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Structure and Conformation of Pseudouridine Analogues†

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ABSTRACT: The structural and conformational features of the "anomeric" DL-*trans*- and DL-*cis*-5-(3-hydroxytetrahydrofuran-2-yl)uracils (**3a**, **4a**) and five similar analogues were studied in order to determine their applicability as models of β- and α-pseudouridine. The 270-MHz proton NMR spectra were measured for all analogues to define their ring geometries in solution and to estimate the solution population of *model* N, S conformers in a two-state dynamic equilibrium treatment. Two sets of calculations were employed to evaluate the relative contributions of these states to the observed vicinal coupling constants related to the C(3′)–C(4′) fragment. In the first, similar geometries were assumed for each pair of conformers, while in the second, limited to **3**, the geometries were those derived from X-ray crystallographic data; both gave comparable results. The cis analogues **4a** and **4b** are excellent conformational models for α-pseudouridine. In the trans series (**3a–c**), the equilibrium is weighted toward the N conformer (~80%), differing from that found in β-pseudouridine for which each model conformer is equally populated. Possible implications of the conformational effects upon the pairing properties of pseudouridine in tRNA are discussed.

Pseudouridine (ψ, **1**; Figure 1), an isostere of uridine (U), is unique among the numerous naturally occurring pyrimidine or purine nucleosides in being the only C-nucleoside constituent of the nucleic acids (RNA) of both procaryotes and eucaryotes. It has been found as a component of the "constant" tetranucleotide TψCG(A)[1] in loop IV of almost every transfer RNA (tRNA) that is active in the elongation step of protein biosynthesis (Zamir et al., 1965; Sprinzl et al., 1978). In addition, two vicinal ψ residues have been identified at the 3′ end of the anticodon loop in certain tRNAs. The sequences ψψ or ψGψ, which are derived from UU or UGU, respectively, through site-specific enzymatic transitions at the macromolecular level, are required for the extended function of such tRNAs in gene repression (Turnbough et al., 1979; Bossi & Cortese, 1977; Cortese et al., 1974a,b; Singer et al., 1972; Allaudeen et al., 1972). The presence of ψ instead of U at these specific sites indicates that certain physicochemical properties unique to the C-nucleoside structure of ψ are critical to the biological function of tRNA molecules. Two features of ψ have been considered as possible contributors to its role in tRNA: (i) its potential to hydrogen bond with adenosine (A) via either the N(1),C(2) or the N(3),C(2) sites (Hurd & Reid, 1977) and (ii) its ability to form a covalent adduct with cysteine at the allylic C(1′) position (Lipnick & Fissekis, 1977).

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[1] Abbreviations used: ψ, β-pseudouridine; A, adenosine; G, guanosine; U, uridine; T, thymidine; C, cytidine; tRNA, transfer RNA; NOE, nuclear Overhauser effect; CNDO, complete neglect of differential overlap; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate.

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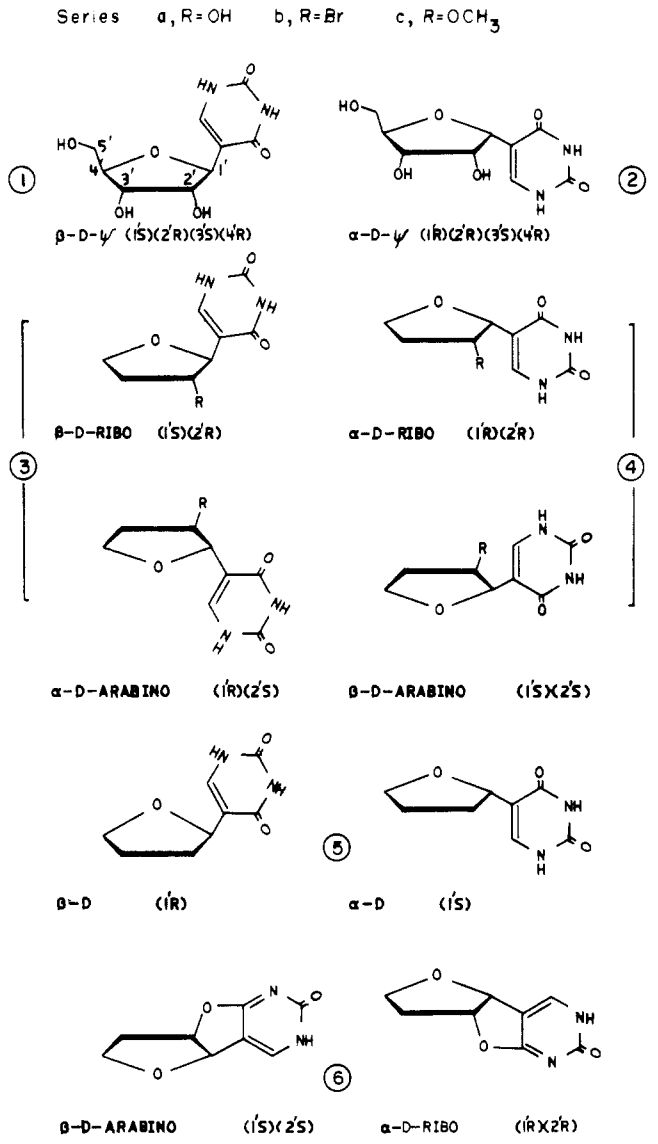

FIGURE 1: Structures of anomeric pseudouridines and of simpler tetrahydrofuran-2-yl analogues. For comparison and to clarify the discussion, the "glycosyl" moieties in the latter are regarded as being related to the D-ribose or D-arabinose.

In earlier studies, the relative acidities of the two lactam hydrogens of pseudouridine and the effects of structural modifications on these properties were investigated. The anomeric β- and α-pseudouridines (**1** and **2**; Figure 1) show a marked difference in the relative proportions of the two monoanionic species present in the equilibrium (equation 1;

$$[N(1)H,N(3)]^- \rightleftarrows [N(1)H,N(3)H] \rightleftarrows [N(1),N(3)H]^- \quad (1)$$

Chambers, 1966) which is established under conditions affording only single ionization of the pyrimidine (Kwiatkowski & Pullman, 1975; Elguero et al., 1976). This difference occurs despite the presumably identical inductive effects of the "glycosyl" moiety at C(5) in the two anomers of ψ.

