Conformational characteristics of the hexanucleoside pentaphosphate AUAUUAU: a 2D NMR study at 500 MHz

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ABSTRACT

All 36 ribose proton resonances and most of the base proton resonances of the hexanucleoside pentaphosphate AUAUUAU have been assigned unequivocally using 2D J-resolved spectroscopy, spin echo correlated spectroscopy (SECSY) and 2D NOE spectroscopy (NOESY).

The NMR parameters of AUAUUAU are compared with those of smaller fragments that contain methylated adenine bases: m_2^5AU , $m_2^6AUm_2^5A$, $m_2^6AUm_2^5AU$ and $m_2^6AUm_2^5AUm_2^5A$. Previous studies on this series of compounds have shown that in all these cases purine-pyrimidine-purine sequences prefer to adopt a mixture of states which have as common feature that the interior pyrimidine residues are bulged out, whereas the purine residues stack upon each other.

Chemical shift data, proton-proton coupling constants, as well as the observation of imino-proton resonances for AUAUUAU show unambiguously that upon lowering the temperature the high-temperature "bulged out" situation reverts to a normal A-RNA-like double helix.

INTRODUCTION ^{1,2}

The advent of modern two-dimensional NMR techniques has enabled the assignment of oligonucleotides of ever increasing length. Since 1982 2D techniques^{3,4}, such as COSY, SECSY, J-resolved spectroscopy and NOESY are commonly employed to assign the non-exchangeable proton resonances of oligoribonucleotides⁵⁻⁷, as well as of oligodeoxyribonucleotides⁸⁻¹⁴, and of a DNA-RNA hybrid¹⁵. In the case of NMR spectra of deoxyribonucleotides complete assignments have been reported up to the octamer level¹⁴, and a partial assignment (excluding the H5' and H5'' proton resonances) of a dodecanucleotide has been published¹⁶. However, in the case of oligoribonucleotides complete assignments are rare, and to the best of our knowledge no such assignments of spectra of hexamers have been reported as yet. Of course, in general the proton spectra displayed by RNA fragments are more complicated than those of DNA oligomers of comparable length. This is due to the fact that H2' of the former resonates within the same crowded spectral region as H3', H4', H5' and H5''. With a few exceptions^{8,9,13,15} thus far no sets of coupling constants have been reported in NMR studies of oligonucleotides containing six or more residues.

From a conformational point of view this situation is disappointing, because coupling constants represent a valuable source of information concerning the geometry of nucleic acid constituents.

In the present paper an NMR study of the RNA hexanucleoside pentaphosphate AUAUAAU is presented. This compound is of considerable interest, as it contains two purine-pyrimidine-purine base sequences. Lee and Tinoco¹⁷⁻¹⁹ have suggested from an NMR study on thirteen trinucleoside diphosphates that those three examples with a purine-pyrimidine-purine type of base sequence prefer to adopt a conformation in which the two purines are stacked and the pyrimidine is bulged out of the stack.

In several previous studies from this laboratory the phenomenon of "bulged bases" was investigated thoroughly using the series $\overline{AU}\overline{A}$ ⁵, $\overline{AU}\overline{AU}$ ²⁰ and $\overline{AU}\overline{AU}\overline{A}$ ⁷ ($\overline{A} = m_2^6A$) as model compounds. $\overline{AU}\overline{A}$ represents an example of a trinucleotide with a bulged interior U-residue; in $\overline{AU}\overline{AU}$ the $\overline{AU}\overline{A}$ -part has similar characteristics as the trimer, however, the 3'-terminal $\overline{AU}$ moiety is involved in a regular stacking interaction; in the pentanucleotide $\overline{AU}\overline{AU}\overline{A}$, two bulged U-residues exist side by side (Figure 1). In contrast to these $\overline{A}$ and U containing oligonucleotides, the hexanucleotide AUAUAAU contains non-methylated adenine bases. Therefore, the hexamer in principle is capable of forming a classical Watson-Crick-type RNA duplex.

At this point the question arises whether or not the capability of forming interstrand hydrogen bridges disturbs the usual pattern of bulging U residues. This question is of some importance, as several studies have indicated that bulged residues may occur in double helical RNA fragments when extra non-Watson-Crick bases are inserted into a double helical fragment^{21,22}. Moreover, single bulged residues frequently occur in natural ribosomal RNA^{23,24}, and it has been suggested that these residues play an important role in the formation of stable 5S RNA-protein complexes²⁵.

