essential imidazolium to the unstable tetrahedral intermediates produced from amides where the N is an aliphatic one, the proton transfer is thermodynamically favorable, and in the case of anilides is either thermodynamically neutral or only slightly uphill.

The second and less obvious feature of imidazole which makes it unusually suitable for general-acid or -base catalysis relative to an aliphatic amine (ammonium) has to do with the molecular shape. Because the proton transfer in imidazole occurs at the sp^2 N, and in the plane of the ring, there is minimal steric hindrance in transfer to sterically congested tetrahedral intermediates.

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Supplementary Material Available: Tables of observed rate constants vs pH for attack of thiols **1b**, **5a–d**, and **6a–d** on amide **4** and characterization of thiol ester **8b** and amide **9a** (7 pages). Ordering information is given on any current masthead page.

Chemistry and Structure of Modified Uridine Dinucleosides Are Determined by Thiolation

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Abstract: The structural determination of modified nucleosides is important for understanding the chemistry, structure, and functional changes that they introduce to the nucleic acids in which they occur. Thiolation of transfer RNA wobble position uridine produces an energetically stabilized conformation of the nucleoside in solution at ambient temperature that is independent of the nature of the 5-position substituent and is of biological significance to tRNA selection of only those codons ending in adenosine (Sierzputowska-Gracz, H.; Sochacka, E.; Malkiewicz, A.; Kuo, K.; Gehrke, C.; Agris, P. F. *J. Am. Chem. Soc.* **1987**, *109*, 7171–7177. Agris, P. F.; Sierzputowska-Gracz, H.; Smith, W.; Malkiewicz, A.; Sochacka, E.; Nawrot, B. *J. Am. Chem. Soc.*, in press). Dinucleoside monophosphates have been synthesized as models for investigating the conformations and structures of wobble position uridine-34 that is thiolated and differently modified at position-5 and that is either 3′-adjacent to the invariant uridine-33 in tRNA or 5′-adjacent to the second anticodon position uridine-35. The structures and conformations of 11 dinucleoside monophosphates were analyzed by ^1H, ^{13}C, and ^{31}P magnetic resonance (NMR) spectroscopy. Within the dinucleosides, the individual modified uridine structures and conformations were very similar to those of their respective mononucleosides. The 2-position thiolation, and not the 5-position modification, produced a significantly more stable, C(3′) endo, gauche$^+$, anti conformer. However, within those dinucleosides in which the 2-thiouridine was 5′ to the unmodified uridine, the nucleic acid backbone torsion angles of the unmodified uridine 5′-phosphate were affected, as determined from the scalar coupling constants J_{H^1H}, $J_{H^{31}P}$, and $J_{^{13}C^{31}P}$. In contrast, uridines that were only 5-position modified did not affect the conformation of the 3′-adjacent unmodified uridine phosphate. The structural data obtained and the nucleoside conformations derived from the data support the "modified wobble hypothesis" (Agris, P. F. *Biochimie* **1992**, *73*, 1345–1349); i.e., the tRNA wobble position-34 nucleoside is modified in such a way as to constrain not only its own conformation but also the structural conformation of the anticodon, thereby producing a specific codon selection during protein synthesis.

Introduction

Uridines (U) in the first position, or wobble position-34, of eucaryotic and procaryotic transfer RNA (tRNA) anticodons that are specific for such amino acids as glutamine, glutamic acid, and lysine are many times found to be naturally thiolated at C(2).[1-4] Thiouridine-34 (s^2U) is almost always found to be additionally modified at C-5.[2] There are a variety of naturally occurring 5-position derivatives: (methylamino)methyl, methoxy, (methoxycarbonyl)methoxy, (methoxycarbonyl)methyl, [(carboxymethyl)amino]methyl, and others.[5] The structures and conformations of the 2-thiolated, 5-derivatized uridines at ambient temperature are dictated by the presence of the thio group and barely influenced by the chemical or structural nature of the 5-substituent.[6] Therefore, all 2-thiouridines have been found to be predominantly anti, C(3′) endo, gauche plus,[6,7] whereas nonthiolated 5-position derivatives can be either syn or anti and have no significant preference for either the C(3′) endo or C(2′) endo conformations.[7] The 2-thio-5-derivatized uridines are thermodynamically more stable than their nonthiolated counterparts.[8]

As an explanation of the codon selectivity exhibited by tRNAs with s^2U, its derivatives, and certain other wobble position modified nucleosides, we have offered a "modified wobble hypothesis" based

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on the conformation and thermodynamic stability of structures produced by the modifications of nucleoside-34.[9] We have synthesized dinucleotides to model the invariant U-33 bound to the wobble position modified U-34, and the wobble position modified U-34 bound to the second nucleoside of the anticodon for $tRNA^{Glu}$ or $tRNA^{Lys}$, U-35. In this paper we report the structures of 10 such dinucleosides in comparison to UpU.

Experimental Section

Synthesis of Dinucleosides. The phosphotriester method was used for the preparation of the uridine dinucleotide monophosphates in which the modified nucleoside (U*) was either at the 3′-position (UpU*) or 5′-position (U*pU).[10] The oligomers s^2mnm^5UpU and s^2mcm^5UpU were synthesized according to reported procedures[11,12] as were the precursors, 5′-*O*-(monomethoxytrityl)-2′-*O*-(tetrahydropyranyl)uridine (as the homogeneous diastereoisomers) and 2′,3′-*O*-(methoxymethylidene)-uridine.[13,14] The nucleoside derivatives and protected oligoribonucleotides were purified by "flash chromatography" (silica gel 60F, Merck; $CHCl_3$/MeOH gradient, 100–90%). The structure and homogeneity of protected nucleosides and oligomers were confirmed by analysis of ^1H NMR, ^{19}F NMR (for *N*-trifluoroacetylated derivatives), ^{31}P NMR (Bruker MSL, 300 MHz), and mass spectra (GC-MS LKB 2091).

1. Synthesis of 5′-*O*-(monomethoxytrityl)-2′-*O*-(methoxytetrahydropyranyl)-protected (MTHP-protected) derivatives of the modified uridines: The 2-thiouridine,[15] 5-[[*N*-(trifluoroacetyl)-*N*-[(methoxycarbonyl)methyl]amino]methyl]uridine,[16] and 5-methoxyuridine[17] precursors to the dimers were transformed into 2′-*O*-methoxytetrahydropyranyl derivatives (ca. 40% yield) according to the procedure of Markiewicz.[13] In the next step of synthesis, they were reacted with monomethoxytrityl (MMTr) chloride under standard conditions[14] to give the trityl derivatives in 30–35% total yield.

2. Synthesis of 2′,3′-*O*-methoxymethylidene-protected derivatives of the modified uridines: The precursors 2-thiouridine,[15] 2-thio-5-[(methoxycarbonyl)methyl]uridine,[15] 2-thio-5-[[*N*-(trifluoroacetyl)-*N*-methylamino]methyl]uridine,[11] 2-thio-5-[[*N*-(trifluoroacetyl)-*N*-[(methoxycarbonyl)methyl]amino]methyl]uridine,[16] 2-thio-5-methoxyuridine,[17] 5-methoxyuridine,[15] and 2-thio-5-[(methoxycarbonyl)methoxy]uridine[16] were condensed with 2,2-dimethoxypropane in the presence of trimethylsilyl chloride[18] to produce the desired derivatives in 75–80% yield.

3. Phosphorylation of the partially blocked nucleosides: The 5′-MMTr-2′-MTHP derivatives of uridine and 2-thiouridine were phosphorylated with 2-chlorophenyl phosphorodichloridate according to Narang's procedure.[19] Crude products were purified by precipitation with hexane (90–95% yield), and their homogeneity (ca. 95%) was verified by analysis of ^{31}P NMR spectra.

4. The scheme for the coupling of the protected modified and unmodified mononucleosides is shown in Figure 1. The 3′-*O*-2-chlorophenyl phosphates were allowed to react with the 2′,3′-protected nucleosides in the presence of mesitylene sulfonyl tetrazolide[20] (molar ratio 1.2:1.3, respectively) for 20 min. The reaction was terminated by addition of water, the product extracted with $CHCl_3$, and the organic layer washed with 0.1 N TEAB (triethylammonium bicarbonate) buffer. The solvent was evaporated under vacuum and the residue coevaporated with toluene. Finally, the product was purified by chromatography, which yielded the fully protected oligomer in 60–75% yield. The homogeneity of the synthesized material was confirmed by chromatography and ^{31}P NMR spectroscopy.