This phenomenon of ionization dependence on anomeric configuration in ψ led to the following considerations. The polarization of a covalent bond X←H affects the processes of hydrogen bonding and of acid dissociation from a XH donor site in a single manner. Specifically the formation of a hydrogen bond from a XH donor site is intimately identified with the concurrent gradual polarization of the respective covalent bond (Murthy & Rao, 1970; Kollman & Allen, 1972), and the acidity of a hydride XH is, in general, directly correlated with the polarity of the X←H bond.[2] It then follows that for a given molecule under defined conditions, the acidity of the hydrogen atom of an XH site provides a qualitative measure of the potential hydrogen donor ability of that site toward the formation of an X··H··Y bond with a suitable electron donor Y.

In the specific case of the uracil series, the relative acidities of the hydrogens at N(1) and N(3) should reflect the relative ability of the respective site to participate as a proton donor in hydrogen bonding. This premise is supported by several lines of evidence.[3] The various factors then, including those of conformational origin, that affect the relative ionization properties of the two acidic sites in the uracil moiety of ψ contribute to the modulation of the pairing properties of the latter.

To evaluate the contributions of individual molecular descriptors to the establishment of the equilibrium 1 as well as to the regulation of the hydrogen-bonding affinities of the two alternate sites in the uracil moiety of ψ, we prepared two series of analogues that lack specific groups on the "glycosyl" residue. These include the 5-(3-substituted-tetrahydrofuran-2-yl)uracils **3**–**6** (Figure 1) (Fissekis et al., 1976)[4] and some related cyclopentane derivatives (Playtis & Fissekis, 1975). The relative acidities of the N(1)H and N(3)H sites were then compared within each series and between these series and ψ in an effort to identify a mechanistic basis for the observed difference in the equilibrium of the monoanion of the β and α anomers of pseudouridine (eq 1). Earlier experiments demonstrated that the annular oxygen within the "glycosyl" moiety is required to produce a tautomeric mixture in favor of the $[N(1),N(3)H]^-$ ion as observed in the case of β-pseudouridine. The ability of this annular oxygen to influence the equilibrium was attributed to the field effect of its lone pair of electrons possessing π character (Playtis & Fissekis, 1975). Such an interaction appears now to be best described by an overlap between the

[2] In simple molecules the phenomenon is mainly a reflection of the electronegativity of the basic atom. In more complex compounds, it may result from electronic effects transmitted through bonds (Hine, 1962; March, 1977).

[3] Insofar as the nucleic acid bases are amphoteric molecules that usually associate in a binary mode by acting concurrently as hydrogen donors and as acceptors, the strength of a particular hydrogen bond, or the association constant for any given base pair, will depend on the overall charge redistribution within the paired system. These parameters would be determined by the contributions of electrostatic, delocalization (charge transfer), dispersion, and repulsion terms (Murthy & Rao, 1970) to both the hydrogen donor and acceptor sites on each participant base. Nevertheless, there is good correlation, e.g., between the association constants, as measured by IR (Kyogoku et al., 1967) and NMR (Katz, 1969), for the interaction of 9-substituted adenine with a series of 1-substituted uracils that includes 5,6-dihydrouracil, uracil, thymine, and the halogeno derivatives 5-bromo- and 5-iodouracil and the order of the acidities of these pyrimidines as predicted from their structure and the electronic effect of the 5 substituent. The pK_a's of 1-methyl-5,6-dihydrouracil (11.9; Janion & Shugar, 1960) and of the nucleosides uridine (9.25; Fox & Shugar, 1952), thymidine (9.68; Fox et al., 1956), 5-bromouridine (8.20; Berens & Shugar, 1963), and 5-iodouridine (8.50; Berens & Shugar, 1963) are consistent with this deduction. Comparative IR studies of the hydrogen bonding affinities of 2,4-dithiouridine have also been interpreted to demonstrate that the increased acidity of the N(3)H results in the strengthening of the hydrogen bond from that site to the adenine nitrogen (Pitha & Scheit, 1975). In a related NMR study of aromatic hydroxyl compounds, a linear dependence was shown to exist between the dissociation constant of the compound and the chemical shift of the hydroxy proton resonance in Me_2SO but not in CCl_4. This was interpreted to demonstrate the direct correlation between the acidity of the phenolic hydrogen and its hydrogen bonding affinity to the solvent (Socrates, 1970).

[4] These compounds may be regarded as analogues of β- and α-pseudouridine lacking the 3'-hydroxyl and 4'-hydroxymethyl groups.

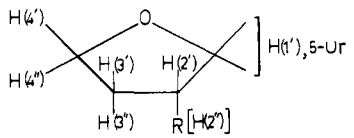

FIGURE 2: Numbering system employed in this study for analogues **3–6**.

antibonding orbital of the C(1′),O(1′) bond and the π orbital of the uracil monoanion. Its magnitude would be expected to exhibit a conformational dependence which, in turn, would be influenced by the net contributions of the substituents on the "glycosyl" moiety.

We have attempted to analyze this conformational phenomenon by a careful study of a set of structurally simpler analogues. The general method we have been employing in our studies of ψ thus far is based upon correlations of physicochemical properties within a series of simplified analogues as well as between such series and ψ. An underlying assumption of such an approach is that despite the structural deletions made, these compounds establish an equilibrium in solution between conformational species of a type similar to that of the anomeric pseudouridines. In this work we demonstrate that the conformational equilibrium of the pseudouridine anomers and the two series of the analogues depicted in Figure 1 is governed in each case by the same criteria. This conformational similarity provides a sufficient theoretical basis for comparing other physicochemical descriptors of such classes of analogues to those of the parent molecules. This will be the subject of a subsequent publication.

Experimental Procedures

The synthesis of compounds **3–6** has been previously described in a preliminary report (Fissekis et al., 1976). The obtained materials were racemic (50/50) mixtures as expected from the synthetic routes employed. For **3**, **4**, and **6**, the enantiomers have been arbitrarily designated as "D-ribo" and "D-arabino" derivatives, respectively (Figure 1). Alternatively, the latter could be visualized as "L-ribo" analogues.

For simplification, the discussion and the ensuing arguments are confined to the "D-ribo" isomers of **3–6**. However, for each of these compounds, we have measured the coupling constants of a mixture of enantiomerically related molecules in solution. The presence of both enantiomers does not affect the theoretical treatment of the results nor interferes with the meaning of the S $\rightleftarrows$ N type of equilibrium, as defined by Altona & Sundaralingam (1973).

