Conformational analysis of a ribopentannucleoside tetraphosphate in aqueous solution. A two-dimensional NMR study at 500 MHz¹

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ABSTRACT

The 30 ribose proton resonances of the pentaribonucleoside tetraphosphate $m_2^6AUm_2^6AUm_2^6A$ have been assigned unequivocally by means of spin-echo-correlated spectroscopy, 2D J-resolved spectroscopy and Nuclear Overhauser difference spectroscopy, carried out at 500 MHz. A detailed comparison of the conformational properties of the title compound with its constituent fragments $m_2^6AUm_2^6AU$, $m_2^6AUm_2^6A$, m_2^6AU and the relevant monomers is given.

Chemical shift data indicate the existence of a doubly "bulged out" conformer, in which the two interior U-fragments are not involved in regular nearest neighbour stacking interactions. The coupling constants of the ribose-ring are interpreted in terms of the N/S equilibrium, and population distributions along the backbone angles β and γ are presented. The combined data suggest a strong similarity between the 5'-terminal triplets in $m_2^6AUm_2^6AUm_2^6A$, $m_2^6AUm_2^6AU$ and $m_2^6AUm_2^6A$.

INTRODUCTION

It is long known that short oligonucleotide fragments in solution are capable of forming stable, single-stranded helices. In the case of RNA fragments, detailed NMR and CD studies on compounds that stack in a regular way, such as AA^3 , AA^4 , UAU^5 and $AACC^6$ have been carried out in our laboratories. These studies indicate that the geometry of the right-handed single helix is very similar to the geometry of double helices as determined by X-ray crystallography and fiber diffraction⁷. Not all base sequences conform to this pattern, however. In particular, Lee and Tinoco⁸ have demonstrated from an NMR study on thirteen trinucleoside diphosphates that the purine-pyrimidine-purine base sequences: ACG, AUG and GUG display unexpected chemical shift behaviour: the interior pyrimidine base protons and 1' protons are less shielded than expected from the constituent dimers and monomers, whereas the terminal purine residues show an excess of shielding. These facts led the authors⁸ to propose a conformational state in which the interior pyrimidine residue is "bulged out", concomitant with a stacking interaction taking place between the next nearest neighbouring purine residues. A similar conclusion was reached by us from in-depth NMR studies of

Fig 1: Nomenclature of oligonucleotide backbone angles.

the trinucleotide $\bar{A}\bar{U}\bar{A}$ ⁹, and of the higher homologue $\bar{A}\bar{U}\bar{A}\bar{U}$ ¹⁰. Both compounds contain pu-py-pu base sequences, and show little shielding of the U(2) residue, concomitant with excess shielding of the $\bar{A}$ (1) and $\bar{A}$ (3) residues. A detailed description of the conformations of the sugar ring and of the backbone angles has been presented for both the trimer and the tetramer. The amount of N-type sugar ring turned out to be surprisingly low ($\approx 30\%$) for the two residues at the 5' terminal of $\bar{A}\bar{U}\bar{A}$ and $\bar{A}\bar{U}\bar{A}\bar{U}$; the 3'-terminal $\bar{A}\bar{U}$ moiety of $\bar{A}\bar{U}\bar{A}\bar{U}$ was shown to be involved in a regular right handed stack with both ribose rings preferring to adopt an N-type conformation. It was concluded¹⁰ that the bulged out situation, in contrast to the earlier proposal⁸, must be viewed to consist of a time-average blend of bulge conformations. It was speculated¹⁰ that the resulting gain in entropy-of-mixing, together with the (1)...(3) stacking of the purine bases, suffices to offset the natural tendency for nearest neighbour stacking. Thus, perhaps bulging out might be viewed as a local disturbance of the regular stack.

Seen in this light the interesting question arises whether or not two bulges can exist side-by-side. In order to answer this question we here report the complete assignment of the ¹H NMR spectrum of $\bar{A}\bar{U}\bar{A}\bar{U}\bar{A}$, the next higher homologue in the series $\bar{A}\bar{U}$, $\bar{A}\bar{U}\bar{A}$, $\bar{A}\bar{U}\bar{A}\bar{U}$. An interpretation of the chemical shifts and coupling constants in terms of conformational preferences is presented. Moreover the extreme usefulness of ³¹P decoupling in two-dimensional NMR experiments on nucleic acid fragments is demonstrated.

