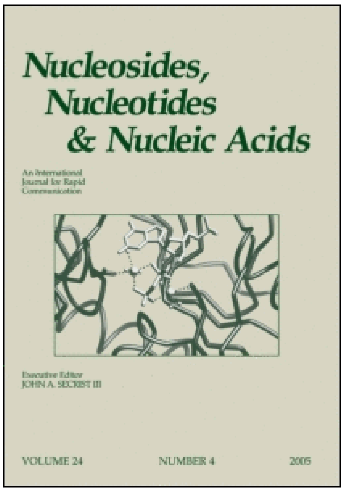

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RNA MODIFIED URIDINES VI:
CONFORMATIONS OF 3-[3-(S)-AMINO-3-CARBOXYPROPYL]URIDINE (acp^3U) FROM tRNA AND 1-METHYL-3-[3-(S)-AMINO-3-CARBOXYPROPYL]PSEUDOURIDINE ($m^1acp^3\Psi$) FROM rRNA

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Abstract: The conformation of chemically synthesized acp^3U is 60% 3'-endo, gauche$^+$, whereas that of $m^1acp^3\Psi$ is 60% 2'-endo, gauche$^+$. We conclude that the difference in conformation probably imparts important local structures to their respective tRNA and rRNA.

Introduction

The chemically related nucleosides 3-[3-(S)-amino-3-carboxypropyl]-uridine (acp^3U) and 1-methyl-3-[3-(S)-amino-3-carboxypropyl]pseudouridine ($m^1acp^3\Psi$) have been found in tRNAs, and rRNAs, respectively. The nucleoside acp^3U has been found in both eucaryotes and procaryotes in the dihydrouridine loop sequences of tRNAs for arg (Ap-acp^3Up-A)[1], asn and val (Dp-acp^3Up-A)[2-6], gly (Ap-acp^3Up-C)[7], met (Cp-acp^3Up-A)[8], and tyr (Gp-acp^3Up-A)[9], and (Gp-acp^3Up-U)[10], and in the extra arm sequences of tRNAs for arg, ile, lys, met, phe, tyr, val (m^7Gp-acp^3Up-C)[11-25] and met$_i$ (m^7Gp-acp^3Up-U).[26] The nucleoside has also been isolated from human urine.[27] The nucleoside $m^1acp^3\Psi$ has been isolated from rRNA of yeast[28], HeLa[28] and Chinese hamster cells.[29] In order to understand the structural and functional differences of these two chemically related modified uridines we have recently developed and published the chemical synthesis of acp^3U[30], and report here the chemical synthesis of $m^1acp^3\Psi$, as well as physical studies on both molecules.

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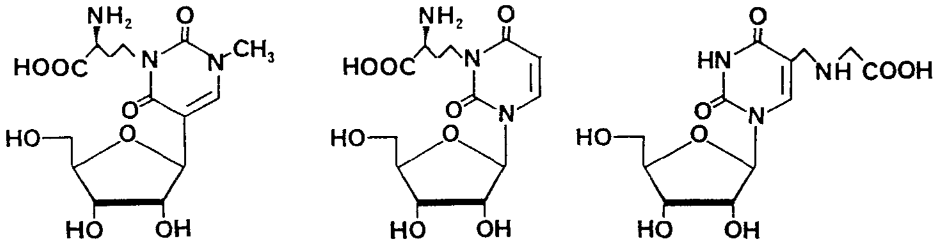