Spectra were measured at 270 MHz on a Bruker HX-270 superconducting NMR spectrometer and for compounds **3**, **4**, and **6** were iteratively analyzed as six-spin systems with LAOCOON III (Castellano & Bothner-By, 1968), employing standard iterative techniques. Compound **5**, containing an additional proton on the tetrahydrofuran ring, was treated as a seven-spin system. The interactions due to H(6) and 2′-OH [for **3a** and **4a** in dimethyl-d_6 sulfoxide (Me_2SO-d_6)] were neglected from the iterative analysis, and coupling constants involving these nuclei were extracted directly from appropriate repeated spacings as $J/\Delta\gamma \ll 0.1$ in all such cases (Bovey, 1969). In some instances, the assignments of H(2′) $J_{1',2'}$ and $J_{1',2''}$ (for **5**) were checked in a separate NMR determination in which $H_{1'}$ was selectively decoupled.

Chemical Shifts. The numbering system used for the "D-ribo" structures is shown in Figure 2. The two most downfield signals in all cases were assigned to H(6) and H(1′) by analogy to the earlier assignments for α- and β-pseudouridine (Grey et al., 1971). For **3**, **4**, and **6**, the next most downfield signals were assigned to H(4′), H(4″), and H(2′), but not necessarily in that order, each of which is attached to a carbon bearing a heteroatom. For the same compounds, the two most upfield proton signals were assigned to H(3′) and H(3″). For **3** and **4**, the more upfield signal was assigned to H(3″), the proton cis to the 2′ substituent, reflecting the known stereodependent shielding of vicinal –OH, –Br, and –OMe groups which have been examined in a number of model cyclic compounds (Jackman & Sternhell, 1969; DeClerq & Samson, 1975, and references cited therein).[5] This dependence and particularly the relative magnitudes of the two vicinal couplings, $J_{2',3'}$ and $J_{2',3''}$, served to establish the stereochemistry of H(3′) and H(3″) with respect to the 2′ substituent.

In the case of the "cyclo" derivative, **6**, the stereodependent chemical shift relationship does not apply,[6] and the chemical shift assignment was based entirely on the relative magnitudes of $J_{2',3'}$ and $J_{2',3''}$,[7] which can be distinguished unambiguously in this case as $J_{trans} < 1.0$ Hz (Townsend, 1973). For **5**, the assignment of the pair, H(2′),H(2″) was made on the basis of decoupling experiments by selective irradiation of H(1′). The two hydrogens at C(3′) resonate at the same chemical shift, which is consistent wiith the absence of a β substituent. The assignments for H(2′),H(2″) and H(4′),H(4″) were confirmed by decoupling experiments involving the H(3′),H(3″) envelope. For these pairs, the more downfield signal for each set was assigned to that hydrogen, H(2′) or H(4′), cis to the 5-uracilyl substituent since an examination of a molecular model in N or S conformation with $\chi \simeq 0$ similar to that observed in the solid state for **3a** and **4a** reveals that the H(2′) and H(4′) hydrogens are within the deshielding zone of the uracil ring, while the H(2″) and H(4″) protons are not affected. This assignment is also in accord with the similarity of δH(2″) and δH(4″) of 5 to the corresponding δH(2) and δH(4) of unsubstituted tetrahydrofuran (Table I) whose chemical shifts are unaffected by the presence of a cis-vicinal substituent as well as with the observation that for compounds **3**, **4**, and **6** the more downfield signal of the pair H(4′),H(4″) is generated by the hydrogen cis to the 5-uracilyl substituent (see below).

Results

The experimental spectra along with Calcomp plots of the corresponding iterative solution spectra are shown in Figures 3–6 [Figures 5 and 6 are in the supplementary material (see paragraph at end of paper regarding supplementary material)]. The iterative solution values of proton chemical shifts and proton–proton coupling constants are presented in Tables I and II, respectively.

Vicinal Coupling Constants. Assuming similar values for the H–C–H valency angles in the two fragments C(4-)–H,H and C(3′)–H,H, the two pairs of cis couplings should be in all instances qualitatively similar, while for unequal N, S populations the two sets of trans couplings should be different (Lipnick, 1974a).[8] The trans couplings may each assume

[5] An examination of Dreiding models suggests that the deshielding effect of the 5-uracilyl moiety in an anti configuration (N or S conformer) should be least important at C(3′) with respect to the tetrahydrofuran ring.

[6] For compound **6** the proximity of the anisotropic heterocycle apparently exerts a larger effect than the 2′-oxygen.

[7] As we discuss later, these assignments provide self-consistent values of $J_{2',3'}$ over a small range, as expected.

[8] For most derivatives (**3**, **4**, and **6**), the two sets of cis-vicinal couplings, $J_{3',4'}$ and $J_{3'',4''}$, are unequal, with $J_{3',4'}$ being the larger by 1.6–2.9 Hz. This difference may be due either to an inequality in the corresponding valency angles arising from the steric repulsion of H(3″) by the 2′ substituent or to a difference in the electronic effect of the ring oxygen depending upon its syn- or anti-periplanar relationship to one of the coupled protons (Booth, 1965).

Table I: Proton NMR Chemical Shifts of Pseudouridine Analogues 3-6 (ppm)[a,b]

compd	solvent	temp (°C)	$H_{2'}$	$H_{2''}$	$H_{3'}$	H_3	$H_{4'}$	$H_{4''}$	$H_{1'}$	H_6[c]	OH
tetrahydrofuran[d]	Me_2SO-d_6	22	1.760	1.760	1.760	1.760	3.604	3.604	3.604		
3a	Me_2SO-d_6	22	4.133		1.910	1.695	3.970	3.819	4.414	7.129	5.043
	D_2O	22	4.421		2.158	1.947	4.180	4.027	4.667	7.455	
	D_2O	80	4.409		2.187	1.955	4.134	4.027	4.630	7.440	
β-pseudouridine[e]	D_2O	30	4.279						4.674	7.660	
4a	Me_2SO-d_6	22	4.737		2.156	1.868	3.753	3.942	4.476	7.069	4.580
	D_2O	22	4.737		2.568	2.259	4.161	4.287	4.909	7.695	
	D_2O	80	4.527		2.345	2.034	3.916	4.079	4.696	7.502	
α-pseudouridine[e]	D_2O	30	4.358						4.991	7.561	
3b	Me_2SO-d_6	22	4.603		2.438	2.130	4.137	3.945	4.877	7.277	
4b	Me_2SO-d_6	22	4.975		2.771	2.359	3.939	4.039	4.605	7.160	
3c	Me_2SO-d_6	22	3.791		1.899	1.845	4.003	3.709	4.575	7.202	
5	Me_2SO-d_6	22	2.148	1.680	1.874	1.874	3.918	3.697	4.606	7.201	
6	Me_2SO-d_6	22	5.204		2.036	2.174	3.889	3.430	5.266	7.974	

[a] For Me_2SO-d_6 solutions, downfield from internal TMS. For D_2O solutions, downfield from internal DSS. [b] See Figure 2 for numbering system used. [c] First order value ($J/\Delta\nu \ll 0.1$) (Bovey, 1969). [d] This work; the value for H(2') and H(2'') given for comparison with 5. [e] Grey et al. (1971). Literature values of H(3') and H(4') are not compared for these compounds because of the absence of substituents on carbons C(3') and C(4') in these analogues.