Nomenclature

Recent recommendations to IU-PAC-IUB on nucleotide conformational nomenclature are adopted in this paper¹¹. The backbone angles are denoted α - ζ and nucleotide units are numbered 1-5, starting at the 5' terminal, as shown in Fig.1.

Fig 2: Structure of $\overline{AUAUA}$.

the 5 mM sample on the 300 MHz spectrometer. For purposes of comparison the monomers used in this study bear one or two terminal methyl ester phosphate groups; thus spectra are recorded under comparable conditions (i.e. same pD, and same ionization state of the phosphate group).

Two-dimensional NMR spectroscopy.

Both SECSY and J-resolved experiments were carried out at 44 °C, in order to place the residual HDO peak in a blank spectral region. A small modification was introduced into the basic pulse sequence of Nagayama et al.¹⁵: the residual HDO peak was suppressed by applying an extra 180° pulse plus the appropriate de-

MATERIALS AND METHODS

The title compound (Fig.2) was synthesized via a modified phosphotriester approach^{12,13}. After purification on a DEAE-Sephadex A-25 column, the sample was treated with Dowex cation-exchange resin (Na⁺ form) to yield the sodium salt.

NMR samples were lyophilized three times from 99.75 % D₂O and taken up in 99.95 % D₂O. D₂O samples of two different nucleotide concentrations were prepared, i.e. 30 mM and 5 mM; a trace of tetramethyl ammonium chloride (TMA) was added as an internal reference, and EDTA was added to both samples to remove paramagnetic contaminations. Finally the samples were degassed on a vacuum line and sealed.

NMR spectra were recorded on a Bruker WM-500 and on a Bruker WM-300 spectrometer. The assignment of the ¹H spectrum of $\overline{AUAUA}$ was achieved by means of 2D J-resolved spectroscopy¹⁴, spin echo correlated spectroscopy¹⁵ and NOE difference spectroscopy¹⁶ at 500 MHz, using the 30 mM sample. Additional chemical shift information was obtained from

lay (τ)¹⁷ prior to the standard SECSY experiment, thus resulting in the following pulse sequence:

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

Simultaneously, ^{31}P - ^1H couplings were removed through heteronuclear broadband decoupling. A number of 256 FIDs consisting of 4K datapoints were recorded. After digital filtering with a shifted sine square window (phase shift: $\pi/6$) the FIDs were zero filled to 1K in the f_1 domain, thus a digital resolution of 2 Hz/point in both dimensions of the frequency domain spectrum was achieved.

For the J-resolved experiment the following pulse-sequence was applied:

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

Again the normal pulse-sequence¹⁸ was preceded by an extra 180° pulse plus the appropriate delay τ for water suppression purposes. Simultaneous ^{31}P decoupling enormously simplified the spectrum (vide infra). A number of 64 FIDs consisting of 8K datapoints was recorded. A $\pi/6$ phase-shifted sine square window was used for resolution enhancement, and zero filling to 16K in the f_2 dimension, and to 256 datapoints in the f_1 dimension resulted after Fourier transformation in a digital resolution of 0.5 Hz and 0.1 Hz respectively.

NOE difference spectroscopy

The NOE difference experiments were performed at 500 MHz and at 0°C . Under these conditions negative NOEs are observed. Peaks of base protons were irradiated for a period of 0.5 seconds using sufficient irradiation power to completely saturate the signal. A control spectrum was recorded with the decoupler offset in a blank spectral region. Each on-resonance FID was subtracted from the off-resonance FID; thus the negative NOEs appear as positive peaks. A linebroadening of 1 Hz was applied in order to improve the signal-to-noise ratio.

RESULTS

Assignment

The unambiguous assignment of the ^1H NMR spectra of RNA oligomers presents an extremely difficult task, due to the fact that all sugar protons, except for 1'-protons, resonate within a small region of the spectrum (1.6-0.6 ppm). In the present case 25 mutually coupled signals are contained within a spectral region of 1 ppm. Even the application of modern two-dimensional NMR techniques at 500 MHz in the first instance did not yield an unambiguous assignment of all high-field multiplets of $\overline{\text{AUAUA}}$. Difficulties arose, mainly in 2D J-resolved spectroscopy, due to the presence of ^1H - ^{31}P coupling constants. Signals of H3', H5' and H5'' which are split by vicinal couplings to ^{31}P , and signals of H2' and

Fig 3: Three-dimensional presentation of the ^{31}P decoupled 2D J-resolved spectrum.