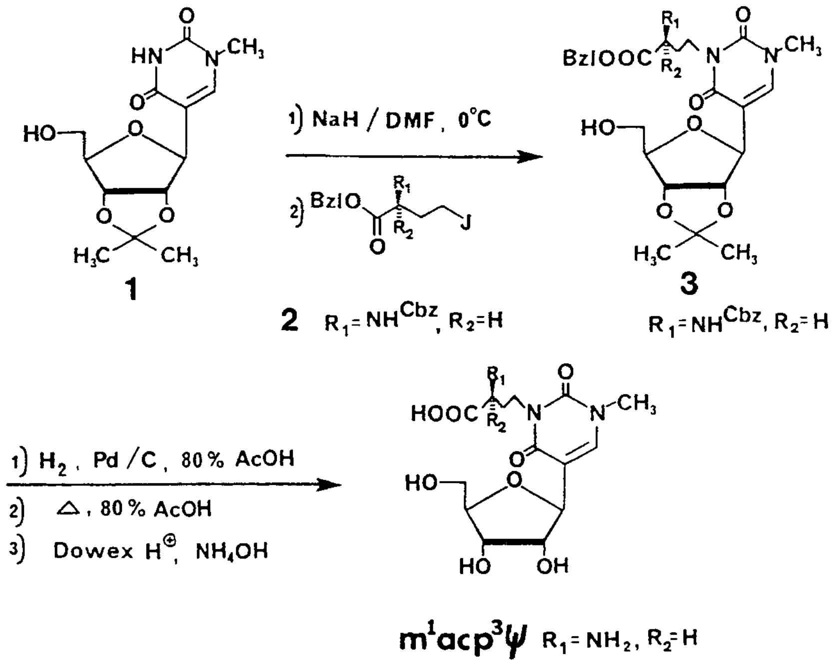
m^{1}acp$^{3}\Psi$ acp^{3}U cmnm^{5}U

FIG. 1. The modified uridines m^{1}acp$^{3}\Psi$, acp^{3}U and cmnm^{5}U.

The N-3 α-amino acid substituted nucleosides acp^{3}U and m^{1}acp$^{3}\Psi$ (Figure 1) could contribute to the dynamic process of RNA folding and structure, and influence thermodynamic parameters of homo- and heterotrophic interactions of the RNA in the cell. The tendency of amino acids to form hydrogen bonds and chelate bivalent cations is well documented,[31] and this is probably also true of the amino acid side chain modifications of nucleosides. In the crystal lattice of cmnm^{5}U (Figure 1), the amino acid residue acts as a donor as well as an acceptor of protons in five hydrogen bonds.[32] We have found in the study of seven different modified uridines that the 5-position modified uridines coordinate strongly with Cu^{2+} and Ni^{2+}, and are weak chelating agents for Zn^{2+}, but do not coordinate with Mg^{2+} (A. Malkiewicz, unpublished results). This is also true for 6-threonylcarbamoyladenosine (t^{6}A).[33] In this context, the study of the conformation and physiochemical properties of oligoribonucleotides carrying amino acids modified nucleosides such as acp^{3}U, m^{1}acp$^{3}\Psi$ cmnm^{5}U[34-37], would be very useful.

Results

Synthesis of m^{1}acp$^{3}\Psi$. The synthesis of m^{1}acp$^{3}\Psi$ follows the protocol we had designed for the preparation of 3-[3-(S)-amino-3-carboxypropyl]-uridine, acp^{3}U, and its 3R epimer.[30] Thus, 2'3'-O-isopropylidene-1-methyl-5-β-D-pseudouridine, **1**[38,39] and benzyl 2-(S)-benzyloxycarbonyl-amino-4-O-(p-toluenesulfnyl)butyrate, **2**[30] were selected for the synthesis of m^{1}acp$^{3}\Psi$, (SCHEME 1). The condensation of the alkylating agent **2** with the monoanion of **1** resulted in the protected nucleoside **3**, which was easily isolated by silica gel column chromatography in good yield (72%). The deprotection of the α-amino acid side chain of **3** was achieved by hydrogenolysis (H_2, 10%Pd/C, SCHEME 1



atmospheric pressure) in 80% acetic acid. After separation from the catalyst, the filtered solution was refluxed for 3 hr to remove the isopropylidene protection. The nucleoside m^{1}acp$^3\Psi$ was eluted from an ion exchange column (Dowex H$^+$) with 10% aqueous ammonia and purified by the paper chromatography. Under the conditions of the deprotection step, no reduction of the pseudouridine heterobase moiety was observed. The structure and purity of nucleoside m^{1}acp$^3\Psi$ has been confirmed by spectral and chromatographic data. Sharp resonances obtained for m^{1}acp$^3\Psi$ in the proton NMR spectrum indicated clearly the high purity and lack of diastereomers for this synthetic nucleoside. The UV as well as CD and ORD spectra of nucleoside m^{1}acp$^3\Psi$ (FIGURE 2) are distinctly different from those reported for acp^{3}U[1,30].