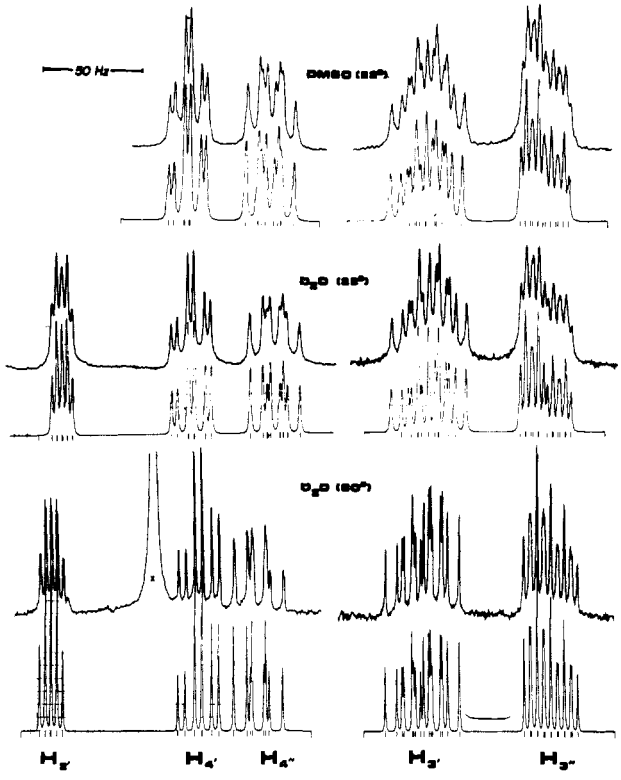

FIGURE 3: Observed and simulated 270-MHz spectra of **3a** under the conditions indicated. In each case, the experimental spectrum is the upper one. The residual HOD signal observed at 80° is marked with an ×. For simplicity only the "D-ribo" form is depicted.

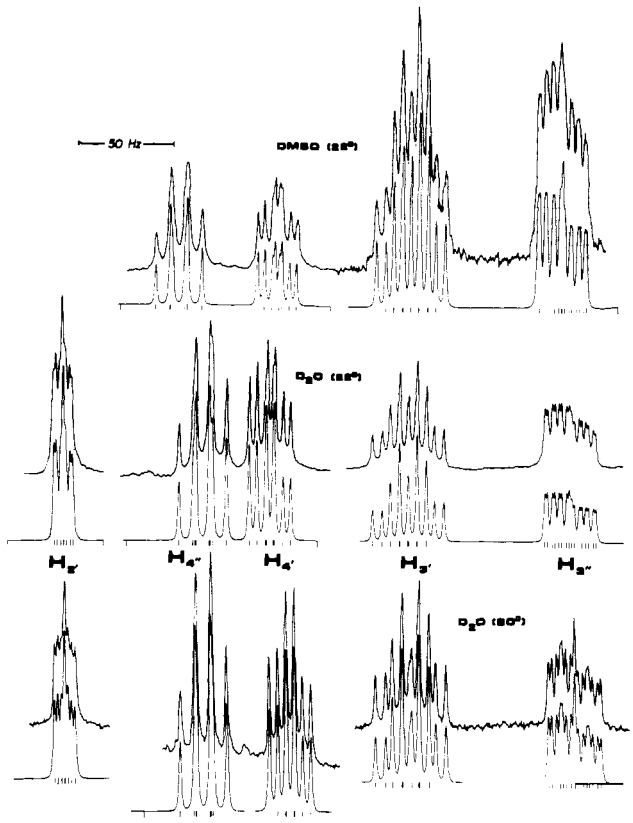

FIGURE 4: Observed and simulated 270-MHz spectra of **4a** under the conditions indicated. In each case, the experimental spectrum is the upper one. For simplicity only the "D-ribo" form is depicted.

values between 0 and 12 Hz. Their sum, however, would remain nearly constant,[9] irrespective of the state of the equilibrium. For compounds **3**, **4**, and **6**, $J_{3'',4'}$ was sufficiently smaller than any of the other three parameters to allow this to be assigned trans, thereby determining the other three. For the same series, the two couplings observed across the C(2')–C(3') fragment range between 4.9–5.8 Hz and 0.6–3.0 Hz. The range defined by the larger couplings compares well with that (4.8–6.4 Hz) previously established for $J_{2',3'}$ cis in nucleosides (Altona & Sundaralingam, 1973) and, in particular, the value (5.6 Hz) for $J_{2',3'}$ cis in 3-deoxyadenosine (Westhof et al., 1977). Therefore, in each case the larger observed coupling was assigned cis and the smaller trans, with the more upfield signal corresponding to the hydrogen cis to the 2' substituent.

A cis $J_{1',2'}$ should be essentially independent of the N ⇄ S equilibrium while a trans $J_{1',2'}$ should vary with the latter. Between 22 and 80 °C, $J_{1',2'}$ changes by 0.6 Hz for **3a** but only by 0.1 Hz for **4a**. The temperature dependency of this parameter for **3a** and **4a** is thus in accord with the cis and trans stereochemistry of the uracil moiety vs. the 2' substituent as determined in the crystallographic study (H. M. Berman, private communication) of these compounds.

The stereochemistry at the C(1')–C(2') fragment in **3b**, **3c**, and **4b** cannot be defined from the magnitudes of the respective, $J_{1',2'}$ alone as in no case is this parameter smaller than 1.0 Hz (Townsend, 1973). However, the self-consistency of

[9] For tetrahydrofuran, only one trans coupling, the average, is obtained. In this case, $(J_{3',4''})_{exp} + (J_{3'',4'})_{exp} = 2J_{trans}$.