5. The dinucleoside monophosphates were deprotected in the following manner. The fully protected oligomers bearing the [[(methoxycarbonyl)methyl]amino]methyl substituent ($mcmnm^5$) were treated with 0.1 N NaOH (aqueous)/dioxane, 1:4, and the solution was subjected to chromatography on Dowex 50 W X-4 (NH_4^+). All other fully protected

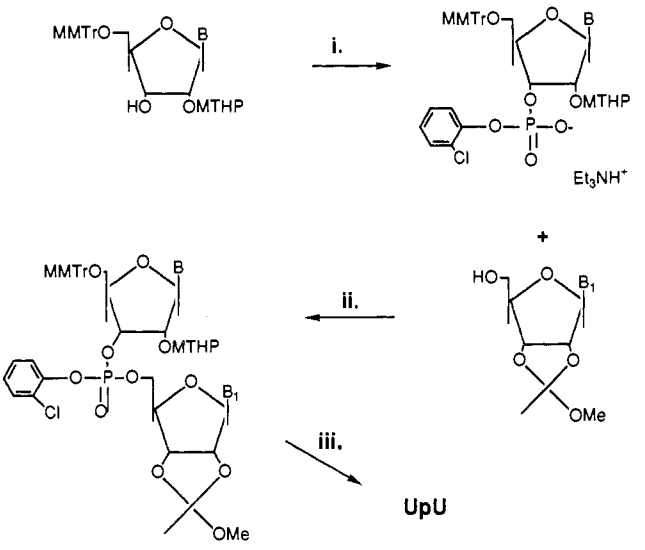

Figure 1. Scheme for the synthesis of modified uridine dinucleoside monophosphate. The reagents and conditions were (i) 2-chlorophenyl phosphorodichloridate, 1,2,4-triazole, pyridine (room temperature, 1 h); (ii) mesitylenesulfonyl tetrazolide, pyridine (room temperature, 25 min); and (iii) 4-nitrobenzaldoxime, N^1,N^1,N^3,N^3-tetramethylguanidine, acetonitrile (room temperature, 2 h), then 0.02 N HCl/H_2O, 1:1 (room temperature, overnight).

oligomers were treated with the N^1,N^1,N^3,N^3-tetramethylguanidinium salt of 4-nitrobenzaldoxime in dry acetonitrile.[21] The crude products were precipitated with dry diethyl ether, and the precipitates were dissolved in water and then chromatographed on Dowex 50 W X-4 (NH_4^+). The eluted and UV-absorbing fractions from the Dowex chromatography were evaporated under vacuum, and the resulting residue was treated with 0.02 N HCl (water/methanol, 1:1) for 12 h. This solution was neutralized with triethylamine, concentrated to a small volume, and applied to a DEAE-32 cellulose column. The product was eluted with a linear gradient (0.005–0.2 N) of TEAB buffer. Final purification was achieved by reverse-phase HPLC (10 × 250 mm Dynamax C18 10 nm) with a gradient elution (0.1 N TEAB and 70% acetonitrile/water; from 0 to 50% of the latter in 60 min). This procedure produced a partial deprotection of the carboxylic function of s^2mcmo^5U; therefore, Ups^2cmo^5U and Ups^2mcmo^5U were isolated in the ratio 1:1. All of the dinucleoside monophosphate samples were lyophilized for storage at −20 °C.

NMR Spectroscopy. The lyophilized samples were dissolved at a concentration of ~8 mM in 98% D_2O. Spectroscopy was accomplished on a GE Omega 500 at ambient temperature, unless otherwise noted. One-dimensional ^1H spectra were typically collected with 8K data points and enough scans to obtain a good signal-to-noise ratio.

^{31}P Decoupling. Phosphorus-decoupled proton spectra at 500 MHz were obtained with a dedicated reverse polarization transfer probe. The spectral width was 5000 Hz over 8K or 16K data points. The phosphorus frequency was determined by collecting a spectrum at 202 MHz with detection through the decoupling coil. For the ^{31}P-decoupled spectrum broad-band phosphorus decoupling with WALTZ 16 modulation was applied for the duration of the experiment.

^{13}C Spectra. One-dimensional ^{13}C spectra were recorded with a dedicated carbon probe at 125 MHz. The spectral width was 25 000 Hz with 8K data points, and broad-band proton decoupling with WALTZ modulation was applied for the duration of the experiment. In order to measure ^{13}C–^{31}P coupling constants, we improved the resolution by zero-filling to 16K data points and carrying out the Fourier transformation without apodization.

Homonuclear Two-Dimensional Experiments. Two-dimensional (2D) phase-sensitive COSY and NOESY spectra were obtained with presaturation of the residual HDO peak and with hypercomplex phase cycling. The data were acquired with 32–128 scans and a 90° pulse of 7-μs duration in 512 blocks, 1024 data points per block. The spectral window was 5000 Hz. The data were typically processed with GE Omega software using a phase-shifted sine squared function in the evolution dimension and exponential apodization in the acquisition dimension. The data were zero-filled twice to give a final data set of 1K × 1K.

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Table I. Proton Assignments[a]

	UpU	s^2UpU	Ups^{2}U	Ups^{2}mo^{5}U	Upmo^{5}U	mo^{5}UpU	Ups^{2}mnmU[b]	s^2mnm-UpU	Ups^{2}mc-mo^{5}U	cmnm^{5}UpU	Ups^{2}cm-nm^{5}U
						5′ Nucleoside					
H5	5.85	6.10	5.82	5.84	5.87		5.82		5.85		5.85
H6	7.89	8.21	7.92	7.90	7.89	7.64	7.84	8.54	7.72	8.18	7.88
H1′	5.87	6.31	5.82	5.86	5.88	5.90	5.86	6.23	5.87	5.87	5.88
H2′	4.43	4.56	4.78	4.45	4.43	4.46	4.46	4.58	4.45	4.44	4.49
H3′	4.53	4.45	4.51	4.56	4.57	4.57	4.56	4.46	4.59	4.55	4.59
H4′	4.30	4.36	4.32	4.30	4.31	4.33	4.29	4.36	4.31	4.32	4.31
H5′, 5″	3.81, 3.91	3.89, 4.03	3.83, 3.95	3.81, 3.91	3.81, 3.90	3.86, 3.95	3.79, 3.89	3.92, 4.08	3.82, 3.91	3.84, 3.94	3.82, 3.92
CH_2								3.96		4.01	
CH_3						3.70		2.79		3.62	
						3′ Nucleoside					
H5	5.87	5.79	6.14			5.86		5.74		5.86	
H6	7.90	7.94	8.11	7.47	7.31	7.91	8.25 (m)	7.97	7.89	7.90	8.32
H1′	5.93	5.93	6.59	6.71	5.91	5.89	6.62 (m)	5.94	6.63	5.94	6.56
H2′	4.32	4.27	4.35	4.39	4.38	4.30	4.35 (m)	4.26	4.38	4.32	4.40
H3′	4.32	4.32	4.28	4.31	4.34	4.30	4.26 (m)	4.31	4.28	4.32	4.30
H4′	4.24	4.23	4.28	4.31	4.25	4.24	4.26 (m)	4.22	4.30	4.24	4.30
H5′, 5″	4.10, 4.24	4.10, 4.34	4.15, 4.34	4.19, 4.33	4.15, 4.24	4.11, 4.29	4.14, 4.34 (m)	4.08, 4.36	4.19, 4.29	4.11, 4.25	4.17, 4.39
CH_2							3.97 (m)		4.71		4.09
CH_3				3.82	3.76		2.69 (m)		3.80		3.63

[a] Proton chemical shifts are given in ppm from DSS as the internal reference at 0 ppm. [b] The designation "m" refers to the modified uridine, corresponding to the labels in the figures.