In the present study the complete assignment of all resonances (except three H2 resonances of adenine) by means of two-dimensional NMR is described. Moreover, a virtually complete set of $^3J_{HH}$ coupling constants is presented, which enables the determination of the preferred conformations of the sugar rings and the backbone angles α .

MATERIALS AND METHODS

NMR samples

The title compound was synthesized via an improved phosphotriester approach²⁶ and treated with Dowex-cation exchange resin to yield the sodium salt. 16 mg hexanucleotide was lyophilized three times from 99.75% D₂O and taken up

Figure 1: Schematic representation of the preferred conformations of $\bar{A}U$, $\bar{A}U\bar{A}$, $\bar{A}U\bar{A}U$ and $\bar{A}U\bar{A}U\bar{A}$.

in 0.4 ml 99.95% D_2O ; the pH was adjusted to 7.5 ± 0.5 (meter reading); tetramethyl ammonium chloride (TMA) was added as an internal reference and ethylenediaminetetraacetic acid was added to neutralize paramagnetic contaminations. Finally, the sample was degassed and sealed under dry nitrogen gas.

A second sample was prepared from 4 mg of hexanucleotide, which was dissolved in 0.4 ml of a 85/15 H_2O/D_2O mixture in order to observe the imino proton resonances.

NMR spectroscopy

NMR spectra were acquired on a Bruker WM-500 spectrometer, equipped with a probe for triple resonance purposes. 1D spectra were recorded on 8K datapoints. Resolution was enhanced by application of a Gaussian window and FIDs were zero-filled to 32K before Fourier transformation.

The SECSY spectrum was recorded using the basic pulse sequence of Nagayama et al²⁷ with the following modifications (eq. 1): the pulse sequence was preceded by a 180° pulse and an appropriate delay in order to suppress the residual HDO peak²⁸. Moreover, the usual second 90° pulse was replaced by a 45° pulse in order to obtain a better resolution near $f_1 = 0^{29}$. Simultaneous ^{31}P decoupling greatly improved the resolution of cross-peaks. 256 FIDs consisting of 2K datapoints were recorded, a sine-bell window was applied in both directions. Before Fourier transformation the spectrum was zero-filled to 1K (f_1) and 4K (f_2), respectively.

$$[180^\circ - \tau - 90^\circ - 1/2t_1 - 45^\circ - 1/2t_1 - t_2]_n \quad (1)$$

The J-resolved spectrum was recorded using the pulse sequence of Nagayama et al³⁰, with a preceding 180° pulse and simultaneous ^{31}P decoupling (eq. 2). 64 FIDs consisting of 8K datapoints were recorded. A phase shifted ($1/6 \pi$) sine-square window was applied in the f_1 direction and a sine-bell window was used in the f_2 direction. Before Fourier transformation the spectrum was zero-filled to 256 and 16K datapoints, respectively.

$$[180^\circ - \tau - 90^\circ - 1/2t_1 - 180^\circ - 1/2t_1 - t_2]_n \quad (2)$$

The above-mentioned 2D spectra were recorded at $32^\circ C$. This temperature was selected as it provides the most favourable conditions for the complete assign-

Figure 2: Conformational nomenclature.

ment of the hexamer. At this particular temperature the residual HDO peak resonates within a blank spectral region and at the same time overlapping of high-field ribose signals is reduced to a minimum.

The NOESY³¹ spectrum (eq. 3) was recorded at 27 °C. The residual solvent peak was suppressed by selective saturation of the HDO signal. A mixing time of 0.8s was chosen. Although this mixing time certainly does not exclude magnetization transfer by "spin diffusion", and therefore cannot provide reliable distance information, it serves well for assignment purposes in nucleic acid chemistry.

$$[90^\circ - t_1 - 90^\circ - t_m - 90^\circ - t_2]_n \quad (3)$$

The imino proton spectra were measured in a H₂O/D₂O (85/15 v/v) mixture. In order to suppress the strong solvent signal, the solvent suppression technique as described by Haasnoot³² was used. This technique essentially rests upon the combined use of time-shared long pulses and a data handling technique.