Conformations of m^{1}acp$^3\Psi$ and acp^{3}U. The conformations of the modified uridines acp^{3}U and m^{1}acp$^3\Psi$ were determined by NMR spectrometry. Structural comparisons of the two nucleosides with uridine (U) and pseudouridine (Ψ) 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. Proton signals were assigned (Table 1) through chemical shift considerations and scalar correlations by

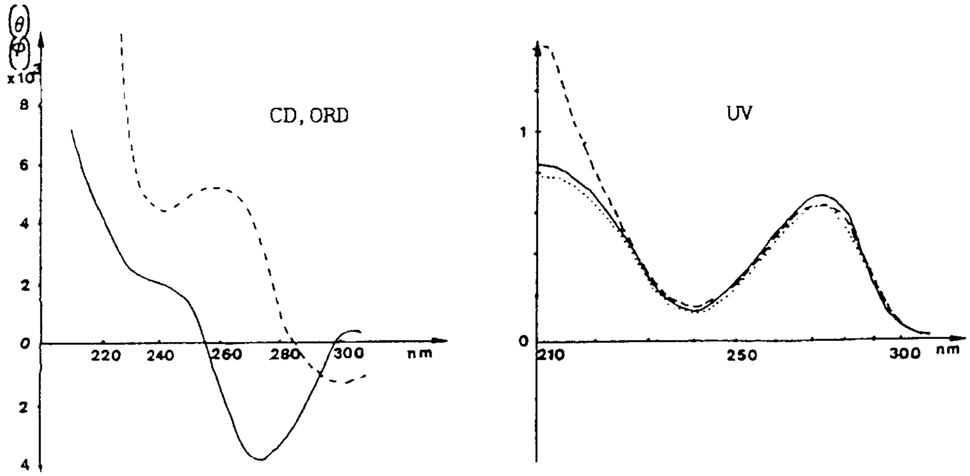


FIG. 2. The CD, ORD and UV spectra of **$m^1acp^3\Psi$**.
CD: pH 7 (-), ORD: pH 7 (---), UV: pH 7 (-), pH 2 (···), pH 12 (---)

TABLE 1. Chemical Shifts[a] of acp^3U, $m^1acp^3\Psi$, U and Ψ.

	U[b]		acp^3U		$m^1acp^3\Psi$		Ψ[c]	
	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
CH3(1)	--	--	--	--	3.40	36.95	--	--
CH2(α)	--	--	4.05	37.27	4.07	37.36	--	--
CH2(β)	--	--	2.20	28.03	2.20	27.97	--	--
CH(γ)	--	--	3.68	52.29	3.69	52.25	--	--
CO(δ)	--	--	--	174.19	--	174.02	--	--
2	--	152.34	--	151.59	--	152.35	--	151.10
4	--	167.30	--	165.01	--	163.70	--	163.57
5	5.90	103.40	5.93	101.36	--	109.76	--	108.75
6	7.90	143.00	7.85	139.66	7.74	143.98	7.64	140.05
1′	5.85	90.60	5.90	90.14	4.70	79.24	4.66	77.45
2′	4.32	74.80	4.34	73.50	4.26	73.23	4.28	71.43
3′	4.21	70.60	4.20	68.97	4.13	70.35	4.13	69.27
4′	4.11	85.50	4.12	83.79	4.00	82.84	4.00	81.75
5′	3.90, 3.76	62.00	3.91, 3.79	60.36	3.85, 3.73	61.06	3.83, 3.71	59.86

[a] Chemical shifts for the nucleosides in 98% D_2O, are referenced to TMS
[b] From reference 34 (in phosphate buffered saline solution, 98% D_2O)
[c] From authors' work and in verification of reference 40.

analysis of the two dimensional homonuclear COSY and HOHAHA spectra. The NOESY experiment was helpful in the assignment of the ^1H resonances. Previously reported chemical shifts for uridine and the other mononucleosides[34,41] aided in the resonance assignments.