Heteronuclear Two-Dimensional Experiments. Two-dimensional reverse-detected heteronuclear shift correlation (HMQC) spectra with hypercomplex phase cycling were collected using the reverse polarization transfer probe. The data were optimized for a one-bond scalar coupling constant of 160 Hz. The experiment was carried out with a proton observation pulse of 10 μs; carbon modulation was carried out with a 90° decoupling pulse of 35 μs. The spectral window in the proton dimension (f2) was 3000 Hz; in the carbon dimension (f1) the spectral window was 21 000 Hz. Five hundred twelve blocks of 64 acquisitions each were collected over 1024 data points. The data were processed with a phase-shifted sine squared function in both dimensions and zero-filled to a 1K × 1K square matrix.

Two-dimensional reverse-detected multiple-bond heteronuclear shift correlation (HMBC) was carried out in the magnitude calculation mode. The dedicated reverse polarization transfer probe was used. The 90° pulses on the proton-observation and carbon-decoupling channels were 10 and 35 μs, respectively. The experiment was set for a one-bond scalar coupling interaction of 160 Hz and a multiple-bond interaction of 7 Hz. No ^{13}C decoupling was applied during acquisition. The spectral window in the proton dimension was the same as in the HMQC experiments; in the carbon dimension, a larger window, 25 000 Hz, was used in order to include quaternary carbons. Two hundred fifty-six blocks of 256 acquisitions each were collected over 1024 data points. The data were processed with a phase-shifted sine squared function in both dimensions and zero-filled to a square matrix.

Coupling Constants. The determination of ^1H–^1H scalar coupling constants was aided by spectral simulation using the PMR program (Serena Software).

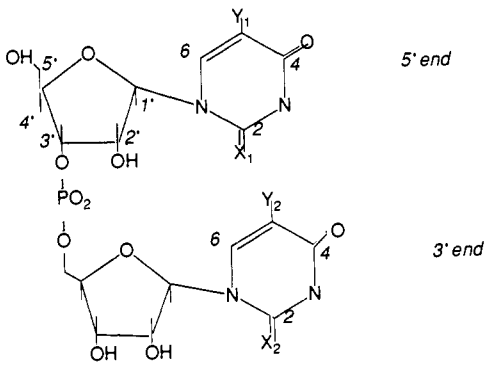

Figure 2. Diagram of modified uridine dinucleoside monophosphates. The abbreviations given correspond to those used in the tables. UpU: X_1 = O, Y_1 = H, X_2 = O, Y_2 = H. Ups^{2}U: X_1 = O, Y_1 = H, X_2 = S, Y_2 = H. s^2UpU: X_1 = S, Y_1 = H, X_2 = O, Y_2 = H. Upmo^{5}U: X_1 = O, Y_1 = H, X_2 = O, Y_2 = OCH_3. Ups^{2}mo^{5}U: X_1 = O, Y_1 = H, X_2 = S, Y_2 = OCH_3. mo^{5}UpU; X_1 = O, Y_1 = OCH_3, X_2 = O, Y_2 = H. Ups^{2}mnm^{5}U: X_1 = O, Y_1 = H, X_2 = S, Y_2 = CH_2NHCH_3. s^2mnm^{5}UpU: X_1 = S, Y_1 = CH_2NHCH_3, X_2 = O, Y_2 = H. Upmcmo^{5}U: X_1 = O, Y_1 = H, X_2 = O, Y_2 = $OCH_2CO_2CH_3$. Ups^{2}mcmo^{5}U: X_1 = O, Y_1 = H, X_2 = S, Y_2 = $OCH_2CO_2CH_3$. cmnm^{5}UpU: X_1 = O, Y_1 = $CH_2NHCH_2CO_2H$, X_2 = O, Y_2 = H. Ups^{2}cmnm^{5}U: X_1 = O, Y_1 = H, X_2 = S, Y_2 = $CH_2NHCH_2CO_2H$.

Results

Ten uridine dinucleoside monophosphates were chemically synthesized in order to determine the influence of the naturally occurring 2-thio group and various C(5) derivatives on their respective structures and conformations and those of each oligomer. Each dimer studied was synthesized with a single modified uridine and an unmodified uridine: U*pU or UpU*. A diagrammatic representation of the chemical structures is shown in Figure 2. Structural comparisons of the 10 dinucleoside monophosphates with UpU and with each other were based on the values of several NMR spectral parameters, including proton and carbon chemical shifts, scalar coupling constants, and dipolar connectivities.

NMR Signal Assignments. Signals were assigned by analysis of the two-dimensional homonuclear COSY spectra. Figure 3 is the ribose region of the COSY spectrum of Ups^{2}mnm^{5}U in D_2O. The one-dimensional ^1H spectrum is shown below the two-dimensional map, and the spectrum is labeled with the assignments. The ^1H signal assignments for Ups^{2}mnm^{5}U and all of the other dinucleoside monophosphates are given in Table I. Proton signals of the 3′ terminal modified nucleoside, s^2mnm^{5}U, are labeled with an "m" in the figure and in Table I to designate the dinucleotide used here as an example for determining the NMR signal assignments.

Previously reported chemical shifts for uridine, s^2mnm^{5}U, and the other mononucleosides[6] aided in the resonance assignments for Ups^{2}mnm^{5}U and the other 10 dinucleoside monophosphates. The two peaks furthest downfield are assigned to the olefinic C(6) protons, deshielded by the adjacent heteroatom, on the dimer's two uridines. The COSY cross-peak from ^1H(6) of the unmodified uridine locates the ^1H(5) signal. Of all the protons in the ribose rings, the anomeric proton, ^1H(1′), is shifted downfield since it is on a carbon directly bonded to two heteroatoms. The COSY cross-peaks from ^1H(1′) resonances identify the nearest neighbors, ^1H(2′).

Assignments of the remaining ribose protons are made using the correlations detailed in the expanded portion of the COSY spectrum (Figure 3). For the ribose ring of the unmodified uridine in this example, it is easy to trace the scalar coupling pattern around the ring beginning with ^1H(2′), the assignment of which was identified above, and continuing to ^1H(5′) and ^1H(5″). In cases in which poor resolution in the ribose region interfered with

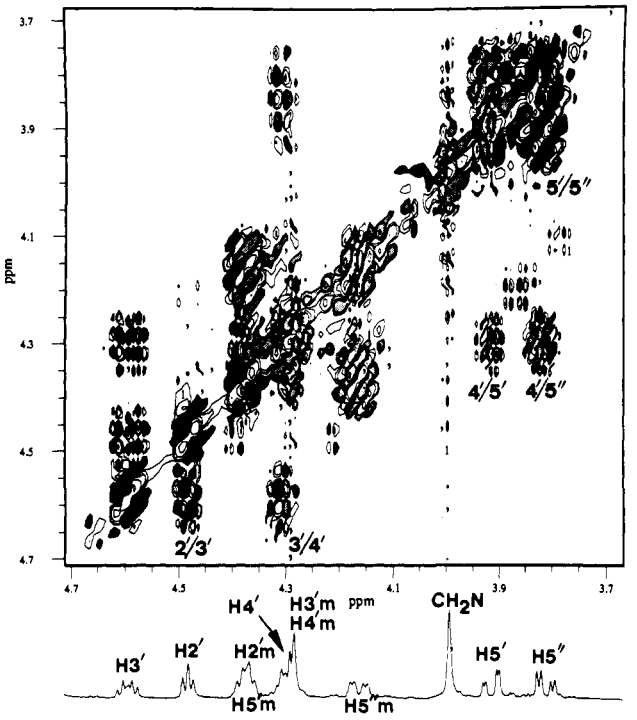

Figure 3. Homonuclear J-coupled (COSY) spectrum of Ups^{2}mnm^{5}U in D_2O. This figure depicts the expanded area for the ribose proton signals of a phase-sensitive COSY spectrum. The single-dimension proton spectrum is labeled below the COSY spectrum contour plot. The spectrum was collected and processed as described in the Experimental Section. The cross-peak assignments are discussed in Results.