H4' which sometimes display four-bond long range ^1H - ^{31}P splitting remain as doublets in the projection of a J-resolved spectrum. The quality of the resulting spectrum thus suffers from residual overlap as well as from smearing-out of the peaks of interest. For this reason the final J-resolved spectrum (Fig.3) as

Fig 4: High-field region of the ^1H spectrum of AUUA with resonances of H2', H3', H4', H5' and H5''. A: Projection of the ^{31}P decoupled J-resolved spectrum. B: ^{31}P decoupled one dimensional spectrum. C: ^{31}P coupled spectrum. D: computer simulation of C.

Fig 5: Contour plot of the ^{31}P -decoupled SECSY spectrum. The plotted region does not include the base protons H8, H2, and H6. Connectivities are indicated between H1' and H2' of all residues.

Fig 6: Expansion of the SECSY spectrum. The high-field ribose region is shown with connectivities between H2', H3', H4', H5' and H5'' of $\bar{A}(1)$ (solid line) and $\bar{A}(3)$ (broken line) indicated.

well as the final SECSY spectrum, were acquired using broad-band decoupling of ^{31}P .

The following strategy was used for the assignment of the pentamer: the ^{31}P decoupled J-resolved spectrum in projection upon the f_2 axis (Fig.4, upper trace) yields the peak positions of all 40 proton signals of the pentamer. Note that the $\text{H4}'(5)$ signal appears as a barely resolved shoulder on the $\text{H2}'(4)$ peak. However, when the resolution of the spectrum is enhanced by means of a sine bell window, this peak appears with better resolution. Attention is drawn to the appearance of a spurious peak, centered between $\text{H5}'(2)$ and $\text{H5}''(2)$, which is due to strong mutual coupling of these protons. Knowledge of the precise resonance positions of all protons is most helpful for the identification of the complex pattern of connectivities appearing in the SECSY plot. Such a plot is shown in Fig.5, where connectivities between the 1' and 2' protons of each ribose residue are indicated. The crowded high-field part of the spectrum can be interpreted from an expanded plot (Fig.6). For the sake of clarity, connectivity patterns of only two ribose residues are indicated; the remaining three residues yield very similar patterns. At this stage all five separate spin systems are identified. Of course, from the experiments described above it cannot be decided which sub-set belongs to which ribose residue. Inspection of the ^{31}P -coupled spectrum partly solves the problem. Because terminal phosphate groups are absent in $\bar{\text{A}}\text{U}\bar{\text{A}}\text{U}\bar{\text{A}}$, the signals of $\text{H3}'(5)$ of the 3'-terminal residue and of $\text{H5}'(1)$ and $\text{H5}''(1)$ at the 5' terminus lack ^{31}P splitting, in contrast to the remaining $\text{H3}'$, $\text{H5}'$ and $\text{H5}''$ signals. In this way residue $\bar{\text{A}}(1)$ and residue $\bar{\text{A}}(5)$ can be assigned unambiguously. Among the remaining three residues, $\bar{\text{A}}(3)$ can be discriminated from $\text{U}(2)$ and $\text{U}(4)$ on the basis of the characteristic low field shift position exhibited by the $\text{H1}'$ of an $\bar{\text{A}}$ residue^{8,10}. This assignment accords with the NOE experiments reported below. At the first sight the discrimination between $\text{U}(2)$ and $\text{U}(4)$ protons seems to be impossible. Fortunately, NOE difference spectroscopy is found to provide for an unambiguous solution of the problem, because both intra- and inter-nucleotide NOEs are readily observed. In Fig.7, the NOE difference spectra that result from irradiation of the low-field H6 and the high-field H6 resonance positions, respectively, are compared to the normal spectrum. Irradiation of the low-field H6 yields intranucleotide NOEs on the low-field H5 , $\text{H1}'$, $\text{H2}'$ and $\text{H3}'$ signals belonging to one and the same U -residue. Moreover, internucleotide NOEs are observed for the $\text{H2}'(1)$ and $\text{H3}'(1)$ resonances of the 5'-terminal $\bar{\text{A}}$ residue. Obviously, on the average, the $\text{U}(2)$ residue has a closer spatial relationship with the $\bar{\text{A}}(1)$ terminal than can be envisaged for the $\text{U}(4)$ residue. Therefore, we conclude that the low-field H6 signal as well as the