Table II: Proton–Proton NMR Coupling Constants (Hz)[a,b] of Pseudouridine Analogues Compared to Those for α- and β-Pseudouridine and Tetrahydrofuran

compd	solvent	temp (°C)	coupling constants (Hz)											
			1',6	1',2'	1',2''	2',2''	2',3'	2'',3''	2'',3'	3',3''	3',4'	3',4''	3'',4'	3'',4''	4',4''	2'-OH
tetrahydrofuran	Me_2SO-d_6	22					8.7[c]	d	d	8.7[c]		8.0[c]	e	8.0[c]		
3a	Me_2SO-d_6	22	1.1	2.1			5.2	2.5		−12.6	8.3	10.0	2.5	6.2	−7.8	4.0
	D_2O	22	1.1	2.3			5.3	2.8		−13.5	8.6	10.2	2.8	6.3	−8.4	
	D_2O	80	1.1	2.9			5.8	3.0		−13.5	8.5	9.4	3.4	6.7	−8.5	
β-pseudouridine[f]	D_2O	30	0.8	5.0			5.0					5.2				
4a	Me_2SO-d_6	22	1.1	3.1			5.0	1.1		−13.0	9.1	8.9	3.5	7.6	−7.7	4.4
	D_2O	22	1.4	3.5			4.9	1.3		−13.8	9.3	9.1	3.8	7.3	−8.5	
	D_2O	80	1.1	3.6			5.3	1.8		−13.6	9.0	8.7	4.8	7.3	−8.4	
α-pseudouridine[f]	D_2O	30	1.3	3.3			4.2					7.9				
3b	Me_2SO-d_6	22	0.9	2.7			5.5	2.8		−14.1	8.1	9.7	2.8	6.1	−8.0	
4b	Me_2SO-d_6	22	1.2	3.3			5.2	0.7		−14.4	9.3	9.4	2.7	6.9	−8.0	
3c	Me_2SO-d_6	22	1.2	1.7			4.8	1.2		−13.1	8.6	11.1	2.0	5.8	−7.8	
5	Me_2SO-d_6	22	1.2	7.0	6.7	−12.3	7.0	7.0	7.3	−12.5	6.5	7.1	6.5	7.1	−7.9	
				(6.8)[g]	(6.8)[g]										(−8.0)[h]	
6	Me_2SO-d_6	22	~0[i]	5.4			5.6	0.6		−13.9	7.6	11.2	1.5	4.7	−8.9	

[a] LAOCOON III probable error for coupling constants is 0.03–0.06; the experimental accuracy is probably closer to 0.1 Hz, so that the numerical values obtained have been rounded off to this decimal place. [b] See Figure 2 for numbering system used. [c] Lambert et al. (1974). [d] $(J_{2',3''} + J_{2'',3'})/2 = 6.3$ Hz. [e] $(J_{3',4''} + J_{3'',4'})/2 = 6.1$ Hz. [f] Grey et al. (1971). [g] First-order value from H(6) decoupled spectrum. [h] First-order value from H(3'),H(3''). [i] No splitting was observed.

the remaining interproton coupling assignments in **3b**, **3c**, and **4b** with respect to **3a** and **4a** is in agreement with the structures assigned previously to these compounds from a comparison of their UV properties.

For **5**, the two sums $J_{1',2'} + J_{3',4''}$ and $J_{3',4''} + J_{3'',4'}$ are larger than the corresponding ones for **3a** and **3b** by 1–2 and for **3c** by 0.5–1 Hz. The cis $J_{1',2''}$ for **5** is also larger by 3.4–3.6 Hz from the corresponding cis $J_{1',2'}$ for **4a** and **4b**. From these correlations it can be inferred that in **5** the tetrahydrofuran ring is flattened along the O(1')–C(1')–C(2')–C(3') segment while the amplitude of puckering within the C(3')–C(4') fragment is increased. Similar considerations suggest that **6** is represented by two rather rigid conformations at equilibrium which are best characterized as C(4')-endo and C(4')-exo, respectively.[10]

Hydroxyl Coupling. The values of $J_{H(2'),OH}$ of 4.0 and 4.4 Hz for **3a** and **4a**, respectively, are similar to those measured in nucleosides in general (Davies, 1978; Davies & Danyluk, 1970, 1974) and suggest no conformational preference around the C(1')–C(2')–O(2')–H torsional angle so that all three staggered rotamers are about equally populated.

Geminal Coupling. For compounds **3**–**6**, the parameter $J_{3',3''}$ falls within the range of −12.5 to −14.4 Hz, in good agreement with the value of −12.8 Hz found at this carbon in tetrahydrofuran (Diez et al., 1974) and with the two values (−12.5 and −12.8 Hz) for geminal ring coupling in methylcyclopentane (Lipnick, 1974a). In the case of **5**, a similar coupling, $J_{2',2''}$, is −12.3 Hz, within the same range. The remaining geminal coupling constant, $J_{4',4''}$, between protons α to the ring oxygen falls within the range of −7.7 to −8.5 for compounds **3**–**5**, which is comparable to the value of this coupling (−9.0 Hz) found in tetrahydrofuran (Diez et al., 1974; Lozach et al., 1975).

In general, J_{gem} adjacent to a single oxygen in a five-membered ring ranges from −9.6 to −7.5 Hz (Cookson et al., 1966). The positive increase of $J_{4',4''}$ over $J_{3',3''}$ and $J_{2',2''}$ (for **5**) is due mainly to inductive withdrawal of electrons by the electronegative oxygen substituent and also to some back-donation from the unshared pairs on the oxygen into the antisymmetric bonding orbitals of the C(4')–H,H methylene group. This latter effect is stereodependent and exerts its maximum positive contribution when the C–O–C plane is perpendicular to the line connecting the nuclei H(4') and H(4'') (Bothner-By, 1965). For **3**–**6**, it appears to make a variable contribution that is least in the case of **6**, in which the C(4')–O(1')–C(1') plane, on the average, deviates most from the perpendicular to the line connecting the nuclei H(4') and H(4'').

Long-Range Coupling. For all compounds except **6**, $J_{1',6}$ = 0.9–1.2 Hz is similar to values reported for α- and β-pseudouridine (Grey et al., 1971; Deslauriers & Smith, 1972; Blackburn et al., 1970) which were attributed to a mostly anti conformation (Grey et al., 1971; Deslauriers & Smith, 1972; Dugaiczyk, 1970).[11] For the "cyclo" derivative, **6**, this parameter is ~0, as expected for allylic coupling for this ster-

[10] Similar values have been reported (Cross & Schleich, 1973) for the corresponding constants in β-D-*O*^{2},2'-cyclouridine, and they were rationalized in terms of a conformational equilibrium between the C(4')-endo and C(4')-exo forms. In contrast, a solution study concluded that the same cyclouridine derivative exists primarily in the C(1')-exo conformation (Guschlbauer et al., 1974). Either interpretation would be consistent with our data.