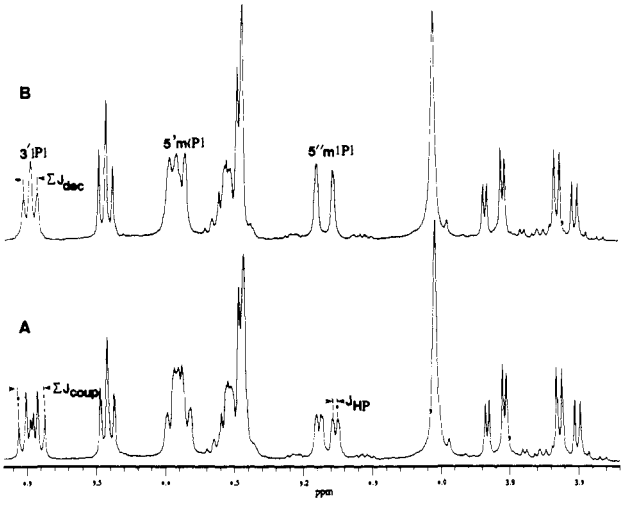

Figure 4. Phosphorus-coupled (A) and -decoupled spectra (B) of Ups^{2}mnm^{5}U. The H(5′)–P and H(5″)–P couplings are noted for the modified nucleoside.

signal assignments, the heteronuclear correlation experiment was helpful in making the assignments (vide infra). The methyl on the 5-position of s^{2}mnm^{5}U is easily assigned to the singlet at 2.69 ppm because of the singlet's intensity and its upfield shift, characteristic of amino methyls.[22] The methylene is assigned here on the basis of chemical shift and intensity.

The phosphate-bound protons can be assigned using broad-band decoupling at the ^{31}P frequency.[23,24] Figure 4 shows the region of the ^{1}H spectrum of Ups^{2}mnm^{5}U where the ribose protons

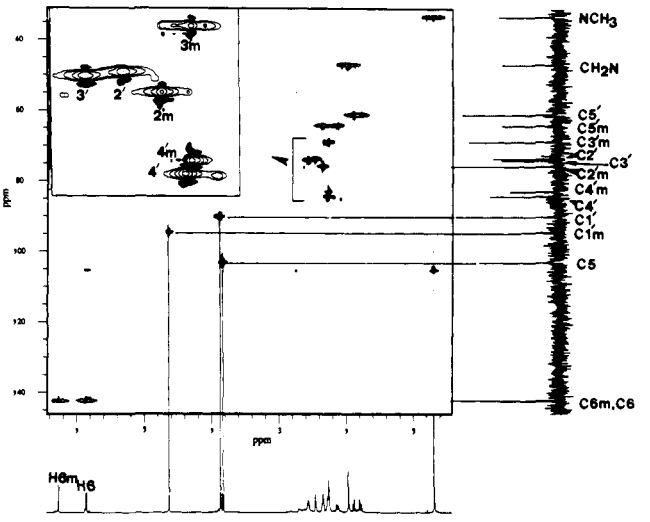

Figure 5. Heteronuclear ^{1}H–^{13}C one-bond (HMQC) correlation spectrum of Ups^{2}mnm^{5}U. The single-dimension ^{1}H and ^{13}C spectra are shown at the bottom and right of the figure, respectively. The assigned carbon signals are noted, and those of the modified nucleoside are designated "m".

resonate. Spectra A and B of the figure were collected identically, except that in spectrum B broad-band phosphorus decoupling was applied. The collapse of the sextet at 4.6 ppm to a triplet clearly identifies the 3′ proton on the unmodified uridine unit. Similarly, the doublet of doublets at 4.15 ppm collapses to a doublet, identifying one of the 5′m protons on the modified uridine. The ^{1}H(5″m) signal is coresonant with the signal previously assigned to ^{1}H(2′m), but with ^{31}P decoupling the collapse of the multiplet pattern is easily seen, thus aiding in the identification of the signal. Since this dinucleoside monophosphate was synthesized with the modified uridine at the 3′ end of the dimer, this information allows assignment of the spin networks, identified with the analysis of the COSY spectrum, to their proper ribose rings. The phosphorus-decoupling experiment is also useful in estimating ^{1}H–^{31}P (three-bond) coupling constants as described below.

A heteronuclear correlation experiment was used to complete the proton assignments of ^{1}H(4′m), ^{1}H(5′m), and ^{1}H(5″m), strengthen the previously made assignments, and assign the ^{13}C spectrum. The two-dimensional HMQC spectrum of Ups^{2}mnm^{5}U is shown in Figure 5 with the one-dimensional proton and carbon spectra plotted along the horizontal and vertical axes, respectively. This figure also illustrates the additional resolution that is realized by modulation in the carbon frequency. The ^{13}C chemical shifts of ^{13}C(6m) and ^{13}C(6) are almost identical, but since the ^{1}H(6) and ^{1}H(6m) peaks are well separated, the cross-peaks are well resolved and the ^{13}C chemical shifts can be obtained from the ^{13}C (or Y) coordinate of the cross-peaks. In the ribose region, the incompletely resolved multiplet at 4.3 ppm (^{1}H) is spread in the carbon dimension and can be seen to be composed of three protons (inset in Figure 5). The chemical shifts of the correlated carbon resonances also provide a useful check of the proton assignments because carbon chemical shifts more reliably reflect the electronic environment.[25] For example, the ^{13}C(4′) chemical shifts are always very similar, while the phosphate-bound ^{13}C(5′) and ^{13}C(3′) signals are shifted downfield of the free ^{13}C(3′) and ^{13}C(5′) resonances.[26] Carbon chemical shifts of the C(2′) and C(3′) of DNA nucleosides studied by solid-state NMR have been shown to be predictive of sugar pucker.[27] We do not find that to be the case with the uridine dinucleosides.

This completes the assignment of all protons and all proton-bound carbons. To assign the five quaternary carbons, we performed a multiple-bond heteronuclear correlation experiment,

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Table II. Carbon Assignments[a]

	UpU	s^2UpU	Ups^2U	Ups^2mo^5U	$Upmo^5U$	mo^5UpU	Ups^2mnm^5U[b]	s^2mnm^5 UpU	Ups^2mcmo^5U	$cmnm^5$ UpU	Ups^2cmnm^5U
						5′ Nucleoside					
C2	151.41	175.85	151.19	151.36	151.39	149.55	152.14	175.53	151.32	150.93	152.75
C4	165.85	162.25	165.54	165.86	165.92	161.06	166.72	162.65	165.81	164.47	167.26
C5	102.12	106.13	101.65	101.99	102.03	136.23	102.43	108.43	102.23	104.86	103.34
C6	141.39	141.35	141.08	141.28	141.24	118.54	141.70	141.95	141.49	142.48	142.84
C1′	89.10	93.95	89.45	89.15	88.96	88.95	89.40	94.15	89.33	89.20	90.52
C2′	73.36	73.16	72.60	72.71	72.72	72.69	72.87	73.87	72.79	73.07	73.94
C3′	73.36	71.05	72.06	72.71	72.88	72.19	73.29	70.34	73.03	72.69	74.17
C4′	84.24	82.75	82.78	83.10	83.21	82.66	83.62	82.53	82.56	83.76	84.54
C5′	60.15	58.87	59.59	60.01	60.08	59.20	60.42	58.19	60.43	59.86	61.34
CH_2								45.15		43.35	
CH_3						56.57		32.15		48.40	
CO										170.90	
						3′ Nucleoside					
C2	151.46	151.45	175.70	171.51	150.08	151.15	178.77 (m)	151.22	172.08	151.31	177.51
C4	165.92	165.45	162.35	159.42	161.29	165.52	167.62 (m)	165.36	158.31	165.64	163.71
C5	102.32	101.82	106.18	140.84	136.75	101.81	109.22 (m)	101.36	138.28	102.03	110.60
C6	141.24	141.00	140.98	119.54	119.42	140.91	141.70 (m)	141.10	124.27	141.13	143.33
C1′	88.71	89.03	93.14	93.37	88.54	88.80	93.86 (m)	88.99	93.45	88.81	94.79
C2′	74.14	73.97	74.20	74.37	73.16	73.42	75.08 (m)	72.96	74.57	73.85	75.78
C3′	69.28	68.58	67.65	68.41	69.49	68.64	68.09 (m)	68.33	68.85	69.19	69.01
C4′	83.47	81.93	81.75	82.37	82.84	82.07	82.19 (m)	81.81	83.07	82.98	83.38
C5′	64.42	63.61	63.02	63.83	64.72	63.88	63.50 (m)	63.39	64.14	64.53	64.44
CH_2							46.34 (m)		67.15		44.94
CH_3				57.15	57.52		32.58 (m)		52.71		50.10
CO									170.33		172.32

[a] Carbon chemical shifts are given in ppm from DSS as the internal reference at 0 ppm. [b] The designation "m" refers to the modified uridine, corresponding to the labels in the figures.