Carbon signal assignments (Table 1) were accomplished by correlation to the proton chemical shifts using 2D-heteronuclear correlation spectroscopy (HMQC). The chemical shifts of the correlated carbon resonances also provided a useful check of the proton assignments because carbon chemical shifts more reliably reflect the nucleoside's electronic environment.[41,43] The C1'-C5 linkage of $m^1ac^3p\Psi$, compared to the N1-glycoside of acp^3U, predictably effects the chemical shifts of C5 and C6, shifting both downfield with respect to acp^3U. To assign the five quaternary carbons we used a multiple bond heteronuclear correlation experiment, HMBC. Each proton at position-6 exhibited 3-bond correlations to its respective carbonyl carbons, $^{13}C(2)$ and $^{13}C(4)$.[34] Small correlations from the $^1H(1')$ to the $^{13}C(2)$ were detected and these too aided in the identification of the quaternary carbons. The ^{13}C carbonyl on the propyl chain was identified by its correlations with $^1H(\gamma)$ and $^1H(\beta)$. The base $^{13}C(5)$ in $m^1acp^3\Psi$ showed correlations from $^1H(6)$ and $^1H(1')$. The proton and carbon chemical shifts of U and Ψ are listed in Table 1 for comparison to the two modified derivatives.

Coupling Constants. Scalar ^1H-^1H coupling constants of acp^3U and $m^1acp^3\Psi$ were determined from the spectra and are given in Table 2 along with that for U and Ψ. 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 spectrum. 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). Specific scalar couplings, again, seem to be effected by whether the base is N-linked or C-linked to the ribose.

Nucleoside Conformation. The conformations of the individual nucleosides are shown in Table 2. The predominating conformation around the glycosidic bond (χ) was determined qualitatively 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[44]. Thus, for N-glycoside bonds when the dipolar correlation from H(6) to H(2') is stronger than that from H(6)-H(1'), the anti conformation is indicated for acp^3U and U. When the relative intensity of the correlations is reversed, the syn conformation is indicated. In the case of acp^3U both cross-peaks were observed and were of equal intensity, indicating equivalent amounts of both syn and anti conformations, Table 2.

TABLE 2. Coupling Constants (J_{HH}) and Conformations

	U	acp^{3}U	m^{1}acp$^3\Psi$	Ψ
J1',2'	4.6	3.9	5.1	5.7
J2',3'	5.2	5.2	5.6	5.3
J3',4'	5.2	5.8	5.0	5.3
J4',5'	2.8	2.7	3.0	3.3
J4',5"	4.3	4.4	4.5	4.9
J5',5"	12.5	12.8	12.8	12.6
J$\alpha\beta$	---	6.9	4.0	---
J$\beta\gamma$	---	5.8	5.0	---
J6,1'	---	---	---	0.82
CONFORMATIONS				
%N[a]	50	60	40	30
%γ^{+}[b]	59	66	62	56
χ[c]	anti	anti-syn	anti-syn	anti[43]

[a] With regard to sugar conformation, %N is the proportion of 3'-endo conformer derived as previously for modified uridines and other nucleosides: %N = (J1'2'/6) x 100.[34,42,49]

[b] The ribose %γ^{+} is the proportion of gauche^{+} conformer derived from ^{1}H(4')-^{1}H(5',5") couplings: %γ^{+} = [(13.75-J4'5'-J4'5")/10.05] x 100.[34,42,49]