[11] From NOE studies and CNDO calculations, it was concluded that equal populations of syn and anti conformations of β-pseudouridine exist in rapid equilibrium (Nanda et al., 1974). On the contrary, T_1 proton relaxation experiments (Neumann et al., 1977) indicated a strong predominance of the syn conformation.

Table III: N, S Conformer Composition for Pseudouridine Analogues

compd	solvent	temp (°C)	$(J_{3',4''} + J_{3'',4'})$	derivation I[a]		derivation II[b]				
				% N	% S	% N	% S	A[c]	B[c]	error[d]
3a	Me_2SO-d_6	22	12.5	80	20	89	11	12.2	2.7	0.019
	D_2O	22	13.0	79	21	87	13	12.7	2.8	0.002
		80	12.8	73	27	82	18	12.8	2.5	0.252
4a	Me_2SO-d_6	22	12.4	71	29					
	D_2O	22	12.9	70	30					
		80	13.0	67	33					
1b	Me_2SO-d_6	22	12.5	78	22					
4b	Me_2SO-d_6	22	12.1	78	22					
1c	Me_2SO-d_6	22	13.0	85	15					
5	Me_2SO-d_6	22	13.6	52	48					
6	Me_2SO-d_6	22	12.7	88[e]	12[e]					
α-ψ[f]	D_2O	22		79	21[g]					
β-ψ[f]	D_2O	22		50	50[g]					

[a] Based on eq 2. [b] Based upon the equations under Appendix. [c] Karplus coefficients derived from least-squares solution of eq 5–8. [d] Minimum error from least-squares solutions (eq 9). [e] In this case, the two conformers in equilibrium are C(4')-endo and C(4')-exo, designated arbitrarily as S and N, respectively, in this table. [f] ψ abbreviated for pseudouridine. [g] See Neumann et al. (1977).

eochemistry (Garbisch, 1964; Hruska, 1971).

In the spectrum of **3a** in D_2O at 80 °C, the signals corresponding to H(3') and H(4') are considerably narrower than those related to H(3'') and H(4''). This phenomenon could be explained by long-range $^4J_{H,H}$ couplings that proceed via the W-pathway (Bhacca & Williams, 1964) to H(1'), each transition of which exhibits substantial broadening. The additional splitting of ~0.6 Hz observed for H(2') at 80 °C could not be satisfactorily explained.

Conformational Analysis. The X-ray crystallographic data (H. M. Berman, private communication) demonstrate that **3a** and **4a** adopt conformations of the N, S type (Sundaralingam, 1969; Altona & Sundaralingam, 1973; Davies, 1978) in the solid state. The NMR solution data for compounds **2–5** are also consistent with a two-conformer equilibrium mixture of the N, S type puckers as observed in nucleosides and in other simpler five-membered rings [Lipnick, 1974a,b,c (and references cited therein); Lambert et al., 1974; Fuchs & Wechsler, 1977; Altona, 1971; Allinger & Chung, 1976]. In nucleosides, the sum $J_{1',2'} + J_{3',4'}$ has been demonstrated to be sensitive to the degree of ring puckering (Davies, 1978). The increased magnitude of the corresponding sum $(J_{1',2'} + J_{3',4''})$ for the analogues studied (~12.4 Hz) over ribonucleosides and C-nucleosides (~10 Hz) suggests either greater puckering or a contribution of additional pseudorotational states for these analogues. For derivatives **5** and **6**, this sum is 14.1 and 16.6 Hz, respectively, and the deviation from standard N, S forms is even greater. The intramolecular cyclization in **6** restricts the conformational freedom to ^4E–^4_3T and $_4E$–^3_4T puckered forms.

We have analyzed the conformational equilibrium for all analogues, making the usual assumption of a two conformer mixture, and calculated the mole fraction ^NX from eq 2.[12]

$$^NX = \frac{J_{3',4'}{}^{obsd}}{J_{3',4'}{}^{obsd} + J_{3'',4'}{}^{obsd}} \quad (2)$$

This approach is equivalent to that employed previously for ribo- and deoxyribonucleosides (Altona & Sundaralingam, 1973; Davies & Danyluk, 1974; Davies, 1978). In this equation, the sum $(J_{3',4''}{}^{obsd} + J_{3'',4'}{}^{obsd})$ should be nearly constant in a series of compounds containing a five-membered ring system of this type, regardless of the N, S equilibrium composition.[13] Thus, the similarity of this sum in tetrahydrofuran[9] and in **3–6** (12.1–13.6 Hz) suggests similar values for the torsion angle τ_3 in all these systems.

By substituting the corresponding values of J^{obsd} for **3–6** into eq 2, we find that the conformer distribution falls in the range of 52–88% N conformer (Table III, derivation I).

For **3**, the geometries of the most likely N and S conformers were available from X-ray studies (H. M. Berman, private communication). Fortuitously that derivative crystallized in a pattern so that within the same crystal lattice the "β-D-ribo" [(1'*S*)(2'*R*)] isomer was in the N (^3_2T) while the "α-D-arabino" [(1'R)(2'S)] enantiomer was in the S ($_3E$) conformation, respectively. In this case, we applied the Karplus (Karplus, 1959, 1963; Barfield & Grant, 1965) equation:

$$^3J_{i,j}{}^{obsd} = {}^NX(A\cos^2{}^N\theta_{i,j} - B\cos{}^N\theta_{i,j}) + {}^SX(A\cos^2{}^S\theta_{i,j} - B\cos{}^S\theta_{i,j}) \quad (3)$$

where ^NX and ^SX are the mole fractions ($^NX + {}^SX = 1$) of the N and S conformers, respectively, and $\theta_{i,j}$ is the dihedral angle related to the $^3J_{i,j}$ coupling of the set of four vicinal couplings across the C(3')–C(4') fragment.