HMBC. The two-dimensional HMBC spectrum of Ups^2mnm^5U is shown in Figure 6 with the ^1H and ^{13}C one-dimensional spectra plotted along the horizontal and vertical axes, respectively. In this experiment, correlations can be observed between a proton and a carbon three or four bonds away. The sensitivity of the HMBC experiment is such that correlation to a quaternary carbon will be detected, even though in a proton-decoupled ^{13}C spectrum acquired over several hours the same carbon will not be seen. Each proton at position-6 of the dimer's two uracils exhibited three-bond correlations to its respective carbonyl carbons, $^{13}C(2)$ and $^{13}C(4)$. The pertinent cross-peaks are labeled in the figure. Small correlations from $^1H(1')$ to $^{13}C(2)$ were detected [circled resonances at 5.90 and 6.62 ppm (^1H) in Figure 6], and these too aided in the identification of the quaternary carbons. The assignment of these carbonyl signals to their specific positions is based on the literature values for unmodified uridine[22] and on the fact that thiolation shifts the 2-carbon resonance downfield.[6] The substituted $^{13}C(5m)$ is identified by its strong correlation to the methylene protons at the first position of the substituent chain. In addition, strong correlations can be seen across the amine from the methylene to the methyl [circled resonances at 2.69 and 4.00 ppm (^1H) in Figure 6].

Effects of Modification on Chemical Shift. The ^1H and ^{13}C chemical shifts for the 11 uridine dinucleoside monophosphates are listed in Tables I and II, respectively. The assignments for the corresponding monomers have been published previously.[6] With the exceptions of the phosphorus-bound positions, the ^1H chemical shift patterns for the monomers and dimers are quite similar. The proton chemical shifts in the dimers differ from those in the monomers by less than 0.2 ppm for all positions except $^1H(3')$, $^1H(4')$, and $^1H(5')$, in which cases the phosphate group causes a downfield shift of 0.3–0.4 ppm. The similarity of dimer to monomer in proton chemical shifts suggests a lack of stacking interaction in the dimers.

Proton chemical shifts of the modified uridines of the dimers are most strongly influenced by 2-thiolation, as had been previously reported for the mononucleosides.[6] The effect of thiolation can be seen by comparing the chemical shifts of the 5′ nucleosides of UpU and s^2UpU, or those of the 3′ nucleosides of UpU and Ups^2U, in Table I. The modified uridines exhibit downfield shifts of up to 0.6 ppm with respect to the unmodified uridines. The changes in chemical shift are localized, with the largest effect occurring at the ribose $^1H(1')$. Chemical shift changes of $^1H(5)$ and $^1H(6)$ are also evident. The influence of thiolation is sequence dependent, being about 50% bigger when the 3′ terminal uridine is thiolated. For instance, $^1H(1')$ $\delta = 0.66$ ppm for Ups^2U vs UpU, whereas $\delta = 0.44$ ppm for s^2UpU vs UpU.

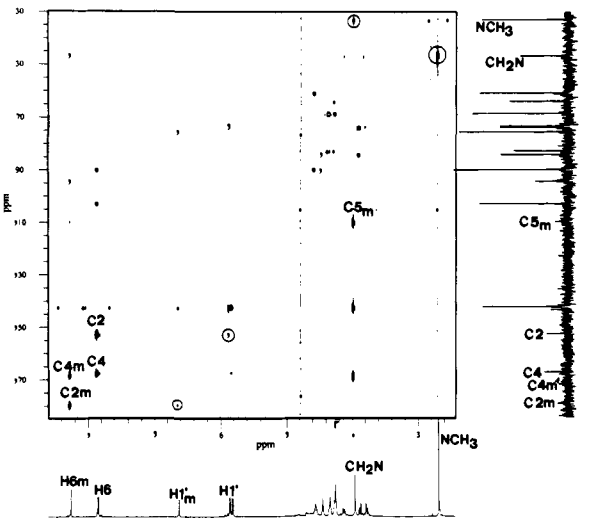

Figure 6. Heteronuclear ^1H–^{13}C multiple-bond (HMBC) correlation spectrum of Ups^2mnm^5U. The single-dimension ^1H and ^{13}C spectra are shown at the bottom and right of the figure, respectively. Both are labeled with assignments, and those of the modified nucleoside are designated "m".

Thiolation obviously affected proton chemical shifts; however, the 5-substitution, regardless of polarity or chain length, had, with one exception, the $^1H(6)$, little effect on the proton chemical shifts. As might be expected, the signal of the $^1H(6)$ proton, adjacent to the substitution, may be shifted upfield or downfield depending on the polarity of the 5-substitution.[28]

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Table III. ^1H–^1H Coupling Constants[a]

	UpU	s^2UpU	Ups^2U	Ups^2mo^5U	$Upmo^5U$	mo^5UpU	Ups^2mnm^5U	s^2mnm^5UpU	Ups^2mcmo^5U	$cmnm^5UpU$	Ups^2cmnm^5U
						5′ Nucleoside					
$J_{1'2'}$	4.4	1.6	3.6	4.6	5.0	3.0	4.7	0.7	4.4	4.0	4.7
$J_{2'3'}$	4.9	4.9	5.0	5.3	4.9	4.0	5.1	4.9	4.6	4.6	5.1
$J_{3'4'}$	5.3	7.4	6.0	5.5	5.0	4.6	5.0	7.5	5.2	5.2	5.1
$J_{3'P}$	7.8	8.0	8.2	8.0	8.4	7.0	8.2	8.2	8.4	7.8	8.3
$J_{4'5'}$	2.7	2.3	2.6	2.6	2.8	2.5	2.8	2.0	3.9	3.5	2.7
$J_{4'5''}$	3.9	3.2	4.0	3.8	3.9	2.5	1.4	2.4	2.6	2.5	3.2
$J_{5'5''}$	12.9	13.4	13.0	12.9	13.0	13.0	12.9	13.4	13.0	13.0	12.7
						3′ Nucleoside					
$J_{1'2}$	3.7	3.5	2.4	3.1	5.1	3.6	2.2	3.0	3.0	3.8	1.5
$J_{2'3'}$	2.0	2.4	4.2	3.8	5.3	4.6	2.6	5.0	3.8	4.0	2.2
$J_{3'4'}$	1.0	3.5	4.0	1.9	4.4	6.2	3.5	6.0	4.2	5.0	1.5
$J_{4'5'}$	2.1	2.4	1.4	2.2	2.8	2.5	1.5	2.7	2.4	3.6	1.2
$J_{4'5''}$	3.9	2.7	1.2	1.9	2.2	2.9	1.2	2.4	2.4	2.4	1.0
$J_{5'5''}$	12.0	11.9	12.0	11.9	11.6	11.4	12.6	11.8	12.6	11.7	11.9
$J_{5'P}$	4.0	2.4	3.6	5.3	3.9	4.4	3.6	2.7	5.6	4.4	4.9
$J_{5''P}$	5.0	2.4	4.2	4.1	5.1	4.4	4.0	2.4	5.6	4.4	4.0

[a] Coupling constants are given as absolute values in hertz.

Table IV. ^{13}C–^{31}P Coupling Constants[a]

	UpU	s^2UpU	Ups^2U	Ups^2mo^5U	$Upmo^5U$	mo^5UpU	Ups^2mnm^5U	s^2mnm^5UpU	Ups^2mcmo^5U	$cmnm^5UpU$	Ups^2cmnm^5U
						5′ Nucleoside					
$J_{C4'P3'}$	4.6	6.8	4.7	5.0	4.0	4.6	4.6	7.5	4.6	4.6	4.8
$J_{C3'P3'}$	4.6	5.1	4.7	5.0	4.3	4.6	4.6	4.5	4.6	4.6	4.8
$J_{C2'P3'}$	3.0	<2.0	3.1	3.7	4.0	3.1	3.0	<2.0	4.6	4.6	3.2
						3′ Nucleoside					
$J_{C5'P5'}$	6.1	5.1	6.3	5.0	4.0	6.1	4.6	4.5	6.1	6.1	4.8
$J_{C4'P5'}$	9.2	10.2	9.4	8.7	9.3	9.2	7.6	9.1	9.2	9.1	9.6

[a] Coupling constants are given in hertz.