[c] Definition of the anti-syn conformation (Ξ) of the glycosidic bond reference 44, and especially for the Ψ C-C linkage is according to reference 45. χ gives the predominant conformation around the glycosidic angle based on the relative magnitudes of the NOE connectivities: H(6)-H(2') > H(6)-H(1') = anti; H(6)-H(1') > H(6)-H(2') = syn.[44]

The conformation around the Ψ glycosidic bond has been previously defined[45]; an H(6) to H(1') dipolar correlation is consistent with an anti conformation, H(6) to H(2') with a syn conformation. The anti conformer of Ψ predominated; the syn conformer was at the limit of detection. However, almost equivalent amounts of the two conformers were found for the nucleoside m^{1}acp$^3\Psi$.

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. The fractional population of nucleoside in the 3' endo conformation can be calculated from $J_{H(1')H(2')}$.[46] Since the 1' protons are usually well-resolved doublets, this value is easily and accurately obtained. The values calculated for acp^{3}U indicated that the nucleoside prefers, at room temperature, the 3'-endo conformation by 60% (average

%N=60) over the 2′-endo conformer. However, $m^1acp^3\Psi$ has a greater fractional population of the 2′-endo conformer, ≈60% (%N=40). The α-β couplings of the two nucleosides are different, indicating that one of the two has an added constraint. The additional constraint could be hydrogen bonding (through H_2O) to one of the carbonyls.[32] For acp^3U, as compared to $m^1acp^3\Psi$, there doesn′t appear to be a difference in the conformation around the C(5′)-C(4′) bond ($\%\gamma^+$).

Discussion

We have found that the expected chemical shifts, intensities and couplings distinguish the N(3) side chain α- and β-CH_2 and γ-CH proton resonances of both acp^3U and $m^1acp^3\Psi$. The CH_2-β was found upfield of the CH_2-α since it has no heteroatom connections. The methylene protons at the α position did not have distinguishable chemical shifts; the same was true at the β position. However, this is in contrast to the α methylene protons of s^2mnm^5U which do exhibit distinguishable chemical shifts.[42] The 0.8-3 ppm upfield shifts of C(2,4,5 and 6) of acp^3U indicated an increased electron density for the ring of acp^3U vs. U. Otherwise, the carbon and proton chemical shifts of the modified nucleosides did not exhibit significant differences when compared to their respective unmodified U and Ψ.

In characterizing the two modified nucleoside conformations relative to their parent nucleosides, it is easy to see that there are few conformational differences. It is interesting to note that the one significant difference between the modified nucleosides and their parent compounds was that the solution structures of the modified nucleosides included equivalent amounts of the syn conformer, whereas the solution structures of the parent compounds did not. We utilized NOE intensities to qualitatively determine the predominant syn/anti conformer; the syn-anti conformation can also be obtained from the $^3J_{C(6)-H(1')}$.[50] Quantitation of the syn and anti conformers from NOESY intensities was not attempted because, although there are calibration standards for purines[51], there are at present none for pyrimidines, especially modified pyrimidines.

In characterizing the two modified nucleoside conformations relative to each other, it is easy to see that there is only one significant conformational difference. The nucleoside acp^3U is 60% 3′-endo, whereas $m^1acp^3\Psi$ was found to be 60% 2′-endo under the same conditions. Modifications of nucleosides, in general, have been shown to be important for the structure-function relationships of tRNAs[47,48]. Modifications of uridines, in particular, have been shown to determine the nucleoside′s local conformation and dynamics[34,49] and, in the case of 2-thiolation, to effect the conformation of the neighboring unmodified uridine.[42] These conformational perturbations through modification have significant implications for function, such as tRNA anticodon recognition of codon[48]. The nucleoside acp^{3}U is found in various sequences of the dihydro-uridine loop of tRNAs, but when found in the extra arm it is always preceded by m^{7}G. Our results indicate that acp^{3}U has the ability to take multiple conformations although the predominant conformer is anti, 3'-endo, gauche$^+$. The conformation could be influenced by local structure and chemistry, which is particularly true when considering acp^{3}U in the extra arm next to m^{7}G. Methyl-7-G has been shown to be positively charged in the 3° structure of tRNA and to be a zwitterion when the triple base interaction of which it is part in yeast tRNA^{Phe} denatures at low temperature[42]. The extra arm is in contact with the aminoacyl-tRNA synthetase[52]. So far acp^{3}U has been found in the extra arms of five of the ten aminoacyl-tRNAs that have Class I synthetases, and in only two of the Class II.

