the comparable uridine in GUUUC. This indicates that 2-thiouridine influences the sugar conformation in a similar fashion for Gs²UUUC as has been described for 2-thiouridine at the nucleoside level.[3] A detailed NMR study describing the effect of sulfur on the RNA structure will be published elsewhere.

In summary, the use of *tert*-butyl hydroperoxide in the synthesis of 2-thiouridine containing oligonucleotides allowed us to incorporate a biologically important modified nucleotide into RNA without the inconvenience of base protection. The high coupling efficiency should make *tert*-butyl hydroperoxide attractive as an oxidant for other modified phosphoramidites susceptible to side reactions with iodine.[19] The efficiency of this protocol makes it attractive for making antisense RNA oligonucleotides containing 2-thiouridine or derivatives of 2-thiouridine where the stabilizing effect of sulfur could be advantageous.

Acknowledgment. This work was supported by American Cancer Society Grant JFRA-405 and NSF Grant MCB-9317196 to D.R.D. The NMR, MS, and DNA and Peptide Synthesis Facilities are supported by NIH Grants P30 CA42014 and S10 RR06262. We thank Jim McCloskey and Steve Pomerantz for mass spectrometry analysis and Richard Griffey and Henri Sasmor for helpful discussions.

Supporting Information Available: Experimental procedures for compounds **4−6**, synthetic Scheme 1, and procedures for the RNA synthesis, deprotection, and purification (5 pages).

JO9514073

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