Carbon chemical shifts of the dimers differ from those of the corresponding monomers noticeably in all the modified uridines. Some of the difference is due to the presence of the phosphate group, whose influence may be propagated through the carbon skeleton. However, thiolation causes a large downfield shift (20–30 ppm) of the $^{13}C(2)$ resonance with respect to the nonthiolated analogues in both the monomeric[6] and dimeric nucleotides. Consider, for example, UpU and s^2UpU vs Ups^2U, $Upmo^5U$ vs Ups^2mo^5U, or s^2mnm^5UpU vs Ups^2mnm^5U (Table II). Thiolation of the 3′ terminal uridine of the dimer produced a slightly larger downfield shift of C(2) (~27 ppm) than thiolation of the uridine at the 5′ terminus of the dimer (~20 ppm). Thiolation also caused a small downfield shift of the anomeric carbon resonance [for $^{13}C(1')$, $\delta \sim 4$ ppm]. The remaining positions in the ribose ring remain insensitive to thiolation and 5-substitution.

The role of the 5-substituent appears to be related to its electronegativity. In dimers in which the 5-substituent includes an oxygen directly bound to the base (i.e., $Upmo^5U$, mo^5UpU, Ups^2mo^5U, and Ups^2mcmo^5U), there is a substantial local effect on carbon shifts. When these dimers are compared with the appropriate unsubstituted dimer, there is an ~30 ppm downfield shift in the resonance for $^{13}C(5)$ and an ~20 ppm upfield shift for $^{13}C(6)$. This effect is seen regardless of whether the substitution occurs on the 5′ terminal nucleotide or the 3′ terminal nucleotide (e.g., mo^5UpU vs UpU, or Ups^2mo^5U vs Ups^2U). In contrast to the large effect of electronegative atom derivatization at C(5), the two substitutions which involved an alkyl carbon directly bound to the base (i.e., Ups^2mnm^5U and s^2mnm^5UpU, or $cmnm^5UpU$ and Ups^2cmnm^5U) affected the $^{13}C(5)$ and $^{13}C(6)$ chemical shifts by less than 10 ppm as compared to the unsubstituted uridine dimer. Other positions were essentially unaffected by the 5-substitution. Similar trends were also observed in the monomers.[6]

Coupling Constants. Scalar ^1H–^1H, ^1H–^{31}P, and ^{13}C–^{31}P coupling constants were determined from the appropriate spectra and are given in Tables III and IV. In most cases preliminary values for ^1H–^1H coupling constants in the ribose ring could be determined from the multiplet splittings of the one-dimensional proton spectrum. In particular, the proton coupling constants $J_{1'2'}$, $J_{4'5'}$, $J_{4'5''}$, and $J_{5'5''}$ could almost always be read directly from the spectrum. As mentioned above, ^1H–^{31}P coupling constants were estimated using the results from broad-band phosphorus decoupling. The difference in hertz between the outer components of a multiplet represents the sum of its coupling interactions, as long as the multiplet is sufficiently removed from its coupling partners.[29] Then the difference between $\sum J$ for the 3′ and 5′ protons in parts A and B of Figure 4 ($\sum J = \sum J_{coup} - \sum J_{dec}$) yields an estimate of the phosphorus coupling. This value of $\sum J$ can be used as a preliminary value in carrying out the spectral simulation.

The various scalar couplings determined from one-dimensional spectra were used as the data for generating an initial spectral simulation with the PMR program (Serena Software). These data were then adjusted in order to match the simulated spectrum to the experimental one. The $J_{^1H^1H}$ values obtained from this process are given in Table III. Specific scalar couplings seem to be effected by thiolation, whereas no particular effects on coupling constants were attributable solely to the 5-position modifications. The $J_{1'2'}$ coupling constant for the thiolated nucleoside, no matter whether at the 5′ or 3′ end of the dimer, was always lower than that of the nonthiolated uridine. However, this was particularly evident for the two thiolated uridines located at the 5′ end of the dimer, i.e., s^2UpU and s^2mnm^5UpU. The same two thiolated uridine dinucleosides exhibited reduced $^1H(5')$–^{31}P and $^1H(5'')$–^{31}P coupling constants relative to the nonthiolated dimer, and the corresponding dimer in which the thio group was on the 3′ terminal uridine. Other effects on proton coupling constants attributable to modification may be noticed. Thiolation at the 5′ nucleoside and any modification at the 3′ nucleoside increased $J_{H(3')H(4')}$. Modification decreased $J_{H(4')H(5')}$ and $J_{H(4')H(5'')}$ on the 3′ nucleoside; thiolation had a stronger effect. While effects of modification can be seen in Table III, it is perhaps more instructive to consider the parameters describing ribose conformation as calculated from coupling constants (vide infra).

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Table V. Nucleoside Conformation[a]

	UpU	s²UpU	Ups²U	Ups²mo⁵U	Upmo⁵U	mo⁵UpU	Ups²mnm⁵U	s²mnm⁵ UpU	Ups²mc-mo⁵U	cmnm⁵UpU	Ups²cm-nm⁵U
						5′ Nucleoside					
% N	52	98	65	48	42	75	47	100	52	58	47
% γ^+	71	82	71	73	71	87	95	100	72	77	78
% ϵt	59	90	61	65	51	59	59	100	59	59	62
χ	anti	anti	anti	anti	anti	anti > syn	anti > syn	anti	anti	anti > syn	anti
						3′ Nucleoside					
% N	63	67	85	73	40	65	88	75	75	62	100
% γ^+	77	86	100	96	87	83	100	86	89	77	100
% βt(^{1}H)	81	100	86	79	81	82	87	95	70	82	81
% βt(^{13}C)	83	92	84	78	83	83	67	81	83	82	86
χ	anti	anti	anti	anti	anti	anti > syn	anti > syn	anti	anti	anti > syn	anti

[a] % N = $[(7.5 - J_{1'2'})/6] \times 100$.[33] % $\gamma^+ = [(13.75 - J_{4'5'} - J_{4'5''}/10.05] \times 100$.[31,42] % $\epsilon t = [(J_{C4'P3'} - 0.4)/7.1] \times 100$.[34] % $\beta t(^1H) = [(25.5 - J_{5'P} - J_{5''P})/20.5] \times 100$.[34] % $\beta t(^{13}C) = [(J_{C4'P5'} - 0.73)/10.27]$.[24] χ is the predominant conformation around the glycosidic angle based on the relative magnitude of NOE connectivities. H6–H2′ > H6–H1′ = anti, H6–H1′ > H6–H2′ = syn.[30,43]