Nomenclature

The proposed IUPAC-IUB nomenclature is used throughout this work. A schematic representation is shown in Figure 2³³.

RESULTS AND DISCUSSION

Assignment of the sugar and base proton resonances

The following strategy was adopted for the assignment of the non-exchangeable proton resonances of the hexamer AUAUUAU. The projection of the ³¹P decoupled 2D J-resolved spectrum recorded at 32 °C (Figure 3 A) yields the accurate peak positions of 44 out of 48 resonances. The remaining 4 resonances cluster at ~1.6 ppm and do not appear as well-resolved singlets, due to strong mutual couplings. Note, that ³¹P decoupling is essential, because other-

Figure 3: High-field region of ^1H spectrum of AUAUUAU. A) Projection of the ^{31}P decoupled J-resolved spectrum. B) One dimensional ^{31}P decoupled ^1H spectrum. C) Computer simulation of B.

Figure 4: Contour plot of the ^{31}P decoupled SECSY spectrum of AUAUUAU. Connectivities between the H1' and H2' protons are shown.

Figure 5: Expanded contour plot of the SECSY spectrum. The high field ribose region is shown, together with the projection of the J-resolved spectrum. Connectivities between the ribose protons of residues 1 and 2 are indicated by a broken line and a solid line, respectively.

wise the projection would consist largely of overlapping doublets, which would effectively block an unambiguous assignment. Through coupling pattern recognition as well as by means of chemical shift considerations, each resonance could be attributed in a straightforward way to a specific type of proton (e.g. H1', H2' etc.). It turned out that the clustering four resonances at ~1.6 ppm arise from two pairs of H2' and H3' protons.

The next step of the assignment procedure consisted of the identification of connectivities due to scalar couplings. These connectivities are apparent from the ^{31}P decoupled SECSY spectrum (Figures 4 and 5). Careful inspection of a contour-plot of this spectrum allows the assignment of all sugar protons to six different independent spin systems. Moreover, the H5-H6 connectivities are revealed. However, the SECSY spectrum does not inform us as to which spin system belongs to which nucleotide residue.

The 5'-terminal residue and the 3'-terminal residue are characterized immediately, because these residues lack a 5' and a 3' phosphate group, respectively. This leads to a characteristic high-field shift of the respective H5', H5'' and H3' resonances, as well as to the absence of the corresponding ^1H - ^{31}P couplings in the ^{31}P coupled spectrum. The remaining residues are assigned from the

Figure 6: Contour plot of a NOESY spectrum of AUAUAU at 300 K.

NOESY spectrum, (Figure 6).

Three cross peaks are seen which connect adenine H8 resonances and H1' resonance positions of the same residue and another set of three cross peaks connect uridine H6 peaks with the corresponding H1' resonance positions. At this point the six sets of spin systems can be allotted to three that belong to adenine residues and three that correspond to uridine residues. A(1) and U(6) are already assigned from the lack of coupling to ^{31}P as described above. Thus, it remains to discriminate A(3) from A(5) and U(2) from U(4). This is accomplished with the aid of interresidue NOEs. The low-field doublet of the three H6 resonances shows a strong cross peak to the known H2'(1). Therefore this H6 signal as well as the NOESY-connected ribose signals are assigned to U(2). The remaining set of uridine resonances therefore belongs to U(4).

In principle, it is possible to assign the two A-residues in a similar way, i.e. from the observation of interresidue NOEs between a specific H8 resonance and the H2' of the 5' linked residue. Unfortunately, the H2'(2) and the H2'(4) signals of AUAUAU are isochronous at 27 °C and cannot be used to discriminate between H8(3) and H8(5). Note, however, that the cross peaks in question indeed are present. The H8/H6 cross peaks are not conclusive either as can be glanced from Figure 6. For these reasons 1D NOE difference experiments were

Table 1: Chemical shifts (ppm) and coupling constants (Hz) of AUAUUAU at 32 °C. Chemical shifts are referenced to the methyl peak of tetramethyl ammonium chloride. For conversion to the DSS scale add +3.18 ppm.