By rearranging eq 3 into the linear form $y_i = m_i x_i + b_i$ where the slope m_i and the intercept b_i are equal to the Karplus parameters B and A, respectively, we obtain

$$\frac{J_{i,j}{}^{obsd}}{{}^NX(\cos^2{}^N\theta_{i,j} - \cos^2{}^S\theta_{i,j}) + \cos^2{}^S\theta_{i,j}} = \frac{B[{}^NX(\cos{}^S\theta_{i,j} - \cos{}^N\theta_{i,j}) - \cos{}^S\theta_{i,j}]}{{}^NX(\cos^2{}^N\theta_{i,j} - \cos^2{}^S\theta_{i,j}) + \cos^2{}^S\theta_{i,j}} + A \quad (4)$$

By substituting the numerical values of the cosine functions of the appropriate torsional angles obtained from the two crystal forms of **3a** in eq 4, we derive four equations containing the three unknown parameters ^NX, A, and B (see Appendix for eq 5–8).

We employed a computer program to calculate the best value of ^NX, A, and B where $0 < {}^NX < 1.0$. For each value of ^NX, the best least-squares fit to the line $y_i = mx_i + b$ is that

[12] This treatment has been extended to the study of the conformational properties of the furanose phosphate backbone in dinucleotides. Under an analogous set of conditions, a relationship similar to eq 2 was derived for the C(4')–C(3')–O(3')–P(3') torsional angle by employing the heteronuclear coupling constants $^3J_{C(2'),P(3')}$, and $^3J_{C(4'),P(3')}$, and $^3J_{C(4'),P(3')}$ (Alderfer & Ts'o, 1977).

[13] By analogy, it has been noted that in the ribofuranosyl nucleosides, the sum of the two trans coupling constants $(J_{1',2'} + J_{3',4'})$ for pyrimidine nucleosides and nucleotides falls in the range 9.5–11.1 Hz, and its mean value (10.3 Hz) is practically independent of the N, S equilibrium composition (Altona & Sundaralingam, 1973).

solution which minimizes the least squares difference error $E(m,b)$ given by (Bevington, 1969)

$$E(m,b) = \sum_{i=1}^{4}(m_i x_i + b_i - y_i)^2 \quad (9)$$

Solution of these equations, using NMR data for **3a** in Me_2SO-d_6 at 22 °C and in D_2O at 22 and 80 °C, leads to the N, S values in Table III (derivation II). Inspection reveals less than a 10% difference between these relative populations and those obtained in the previous calculation employing NMR parameters alone with certain simplifying assumptions.

Discussion

We have performed NMR conformational studies of a series of β- and α-pseudouridine analogues to determine their suitability as stereochemical models of the parent compounds. The relative stereochemistries at C(1′) and C(2′) in **3** and **4** which were previously deduced from correlations of UV absorption properties (Fissekis et al., 1976) have been confirmed by an X-ray crystallographic study of **3a** and **4a**. These stereochemical assignments are consistent with the vicinal coupling constant values extracted in this work.

Our results demonstrate that in solution **3** and **4** exist in an equilibrium of two conformer populations of N and S type species qualitatively similar to that of the parent C-nucleosides. Interestingly, similar conclusions were reached with respect to 1-(tetrahydrofuran-2-yl)uracil in recent studies by X-ray (Verdegall et al., 1979) and NMR (Kruse et al., 1979).

The magnitudes of the cis couplings $J_{2',3'}$ (4.9–5.8 Hz) of the tetrahydrofuranyl analogues **3**, **4**, and **6**, which should be essentially independent of conformer population, are nearly identical with those observed in pentofuranosyl nucleosides (4.8–6.4 Hz; Altona & Sundaralingam, 1973). The H(3′)–C(3′)–C(2′)–H(2′) torsional angle also falls within the same range for both series. That angle varies from 33–43° [14] for both N and S populations in the solid state of pentofuranosyl nucleosides. By comparison, this angle for **3a** was found from the crystallographic studies to vary from 33° in the N conformer to 40° in the S conformer, while for **4a** in an N conformation this angle was found to be 37°.

We have estimated the N, S conformer populations for these analogues by using two approaches. The first (derivation I, Table III) assumes symmetrical model N, S conformers for all analogues. The second (derivation II, Table III) employs H–C–C–H dihedral angles obtained in the solid state, for the *trans*-hydroxyl analogue **3a**, which alone serendipitously crystallized as a mixture of N, S conformers. The two calculations for **3a** under three sets of conditions in solution agree within about 10%. This self-consistency suggests that the calculated equilibrium value is rather insensitive to the model geometries chosen, and it provides additional justification for applying derivation I to the other analogues.[15]

It is noteworthy that the unsubstituted tetrahydrofuran derivative **5** achieves a conformational equilibrium (52% N) similar to that of β-pseudouridine (50% N; Neumann et al., 1977). Evidently no net change takes place in this conformational equilibrium as a result of the collective replacement of the 2′-, 3′-, and 4′-glycosyl substituents in ψ by hydrogen. The population distribution for the *cis*-2′-hydroxyl derivative **4a** favors the N (70%) conformer. Analogous results were obtained for the *cis*-2′-bromo derivative **4b** (78%). This conformer distribution is remarkably similar to that reported for α-pseudouridine (79%) (Neumann et al., 1977). Thus, the cis-2′-substituted-tetrahydrofuranyl compounds are apparently good models for the conformational populations of the ribose ring in α-pseudouridine. The trans isomers **3a**, **3b**, and **3c** exhibit a significant increase in the N conformer population (80%) relative to that of β-pseudouridine which exists as an equimolar mixture of N and S conformers (Neumann et al., 1977). This shift in favor of the N states seen in the "β-D-ribo" series of these analogues most likely results from a tendency to relieve the gauche electrostatic interaction that exists in the S state between the electronegative 2′ substituent and the pyrimidine. In β-pseudouridine this conformational perturbation may be limited by steric repulsion between the C(4′) hydroxymethyl group in the dominant *gg* position and the pyrimidine. The increase in population of S conformers observed for the unsubstituted tetrahydrofuran derivative **5** is consistent with this explanation. The interpretation is also in accord with the fact that 2′-deoxyuridine (Davies, 1978; Schleich et al., 1972) and pyrimidine 2′-deoxynucleosides, in general, exhibit a shift toward the S conformation compared to the respective ribonucleosides.

Within each series of the analogues, **3**–**5**, there is a remarkable similarity of the measured N, S distribution, irrespective of the nature of the 2′ substituent. This suggests that the actual conformational equilibrium for **3a** and **4a**, in both D_2O and Me_2SO, like that of the other compounds, **3b**, **3c**, and **4b**, is not affected by possible intramolecular hydrogen bonding of the 2′-hydroxyl group to the pyrimidine. The "cyclo" derivative, **6**, in contrast to the other derivatives, deviates substantially from the N, S type states and can probably best be described in terms of an ${}^4E$–${}^4_3T \rightleftarrows {}_4E$–${}^3_4T$ equilibrium.