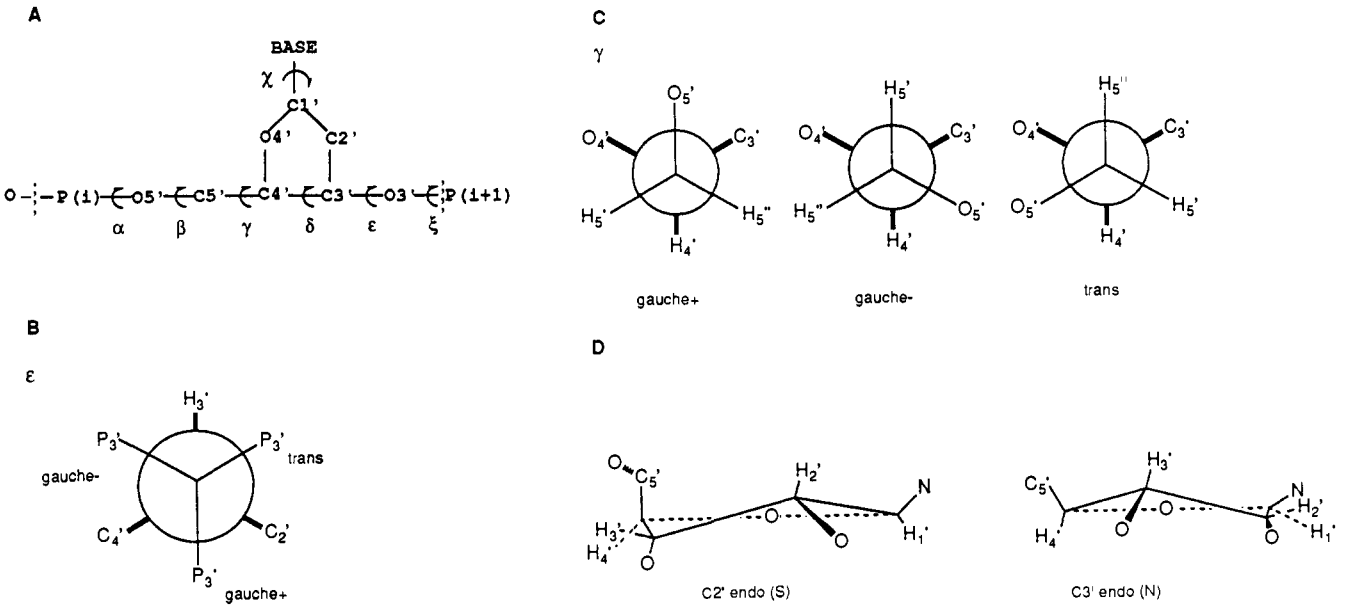

Figure 7. Torsion angles and torsion angle conformations for 5′–3′ ribose. The standard angle designations and the accepted torsion angle conformations are shown.

The ^{13}C–^{31}P coupling constants given in Table IV were determined from proton-decoupled ^{13}C spectra which were processed to increase resolution (see Experimental Section). The coupling constants were then obtained directly from the spectra. These values were also used to determine ribose conformation. The methoxy derivatization of the 3′ nucleoside (Upmo⁵U and Ups²mo⁵U) decreased $J_{C(5')P(5')}$. Otherwise, thiolation was the most consistent effector of carbon coupling constants, as it was with the proton coupling constants. The $J_{C(2')P(3')}$ values of both s²UpU and s²mnm⁵UpU were found to be significantly smaller than those of the control UpU and any of the other dinucleoside monophosphates (Table IV). The same two thiolated dimers demonstrated that thiolation of the 5′ terminal nucleoside affected the carbon–phosphorus couplings of the adjacent nucleoside. The $J_{C(5')P(5')}$ of the two dimers was reduced relative to that of the nonthiolated dimer. Thus, the angles of the phosphodiester backbone of s²UpU and Ups²U are probably different from those of the other nine dinucleotides.

Nucleotide Conformation. The conformations of the individual nucleosides within each dimer are shown in Table V. The conformation around the glycosidic bond was determined from 2D NOESY experiments. The NOE observed from the H(6) proton to the ribose ring can be used as a marker to decide whether the predominant conformation is syn or anti.[30] Thus when the dipolar correlation from H(6) to H(2′) is stronger than that from H(6) to H(1′), the anti conformation is indicated. When the relative intensity of the correlations is reversed, the syn conformation is indicated. Those cases in which both cross-peaks were observed, indicating the presence of both syn and anti conformations, are noted in Table V. In all cases, dipolar correlation from H(6) to H(2′) indicated a predominance of the anti conformation. In a few cases, a less intense correlation from H(6) to H(1′) indicated the presence of an observable percentage of syn conformer. Puckering of the ribose ring was characterized by the torsion angles identified in Figure 7.[31] Also shown in the figure are the conformations corresponding to the nomenclature used in Table V.

Relating experimental coupling constants to the torsion angles requires Karplus-like equations in which the constants are obtained by mathematical regression using experimental data from a large number of related compounds.[32] The fractional population of nucleoside in the 3′ endo conformation can be calculated from $J_{H(1')H(2')}$.[33] Since the 1′ protons are usually well-resolved doublets, the scalar coupling is easily and accurately obtained. The values calculated for UpU indicated that comparable amounts of the 3′ endo and 2′ endo conformers are found in unmodified uridine (average % N = 51). However, thiolation causes a substantial increase in the percentage of 3′ endo conformer in the population to as high as 100%. Interestingly, the size of this effect is *sequence*

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(33) Sierzputowska-Gracz, H.; Guenther, R. H.; Agris, P. F.; Folkman, W.; Golankiewicz, B. *Magn. Reson. Chem.* **1991**, *29*, 885–892.

dependent. With the 2-thio modification at the 5′ end of the dimer in s^2UpU and s^2mnm^{5}UpU, the riboses of the thiolated uridines are almost entirely 3′ endo (% N ~ 100%), whereas in Ups^{2}U, Ups^{2}mo^{5}U, Ups^{2}mnm^{5}U, and Ups^{2}mcmo^{5}U, the average value for % N in the modified 3′ nucleoside, though increased relative to UpU, is 80%. The 5-substitution alone, regardless of length or electronegativity, does not cause any large effect on the percentage of the 3′ endo conformer, independent of the position in the dinucleoside. These results echo those seen for the monomers, but in addition support the possibility of a position effect exhibited by thiolated uridines on 3′-adjacent nucleosides.[9]

Modification appears to affect the conformation around the C(5′)–C(4′) bond of the modified nucleoside; the fractional population of γ^+ conformer increased for all modified nucleosides, regardless of the modified nucleoside's position in the dimer, relative to that of the unmodified uridine. However, only for those dimers in which the 5′ nucleoside was thiolated (s^2UpU and s^2mnm^{5}UpU) was there an increase of γ^+ conformer for the 3′ nucleoside as well. Thiolation of the 5′ end nucleotide caused a substantial increase in the percent of the gauche$^+$ configuration relative to the other possible configurations. Another notable effect of thiolation on the 5′ nucleoside was a significant increase in % ϵ^+, which describes the conformers around the C(3′)–OP bond.[34]

Discussion

Thiolation of the 2-position of uridine has been shown to be the dominating effector of mononucleoside conformation for those 2-thio-5-modified uridines occurring at wobble position-34 in native tRNAs.[6] The aliphatic or electronegative character of the 5-position modification had little or no effect on the mononucleoside conformation. If the physical structure imposed by thiolation of the 2-position of the wobble uridine could so dramatically restrict this nucleoside's conformation and dynamics, perhaps it could also influence those of the 5′ and/or 3′ adjacent nucleosides and, thus, contribute to the structure and dynamics of the pyrimidine-rich tRNA anticodon loops in which it is found.[9] Here we have presented a comparison of the NMR spectral analyses of 11 uridine dinucleoside monophosphates. The results demonstrate that thiolation of uridine position-2 is the predominant influence on the thiolated nucleoside's conformation, even in the presence of a phosphodiester bond and an adjacent nucleoside. The fractional population of 3′ endo uridine in the dimers is increased by thiolation from a low of 52% in UpU to a high of 100% in s^2mnm^{5}UpU. The thiolated nucleosides in these dimers preferred almost exclusively (~90%) the 3′ endo-gauche$^+$-anti conformation, as did the thiolated uridine mononucleosides. Neither the character of the 5-position modification nor the 5′ or 3′ adjacent unmodified uridines constrained the uridine conformation in the dimer as much as did thiolation. Recently, the 2′-O-methylation of uridine has been shown to increase the fractional population of 3′ endo conformers, though the stabilization, from 52% 3′ endo for UpU to 62% for UmpU, is not nearly as dramatic as it is with thiolation.[35] In general the presence of the phosphodiester bond does affect ribose conformation, but contrary to the suggestion that certain 5-position modifications would H-bond with the 3′ or 5′ phosphate and thereby stabilize a ribose pucker,[36] no specific effect on ribose conformation could be correlated to a particular 5-position modification. However, there was some evidence of side-chain interaction which apparently was affected by the orientation of the side chain in the dimer.