proton shift	A(1)	U(2)	Ā(3)	U(4)	A(5)	U(6)
H8	5.013	--	5.070	--	5.132	--
H6	--	4.602	--	4.554	--	4.565
H5	--	2.609	--	2.539	--	2.491
H1'	2.792	2.687	2.730	2.631	2.834	2.642
H2'	1.644	1.135	1.544	1.155	1.548	1.063
H3'	1.592	1.474	1.562	1.431	1.562	1.109
H4'	1.243	1.254	1.339	1.244	1.357	1.069
H5'	0.780	1.032	1.104	1.049	1.109	1.125
H5''	0.678	0.976	1.002	0.969	1.007	0.955
coupling						
1'2'	5.5	5.9	5.1	5.8	4.2	4.1
2'3'	5.1	5.2	5.5	5.1	5.5	5.4
3'4'	4.0	3.7	4.4	4.2	5.3	5.5
4'5'	2.5	2.5	2.6	2.5	2.6	2.7
4'5''	3.3	3.0	3.8	3.0	3.8	2.7
5'5''	-13.0	-11.8	-11.8	-11.8	-11.8	-11.8

performed at 32 °C, at which temperature the H2'(2) and H2'(4) resonances are sufficiently separated. Indeed, irradiation of each of the two H8 resonances in turn yield strong and specific NOEs to the corresponding H2' resonances of the 5' linked U(2) and U(4) residues. This experiment effectively completes the assignment.

In Figure 3C a computer simulation of the ^{31}P decoupled spectrum of AUAUUAU at 34 °C is shown. Chemical shifts and coupling constants, which were acquired from the 2D J-resolved experiment were used (after small adjustments) as input data (Table 1). The agreement between simulation and experiment is seen to be excellent, which supports the correctness of the assignment reported above. No attempt was made to obtain a computer simulation of the phosphorus coupled spectrum, as in this case individual signals are barely resolved. For this reason accurate determination of ^1H - ^{31}P coupling constants is precluded.

Chemical shifts of the non-exchangeable protons

The temperature dependence of proton chemical shifts constitutes a sensitive probe to monitor conformational equilibria. In a previous paper⁷ the temperature dependency of the chemical shifts of $\bar{\text{A}}\text{U}\bar{\text{A}}\text{U}\bar{\text{A}}$ was studied. Chemical shifts of the pentamer were compared with those of the appropriate dimers and monomers. At all temperatures the pentamer displays an excess of shielding of the protons of the $\bar{\text{A}}$ residues, and a lack of shielding of the protons of the U residues. It was concluded that the $\bar{\text{A}}$ bases exert mutual shielding effects, but do not appear to significantly shield the U residues. In other words, the $\bar{\text{A}}$ res-

Figure 7: Chemical shifts vs. temperature profiles of AUAUAU, $\overline{\text{AUUAUA}}$ and $\overline{\text{AU}}$. From the left to the right (starting at the top) H5, H1' of U-residues, H6, H1' of A residues, H8. Symbols H, P and D refer to the hexamer, the pentamer and the dimer, respectively. Thus, for example, H(3) refers to residue 3 of the hexamer.

idues have stacking interactions with each other and the U residues are turned away (bulged out).

In Figure 7 the chemical shift vs. temperature profiles of the 1' protons and of the base protons of AUAUAU are compared to those of $\overline{\text{AUUAUA}}$ and to those of $\overline{\text{AU}}$. It is apparent from Figure 7 that the three H5 protons of $\overline{\text{AUUAUA}}$, which at elevated temperatures have similar chemical shifts as those of the pen-

Figure 8: Imino proton spectra of AUAUAU at -4, -1 and +4 °C.

tamer, become strongly shielded when the temperature is lowered. The same effect is seen for the H1' of the residues U(2), U(4) and U(6) and for the H6 of U(4) and U(6). In all these cases the pattern observed for the chemical shift vs. temperature profiles is similar to the behaviour of the U protons of $\bar{A}U$. It is well known that the latter compound displays a strong preference for a stacked state at lower temperatures³⁴. It is concluded, therefore, that the U bases of the hexamer at high temperature exist in either a bulge-out situation or in a randomly coiled strand. At temperatures below about 35-40 °C the U residues become involved in vertical stacking interactions with the A bases. Judging from the relative steepness of the chemical shift vs. temperature profiles compared to those of the dimer $\bar{A}U$, the stacking process in the hexamer is strongly cooperative. This would be expected if the main driving force for this process is the formation of a double helix.