The present data provide justification for using simplified analogues to study structural and conformational parameters influencing the monoanion tautomerism of pseudouridine. This and our earlier studies (Playtis & Fissekis, 1975) demonstrate that the glycosyl moiety in ψ, and presumably in other C-nucleosides, can exert a subtle effect upon the ionization properties of the aglycon that is dependent on the conformation of the "glycosyl" side chain. More extensive examination of this phenomenon seems important with regard to elucidating the unique biological properties of ψ. Spectrophotometric studies in solution (Chambers, 1966) indicate that in the β-pseudouridine monomer, the acidity of the N(1)H is greater to that of the N(3)H, i.e., $[N(1),N(3)H]^- > [N(1)H,N(3)]^-$ (eq 1). However, solutions of β-pseudouridine contain approximately equal populations of rapidly interconverting N, S conformers in the syn configuration (Neumann et al., 1977). Our data now suggest that the calculated pK_a for each ionization site of the pyrimidine in ψ must reflect the contribution of this conformational term to the equilibrium of eq 1 reached by this nucleoside.

The physical parameters that affect the acid dissociation of ψ at the polymer level rather than those influencing the ionization properties of the monomer are likely to be pertinent to the function of that C-nucleoside in tRNA. In this respect, the ultimate conformation that ψ adopts within the tRNA is of importance. A recent crystallographic refinement of the structure of $tRNA^{Phe}$ (Sussman et al., 1978) showed that the pseudouridine on the anticodon stem (ψ_{39}) adopts a C(3′)-endo (i.e., N type) pucker and the anti form. A similar conformation for that residue of ψ in $tRNA^{Phe}$, and by analogy in other tRNAs where it is present, is likely to persist when the

[14] Calculated from the Karplus equation, $J_i = A\cos^2\theta_i - B\cos\theta_i$, employing the values $A = 10.5$ and $B = 1.2$ (Altona & Sundaralingam, 1973).

[15] Application of derivation I to 1-(tetrahydrofuran-2-yl)uracil results in an estimated value of 64% N which compares favorably with the authors (Kruse et al., 1979) estimated 68% N, as derived from coupling data across the C(1′)–C(2′) fragment.

tRNA is in solution. Furthermore, it is clearly apparent from the crystal structure of $tRNA^{Phe}$ (Holbrook et al., 1978) that ψ_{39} on the anticodon stem is base paired via the N(3)H,C(2)O sites with the adenosine (A_{31}) on the complementary strand. The N(1)H of ψ_{39} appears to be oriented toward the groove and to be exposed. It is probable that in the site-equivalent ψ_{41}:A_{33} pair in $tRNA^{His}$ in the wild-type strain of *S. typhimurium* (Singer & Smith, 1972) ψ similarly assumes the anti disposition. For the corresponding ψ_{41}:G_{31} pair in $tRNA^{Leu}$ in the same strain (Allaudeen et al., 1972) only a "wobble"-type interaction involving the N(3),C(4) sites of ψ is likely, leaving the N(1)H site free. The charged forms of both of these two tRNAs possess a supplemental regulatory (repressor) functions which requires, in each instance, the presence of a pseudouridine residue at the anticodon stem (ψ_{41}) as well as a second such residue at an adjacent position of the anticodon loop (ψ_{40} and ψ_{39}, respectively). Absence of these two U → ψ modifications abolishes the tRNA repressor activity (Turnbough et al., 1979; Bossi & Cortese, 1977; Cortese et al., 1974a,b; Singer et al., 1972; Allaudeen et al., 1972). The N(1)H sites in these pseudouridines might constitute part of a specific recognition or binding site for a regulator, presumably a protein, involved in the modulation of the expression of the respective amino acid operon. This provides an explanation for the requirement for these modified nucleosides.[16] A favoring of the C(3′)-endo pucker, at least for the ψ on the stem, should enhance the relative acidity of the N(1)H and therefore the potential of the latter to serve as a hydrogen-bond donor. Consequently, that particular ψ residue should be capable of interacting concurrently with both the complementary A and, on demand, a regulatory molecule as postulated above. By contrast, a uridine moiety in either such location and in the anti configuration would lack the additional N(H) binding site so that any corresponding undermodified tRNA should also lack a regulatory ability. Experimental observations with His T mutants of *S. typhimurium* are completely in agreement with this prediction (Allaudeen et al., 1972; Turnbough et al., 1979).

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Appendix

Derivation II. For the values ${}^{S}\theta_{3',4''} = 82°$, ${}^{S}\theta_{3'',4''} = 49°$, ${}^{S}\theta_{3'',4'} = 170°$, ${}^{S}\theta_{3',4'} = 39°$, ${}^{N}\theta_{3',4''} = 150°$, ${}^{N}\theta_{3'',4''} = 33°$, ${}^{N}\theta_{3'',4'} = 101°$, and ${}^{N}\theta_{3',4'} = 16°$, we obtain

$$\frac{J_{3',4''}^{obsd}}{0.731^{N}X + 0.019} = \frac{B(1.005^{N}X - 0.139)}{0.731^{N}X + 0.019} + A \quad (5)$$

$$\frac{J_{3'',4''}^{obsd}}{0.273^{N}X + 0.430} = \frac{B(-0.183^{N}X - 0.656)}{0.273^{N}X + 0.430} + A \quad (6)$$

$$\frac{J_{3'',4'}^{obsd}}{-0.934^{N}X + 0.970} = \frac{B(-0.794^{N}X + 0.985)}{-0.934^{N}X + 0.970} + A \quad (7)$$

$$\frac{J_{3',4'}^{obsd}}{0.320^{N}X + 0.604} = \frac{B(-0.184^{N}X - 0.777)}{0.320^{N}X + 0.604} + A \quad (8)$$

Supplementary Material Available

Figures 5 and 6 showing observed and simulated 270-MHz spectra for **3**–**6** (2 pages). Ordering information is given on any current masthead page.

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[16] It has been proposed that the pseudouridine residue on the anticodon stem of regulatory tRNAs pairs in the syn rather than the anti conformation with adenosine. Such an unusual mode of association was suggested to constitute the structural basis for the regulatory function of that ψ:A pair (Hurd & Reid, 1977). The results from the crystallographic study of $tRNA^{Phe}$ (Quigley & Rich, 1976; Sussman et al., 1978) regarding the structure of the ψ:A pair at the lower end of the anticodon stem, if applicable to the regulatory tRNAs, show such a model to be untenable.

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