In several modified dinucleosides with long side chains at the 5-position, the methylene in the side chain was split into a quartet with $J_{^1H^1H}$ = ~14 Hz. It is interesting that, in two of the dinucleosides, Ups^{2}mnm^{5}U and Ups^{2}cmnm^{5}U, the methylene was either a singlet or a partially collapsed quartet, while in dinucleosides with the same side-chain substituent on the other unit in the dimer, the methylene was a clear quartet. The magnitude of the scalar coupling decreased with increasing temperature. Splitting of methylene resonances comes about due to methylene's being adjacent to a chiral center, as in the case of the 5′ methylene protons on the ribose, or to unequally populated rotational conformers.[37] In an earlier paper on queuine, which has an NH_2CH_2 cyclopentyl substituent,[38] the methylene protons are split into a quartet with $J_{^1H^1H}$ = 14 Hz in the proton spectrum. The crystal structure of queuine[39] shows a hydrogen bond between the NH_2^+ on the side chain and the carbonyl oxygen on position-6 of the 7-deazaguanine ring. More importantly in the crystal structure of s^2mnm^{5}U·2H_2O, a hydrogen bond exists between the NH_2 on the 5-position chain, through a water molecule, to the C(4) carbonyl oxygen.[44] Presumably the presence of the hydrogen bond changes the population distribution of the rotational conformers, thereby changing the average chemical shifts of the two methylene protons and enabling scalar coupling between them. This observation is being investigated.

The strong influence of 2-thiolation on the conformational preference of the thiolated uridines within the dimers was transferred in a unidirectional manner, 5′ to 3′, to the dimer's phosphodiester bond and adjacent unmodified uridine. Thiolation of the 5′ nucleoside within the dimer affected the phosphodiester bond and carbon backbone torsion angles resulting in an ϵt, gauche$^+$, βt(^{1}H) conformation, whereas thiolation of the 3′ uridine did not generate a similar structural preference. In comparison to the strong influence of thiolation resulting in what is almost a 100% stabilization of the uridine 3′ endo conformation, the weaker effect reported as 62% 3′ endo for the 2′-O-methylation of uridine[35] does not seem large enough to influence backbone torsion angles.

The chemically synthesized dinucleosides are models for the naturally occurring s^2U and its 5-position-modified derivatives. These nucleosides, found in wobble position-34 of pyrimidine-rich anticodon loops, are 3′-adjacent to the invariant U-33 and 5′-adjacent to the second anticodon position, U-35. Transfer RNAs with wobble position thiolated uridines do not wobble to the degenerate codons for the same amino acid;[9] they select the codon ending in A from a codon family. For example, for tRNA^{Glu}, GAA is recognized preferentially to GAG.[7] The "modified wobble hypothesis",[9] in explaining codon preference in contrast to wobble, suggests that modification of the wobble position nucleoside could physically constrain anticodon conformation and thus, by permitting only certain H bonds to mRNA, direct codon selection during protein synthesis. The "modified wobble hypothesis" is supported by the fact that s^2U and its 5-position modifications not only maintain their strong structural identity within the dimers studied but, when located at the dimer's 5′ terminus, they also constrain the phosphodiester backbone. Thus, the structural constraints imposed by thiolation are transferred only in the 3′ direction toward the second anticodon nucleotide and not toward the 5′-adjacent, invariant U-33.

Transfer RNA wobble position recognition of the third base of the codon is dependent on the wobble nucleoside's base-pairing ability, which is conferred by that nucleoside's conformation and dynamics. Thus, codon recognition by tRNAs containing a wobble

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position s^2U has been found to be a triplet rather than a two-base recognition,[40] and the third base discrimination is conferred primarily by thiolation.[9] Studies in vivo and in vitro[9] have shown that tRNAs with anticodon wobble position thiolated uridine-34 will select codons ending in adenosine and exhibit only a minimal amount of wobble to guanine. Wobble position-34 uridines that are modified only at C(5) recognize G just as well as A (Yokoyama, S., personal communication).

Conformational constraints imposed on particular tRNA anticodons through position-34 modifications would facilitate correct codon recognition. This is particularly important for the pyrimidine-rich anticodons of tRNAs for glutamine, glutamic acid, and lysine. It is just these tRNAs in which the s^2U derivatives are found.[1-4] The anticodon loops of glutamic acid and lysine tRNAs are composed of $U_{33}PyPyPy$, tetrapyrimidine stretches, and those of glutamine tRNAs are composed of $U_{33}PyPyG/A$, tripyrimidine stretches. In contrast to purine-rich anticodons, such as that of $tRNA^{Phe}$,[41] the pyrimidine-rich anticodons probably lack the effective stacking interactions which would facilitate codon recognition and Watson-Crick base pairing with mRNA. In the absence of effective stacking interactions, thiolation of the position-34 uridine may be an evolved mechanism of constraining the anticodon conformation in a 3′ direction for correct codon recognition and base pairing.

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Raman Spectroscopic Studies of the Effects of Substrate Binding on Coenzymes Bound to Lactate Dehydrogenase†

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Abstract: We have studied how known changes in protein structure, i.e., "loop closure" in lactate dehydrogenase brought about by the binding of substrate, affect the Raman difference spectra of both bound NADH coenzyme and other catalytically active analogues. The Raman spectrum of the NADH coenzyme in binary enzyme·NADH complex is compared to its spectrum in ternary enzyme·NADH·substrate analogue complex. As expected, we find that the protein conformational change does not modify the binding patterns of the adenosine moiety of NADH. On the other hand, there are a number of changes in the Raman spectra of the dihydronicotinamide ring of NADH and its dihydropyridine analogue, especially the vibrational modes related with the amide $-NH_2$ of NADH and C=O of PAADH motions. On the basis of our preliminary normal mode analysis of the Raman data and other studies, we suggest that the amide C=O bond of NADH changes from a loose cisoid (to N1 nitrogen) conformation in solution to a tightly hydrogen bonded transoid conformation in LDH. Furthermore, a decrease in the number of accessible conformational states available to NADH is observed when a substrate analogue oxamate binds to the binary complex as judged by the narrowing of certain Raman bands, particularly the C4-H stretch mode of NADH. From this data, we calculate that an entropy loss corresponding to ~0.7 kcal/mol of free energy is associated with this constraint of NADH nicotinamide ring, and another ~0.7 kcal/mol is associated with the constraint of the carbonyl of pyruvate. On the basis of this, we suggest that, of the 4.2 kcal/mol decrease of the transition state barrier for hydride transfer caused by the loop closure,[1] at least 1.4 kcal/mol arises from elimination of various nonproductive conformations of nicotinamide ring and pyruvate upon binding of the substrate.

Introduction

Pyridine nucleotide dependent dehydrogenases catalyze the oxidation of alcohols by the direct transfer of a hydride ion to $NAD(P)^+$. For instance, lactate dehydrogenase (LDH^2), the subject of this paper, catalyzes the direct transfer of the hydrogen from *l*-lactate to the re face of the nicotinamide ring of NAD^+ with a stereospecificity of >99.999999%; thus, *pro*-4R transfer is favored over *pro*-4S transfer by <−10 kcal/mol.[3] The catalytic efficiency of LDH is high, since this enzyme accelerates the hydride transfer step during the oxidation of lactate by NAD^+ by at least 10^{14}-fold relative to the uncatalyzed reaction.[4] This represents a reduction in the height of the transition state barrier of at least 19 kcal/mol. The catalytic mechanism of LDH is predominantly ordered with the cofactor first on and last off (cf. Adams[5]). The presence of the cofactor drives (induces) a subtle conformational change in the active site of the enzyme (cf. Holbrook et al.[6]) that increases the affinity of the enzyme for substrate by about −2 to −3 kcal.[7]

Binding of either the substrate or a substrate analogue induces a conformational change in LDH.[1,8,9] A "loop" of the protein involving residues 98-110 closes over the active site, which moves

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(2) Abbreviations: NADH, reduced β-nicotinamide adenine dinucleotide; NAD^+, oxidized β-nicotinamide adenine dinucleotide; [4,4-D2]NADH, NADH doubly deuterated at the C4 position of the nicotinamide ring; LDH, lactate dehydrogenase; $PAAD^+$, oxidized 3-pyridinealdehyde adenine dinucleotide; PAADH, reduced 3-pyridinealdehyde adenine dinucleotide; OMA, optical multichannel analyzer.

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