The nucleoside m^{1}acp^{3}Ψ, which is chemically related to acp^{3}U, has only been found in rRNAs. It has been found in the 18S rRNA of the mammalian small ribosomal subunit and the 17S rRNA of yeast in the sequence AAC-m^{1}acp^{3}Ψ-CACACG.[28] The function of m^{1}acp^{3}Ψ is unknown. Transfer RNA is known for having a large number of Ψs at various positions. The nucleoside Ψ in tRNA is versatile in hydrogen bonding because both nitrogens and both carbonyls are free as proton donors and acceptors. At the bottom of the anticodon stem Ψ, in a A-Ψ base pair, stabilizes the stem relative to U in the same position[53]. However, the m^1- and acp^3-modifications negate hydrogen bonding to the N1 and N3. Perhaps the modified nucleoside in rRNA is at a location where hydrogen bonding would be disadvantageous. To more fully understand the implications of the modified uridine structure we are beginning to study its conformation in the context of an oligonucleotide sequence.

Experimental

Analytical Instrumentation. Proton NMR spectra for analysis of synthesis: BRUKER MSL (300 MHz) with TMS as the external standard. Electron impact mass spectrometry (MS) at 70eV and 15eV: a GS MS LKB 2091 instrument. CD and ORD spectra: JASCO J-20 Automatic Recording Spectropolarimeter, room temperature, 10 mm cell. UV spectrum: Specord UV-VIS Spectrometer. High-performance liquid chromatography (HPLC) was accomplished with a LDC/Milton Roy equipped with a UV spectroMonitor 3100, λ=254nm; C-18 (Wydac); stainless steel column (250x4.6mm), 0.1 M aqueous triethyl-ammonium bicarbonate (pH 7.5), flow rate 1 ml/min.

Thin layer chromatography (TLC) was performed on silica gel 60 F$_{254}$ (Merck) plates in the solvent systems (v/v): (A) chloroform/methanol 95/5; (B) isopropanol/water/ammonium hydroxide 7/2/1. Silica gel (70-230 mesh) was used for flash column chromatography. Paper chromatography was accomplished with Whatman paper (3MM). Evaporations were carried out under reduced pressure and bath temperature below 40°C.