At high temperatures the H8 protons of the (non-methylated) A bases in the hexamer resonate 0.15-0.2 ppm downfield from those of the m_2^6A bases in the pentamer. Comparison of the H8 chemical shifts of non-methylated vs. methylated monomers³⁵ shows that N6-dimethylation causes a 0.1 ppm downfield shift of H8 signals. After correction for the effect of the CH_3 groups it is seen that the H8 protons of the hexamer are slightly deshielded with respect to those of the pentamer at high temperature. Perhaps this is indicative for a weaker A--A next-nearest-neighbour stacking in the non-methylated AUA sequence, analogous to the weaker A--A nearest neighbour stacking in ApA ($T_m = 295$ K) com-

pared to $\bar{A}p\bar{A}$ ($T_m \approx 318$ K)²⁵.

At lower temperatures (<35 °C) the chemical shift profiles of the H8 signals of the hexamer, notably those assigned to H8(3) and H8(5), deviate from the H8 profiles of the bulged-out pentamer. In the latter case the signals of H8(1), H8(3) and H8(5) move linearly upfield when the temperature is lowered; in the former case a distinct non-linear upfield shift is seen. Remembering that the chemical shift of H8 is rather insensitive to the position of the U bases, it is suggested that the H8 upfield shifts in the hexamer are caused by the ring currents of the A bases in the opposite strand of a double helix which starts to form at low temperatures.

Concomitant with the cooperative shielding effects upon several protons of AUAUUAU a gradual broadening of the ¹H NMR spectra is seen at temperatures below 30 °C. Below 12 °C the spectrum rapidly broadens to an extent that all fine structure is lost and no chemical shifts and coupling constants can be measured. This observation forms another strong indication that AUAUUAU starts to dimerize into a double helix at lower temperatures. Exchange broadening is commonly observed in a temperature range where a significant population of duplex equilibrates with a significant population of single strands, see for instance reference 36. Unfortunately, the melting temperature of the double helix appears to be rather low (≤ 12 °C). Attempts to reach the plateau region of chemical shifts which correspond to 100% duplex were unsuccessful. Even when the nucleotide concentration was increased to 60mM or 1M salt was added the spectra remained broadened. The rules proposed by Borer et al³⁷ predict a value of $T_m \approx 22$ °C for 20 mM AUAUUAU and 1 M NaCl. The chemical shift vs. temperature profiles obtained in the present study were run in the absence of added salt. Therefore, a verification of this prediction is precluded.

Imino proton spectra of AUAUUAU

The imino proton spectra of AUAUUAU measured at -4 , -1 and +4 °C are shown in Figure 8. The appearance of three imino proton resonances at 13.7, 13.8 and 14.0 ppm (downfield from DSS) clearly indicates the presence of a double helix with concomitant formation of A•U base pairs. The imino proton peaks broaden out and disappear when the temperature is increased to +4 °C. The chemical shifts of the imino proton resonances are in good agreement with the values predicted by Patel et al.³⁸ (14.0-14.6 ppm).

Conformational analysis of the sugar ring

The ribose ring can assume either of two geometrical types, the N conformer or the S conformer³⁹⁻⁴¹. It is well known that the ribose rings in A-RNA double helices prefer to assume the N-type conformation, whereas in random

Table 2: Pseudorotational analysis of the six ribose rings of AUAU²AU at 32 °C; an asterisk indicates a constrained value, see text.

residue	$\phi(N)$	P(N)	$\phi(S)$	P(S)	x(N)	rms(Hz)
A(1)	37*	17	37*	158	35	0.011
U(2)	37*	19	37*	155	30	0.011
A(3)	37*	20	37*	152	40	0.175
U(4)	37*	20	37*	152	34	0.152
A(5)	37*	20	37*	153	53	0.216
U(6)	37*	18	37*	154*	55	0.083

coils an equilibrium between the above-mentioned two conformers exists. Populations of the N and S conformers can be calculated from the vicinal coupling constants by means of computer program PSEUROT⁴². In this approach a two-state equilibrium is assumed, and coupling constants of the ribose ring are translated into torsion angles by means of a generalized Karplus equation⁴³, which takes into account the electronegativity and orientation of substituents. The calculated ϕ_{HH} torsion angles are directly related to the pseudorotational parameters of the sugar ring through empirical rules, proposed by de Leeuw et al.⁴⁴.