1-Methyl-3[3-(S)-benzyloxycarbonylamino-3-benzyloxycarbonyl propyl]-2',3'-O-isopropylidenepseudouridine, 3. NaH (48 mg, 2 mmol) was added to the cooled (0°C) solution of 2'3'-O-isopropylidene-1-methyl-5-β-D-pseudouridine 1 (596 mg, 2 mmol), in anhydrous N,N-dimethylformamide (10 ml). Compound 1 had been prepared from pseudouridine according to Watanabe et al.^{38,39} The reaction mixture was stirred for 30 min. After warming to room temperature, a solution of benzyl-2-(S)-benzyloxy-carbonylamino-4-O-(p-toluenesulfnyl)butyrate, 2^{30} (897 mg, 2.1 mmol) in N,N-di-methylformamide (5 ml) was added dropwise to the reaction mixture. The reaction mixture was heated for 3 hr at 60-80°C and kept at room temperature for 20 hr. After removal of the solvent, the residue was co-evaporated twice with toluene and dissolved in chloroform (100 ml). The organic layer was washed with water (20 ml), dried over $MgSO_4$ and concentrated to dryness. The residue was chromatographed over a silica gel column with chloroform/methanol (99/1) as an eluent to give 3 (915 mg, 72% yield). TLC: R_f = 0.4 (solvent system A), [R_f of 2',3'-O-isopropylidene-1-methylpseudouridine = 0.18 (A)]. ^{1}H NMR ($CDCl_3$): δ ppm: 7.36-7.32 (m, 10 H, two phenyl groups), 7.15 (s, 1H, H-6), 5.82 (br s, 1H, NH), 5.12 (s, 2H, CH_2 of benzyl ester), 5.10 (q, J=15.4 Hz, 2H, CH_2 of N-Cbz group), 4.93-4.91 (m, 2H, H-1', H-2"), 4.54 (m, 1H, 5'-OH), 4.41 (d, J=4.7 Hz, 1H, H-3'), 4.25 (m, 1H, N-CH), 4.05 (t, J=7.1 Hz, 2H, N-$\underline{CH_2}$-CH_2), 3.84-3.70 (m, 3H, H-4', H-5', H-5"), 3.30 (s, 3H, N-CH_3), 2.18-2.16 (m, 2H, N-CH_2-$\underline{CH_2}$), 1.57 (s, 3H, CH_3), 1.32 (s, 3H, CH_3); MS (70eV) m/z (rel. int.): 623 (0.35%) M, 490 (2.55%), 400 (2.19%), 91 (100%); 15eV: 623 (3.83%) M, 608 (6.63%) M-15, 605 (12.73%), 532 (10.29%), 490 (23.03%), 488 (10.64%), 400 (22.33%).

1-Methyl-3-[3-(S)-amino-3-carboxypropyl]pseudouridine, m^{1}acp^{3}ψ. Pd/C (10% 100 mg) was added to the stirred solution of 3 (600 mg, 0.96 mmol) in 80% acetic acid/H_2O (50 ml). Hydrogen was bubbled through the reaction mixture for 3 hr at room temperature. The catalyst was removed by filtration and washed with 80% acetic acid. The filtered solution was refluxed for 1 hr, the solvent removed *in vacuo*, and the residue was co-evaporated several times with water. The water solution was transferred to a Dowex H^+ (50WX1 50-100 mesh, 6 ml) column which was washed with water to neutrality. The product was eluted with 10% NH_4OH (50 ml) and purified by Whatman 3MM paper chromatography with isopropanol/water/ammonium hydroxide (7/2/1). Compound m^{1}acp^{3}Ψ was obtained in 75% yield, 248 mg. TLC: R_f=0.23 (solvent system B); positive ninhydrin test; HPLC (min): R_T m^{1}acp^{3}Ψ=5.73 [R_TX=6.14, R_TU=5.24]; UV: (H_2O) λ_{max}=275 nm (ϵ=8.6x10^3), pH 2 λ_{max}=275 nm, pH 12 λ_{max}=275 nm; MS of a tetra TMS derivative (70eV), m/z (rel. int.): 632 (0.42%) M-15, 602 (5.96%), 547 (23.28%), 475 (5.70%), 313 (12.03%), 73 (100%); 15eV: 632 (1.39%) M-15, 602 (31.93%), 547 (100%), 475 (24.67%), 313 (28.78%).

Conformational Analysis by NMR Spectroscopy. The lyophilized samples were dissolved at a concentration of ~8 mmol in 98% D_2O. Spectroscopy was accomplished on a GE Omega 500 at 25°C. One-dimensional ^1H and ^{13}C spectra, two-dimensional (2D) phase-sensitive COSY and NOESY spectra, two-dimensional reverse-detected heteronuclear shift correlation (HMQC) spectra, and two-dimensional reverse detected multiple bond heteronuclear shift correlation (HMBC) spectra, were collected according to previously published methods.[34,42,49]

Coupling constants. Determination of scalar coupling constants was aided by spectral simulation using the PMR program (Serena Software).

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