It was shown earlier⁵ that a full pseudorotational analysis in terms of all relevant parameters is only possible in those cases where several sets of coupling constants have been measured at different temperatures, covering a reasonably large shift of the N/S equilibrium constant. In the present case no such data sets are available. Therefore, the amplitudes of puckering ϕ_N and ϕ_S were constrained to adopt a value of 37°, which corresponds to the mean value obtained earlier for a number of small RNA fragments⁴¹. Table 2 shows the phase angles of pseudorotation P_N and P_S and the relative population of N conformer for each of the six ribose rings at 32 °C.

The calculated phase angles are found within the ranges long known from X-ray crystallography⁴⁴ and deserve little comment. Of more interest are the relative N and S populations. At a temperature of 32 °C, where the population of the duplexed structure is still quite small according to the chemical shift vs. temperature profiles (vide supra), the ribose rings of the hexamer display considerable conformational freedom: 30-40 % N-type rings being indicated for residues (1) to (4) and about 55% for residues A(5) and U(6). The higher N population for the terminal sequence is not unexpected, because a similar situation occurs in the tetramer $\bar{A}U\bar{A}U^{20}$. In the tetramer the 3' terminal $\bar{A}U$ sequence experiences normal base-base stacking with the sugars adopting an N-type conformation when the temperature is lowered, whereas the sugars $\bar{A}(1)$ and U(2) retain conformational flexibility. The present data suggest that some

Table 3: The population of N-conformer as a function of temperature for AUAUUAU, $\bar{A}\bar{U}\bar{A}\bar{U}\bar{A}$ and for $\bar{A}\bar{U}$.

T(°C)	AUAUUAU						$\bar{A}\bar{U}\bar{A}\bar{U}\bar{A}$					$\bar{A}\bar{U}$	
	A(1)	U(2)	A(3)	U(4)	A(5)	U(6)	$\bar{A}$ (1)	U(2)	$\bar{A}$ (3)	U(4)	$\bar{A}$ (5)	$\bar{A}$ (1)	U(2)
11	--	--	75	--	80	66	30	25	43	40	52	81	82
15	--	49	66	--	80	65	30	25	--	--	51	79	80
22	45	38	54	52	66	62	30	25	41	--	51	73	76
32	35	30	40	34	53	55	30	25	40	41	48	66	72
54	33	--	43	31	49	--	30	25	38	--	46	52	65
65	32	28	43	--	48	55	30	--	38	--	46	47	62

stacking between A(5) and U(6) also occurs in the hexamer at 32 °C and only a little between the remaining residues at this temperature.

At this point it becomes mandatory to study the conformational behaviour of the six riboses in the hexamer as a function of temperature. If the present interpretation of the chemical shift profiles in terms of an increasing population of duplex structure at low temperature is correct, then one must expect a concomitant increase of N-type population. This is indeed found. A series of 1D spectra taken at temperatures varying from 11 °C to 65 °C, were analyzed to extract first order $J_{1,2}$ couplings. Using the geometrical parameters shown in Table 2 the mole fractions N-type conformer can be reliably approximated from knowledge of $J_{1,2}$. The mole fractions of N conformer are shown in Table 3, along with the comparable values for $\bar{A}\bar{U}\bar{A}\bar{U}\bar{A}$ and of $\bar{A}\bar{U}$. Striking similarities and differences between the hexamer and the pentamer are immediately apparent. At elevated temperatures, the hexamer displays a similar preference for S-type ribose rings as does the pentamer. Upon lowering the temperature, however, the individual ribose equilibria of the pentamer do not shift at all, whereas the riboses of the hexamer in the interval 32-11 °C adopt significant populations of the N-type conformation. Of special interest is the A(5) residue which more strongly resembles the behaviour of $\bar{A}\bar{U}$.

Table 4: Population distribution of the backbone angle γ of AUAUUAU (32 °C), of $\bar{A}\bar{U}\bar{A}\bar{U}\bar{A}$ and of $\bar{A}\bar{U}$ (43 °C).

res.	AUAUUAU			$\bar{A}\bar{U}\bar{A}\bar{U}\bar{A}$			$\bar{A}\bar{U}$		
	g^+	t	g^-	g^+	t	g^-	g^+	t	g^-
A(1)	78	22	0	76	21	3	76	19	5
U(2)	81	18	0	78	20	2	82	17	1
A(3)	72	27	1	63	33	4	--	--	--
U(4)	81	18	0	77	19	5	--	--	--
A(5)	72	27	1	71	24	6	--	--	--
U(6)	83	18	3	--	--	--	--	--	--

Unfortunately, not all $J_{1,2'}$ couplings could be followed down to 11 °C, but there is no doubt that each individual ribose in the strand follows the same trend. Again, these results point towards the formation of a stacked structure, i.e. a duplex, at low temperature.

Backbone angle O5'-C5'-C4'-C3' (χ)

The conformational situation around χ can be monitored by means of $J(4'5')$ and $J(4'5'')$. The existence of a blend of χ^t , χ^- and χ^+ rotamers is assumed, and the population distribution between these conformers is calculated according to Haasnoot et al.⁴⁵. The results are compared to those obtained earlier for $\overline{AUAUA}$ and $\overline{AU}$ in Table 4. Note that the population distributions of the hexamer are given at a slightly lower temperature than those of the pentamer and the dimer. It can be seen that no strong differences between the hexamer and the pentamer occur. This is true because at 32 °C the population of AUAUAU in the duplex state is still rather small

CONCLUSIONS

From a number of previous studies^{5,7,28} on RNA fragments containing $\overline{A}$ and U bases it is known that oligonucleotides consisting of purine-pyrimidine-purine base sequences prefer to occur in a mixture of conformational states, which have the following common feature: the central pyrimidine residue in these sequences is not involved in vertical base-base stacking, whereas the two flanking $\overline{A}$ bases exhibit some kind of a stacking interaction (Figure 1). Evidence for this so-called "bulge-out" situation stems in the first place from the absence of shielding effects on the chemical shifts of the protons of the central pyrimidine residue, concomitant with an excess of shielding for the protons of the purine residues. In the second place, the absence of regular right-handed stacking in purine-pyrimidine-purine sequences is clearly evidenced by the larger degree of conformational freedom of the backbone torsion angles β , χ and ϵ as well as by the extraordinarily low populations of N conformer exhibited by the sugar rings (25-50%).

The abovementioned $\overline{A}$ bases, by virtue of the N6 methylation, are not capable of forming hydrogen bonds to U bases located in an opposite strand. Therefore, all oligonucleotides of this series studied thus far necessarily occur as single-stranded species. Depending on the particular base sequence these single strands may occur as highly stacked species ($\overline{AU}^{24}$, $\overline{AA}^{25}$, $U\overline{AU}^{26}$ and the 3'-terminal moiety of $\overline{AU\overline{AU}}^{28}$), or as bulged out species ($\overline{AU\overline{A}}^5$, the 5' terminus of $\overline{AU\overline{AU}}^{28}$ and both moieties of $\overline{AU\overline{AU\overline{A}}}$ ⁷).

The present study supplies an answer to the question whether or not the bulged-out situation reverts to a helical situation in the case of non-methylated

self-complementary $[A-U]_n$ sequences. Indeed, a number of observations made on AUAUUAU provides strong evidence for the existence of an A-RNA-like double helix at low temperature.

1. NMR spectra gradually broaden out below 30 °C.
2. Chemical shifts of the protons of the U-residues of AUAUUAU diverge from those of $\overline{AU\overline{AU\overline{A}}}$ below 30 °C, i.e. the former become more shielded.
3. At -4 °C three imino proton resonances are seen at ~14 ppm downfield from DSS.
4. All ribose rings of AUAUUAU display a significant and increasing preference for the N conformer upon lowering the temperature below 30 °C.

Thus, it is concluded that the bulged-out situation, which occurs in purine-pyrimidine-purine base sequences, reverts to a helical state in the case of a self-complementary base sequence, which is capable of forming hydrogen bonds to the opposite strand. Therefore, the formation of an extrahelical base in double-stranded RNA appears to be limited to those cases where an extra residue is inserted within a self-complementary strand.

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2. Some oligonucleotides mentioned in this study have modified A-bases: 6-N-(dimethyl)adenine, these will in the remainder of this work be abbreviated as $\overline{A}$. Thus, for example, 6-N-(dimethyl)adenylyl-(3'-5')-uridine is abbreviated as $\overline{AU}$.
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