Fig 7: NOE difference spectra of $\overline{AUUA}$ resulting from irradiation of the high-field H6 (A), and of the low-field H6 (B). For comparison the control spectrum is shown in the bottom trace (C). The peak due to residual H₂O at 1.8 ppm is omitted from these plots.

Table 1. Proton chemical shifts (ppm) and coupling constants (Hz) of $\overline{AUUA}$ recorded at 44 °C, concentration 30 mM, pH 7. Shifts are relative to TMA, estimated accuracy 0.003 ppm. Coupling constants ± 0.1 Hz, except where indicated by an asterisk (± 0.3 Hz).

proton shift	$\overline{A}(1)$	U(2)	$\overline{A}(3)$	U(4)	$\overline{A}(5)$
8	4.864	--	4.910	--	4.965
2	4.774	--	4.742	--	4.801
6	--	4.577	--	4.516	--
5	--	2.616	--	2.526	--
1'	2.743	2.668	2.702	2.612	2.788
2'	1.599	1.167	1.500	1.155	1.361
3'	1.554	1.477	1.532	1.416	1.251
4'	1.233	1.237	1.331	1.208	1.146
5'	0.765	1.007	1.106	1.039	1.062
5''	0.662	0.980	0.996	0.955	0.971
coupling					
1'2'	5.8	6.2	5.2	5.1	4.6
2'3'	5.2	5.4	5.3	5.1	5.1
3'4'	3.5	3.4	4.2	4.5	5.3
4'5'	2.7	2.6	2.9	2.8	2.9
4'5''	3.3	3.2	4.4	3.2	3.7
5'5''	-13.0	-11.8	-11.8	-11.9	-11.8
2'P	1.1	0.5	0.6	0.5	--
3'P	7.8	8.0	8.0	7.8	--
4'P	--	2.0*	1.8	0.5*	1.5*
5'P	--	4.3*	4.9	4.1	4.8
5''P	--	5.6*	5.3*	3.9*	5.6*

set of low-field ribose proton resonances belong to the U(2) residue. This assignment was unambiguously confirmed by irradiation of the high-field H6 resonance. Indeed, intranucleotide NOEs were observed on the remaining high-field H5, H1', H2', H3' group of resonances, concomitant with internucleotide NOEs on H2'(3) and H3'(3). It is of interest to note that in both cases the H6 protons are spatially closer to the 5' linked sugar ring "above", than to the 3' linked ribose "below".

Finally, the assignment of the respective H8 resonances was also achieved by means of NOE difference spectroscopy. Upon successive irradiation of the three H8 resonances, NOEs were observed on the H1' and H2' of each residue. Unfortunately, H2 resonances could not be assigned in a straightforward manner. The present assignment rests on analogy with the assignment given by Hartel et al¹⁰. Fig.4 shows a computer simulation of the 1D spectrum, based upon the data of Table 1. Coupling constants were derived from the cross sections of the J-resolved spectrum taken parallel to f_1 (Fig.8), except for $^3J_{HP}$ values, which were determined from the spectral simulation. An excellent agreement between the experimental spectrum and the simulated one is noted.

Chemical shifts

Chemical shifts are highly sensitive towards the ring current effect exerted by aromatic bases. Particularly, protons lying below or above the plane of a purine base are strongly shielded. Accordingly, chemical shifts provide a suitable probe to monitor stacking equilibria in oligonucleotides. In Fig.9 the chemical shift vs temperature profiles are collected for the H6, H5 and H1' signals of several compounds. Strong shielding, due to stacking, is observed for the U-protons of $\bar{A}U$ upon lowering the temperature. The U-protons of $\bar{A}U\bar{A}$ and $\bar{A}U\bar{A}U\bar{A}$ behave differently: no shielding effects are observed. On the contrary, H1'(2) in these compounds is even slightly deshielded at lower temperatures. Apparently, the U-residues are positioned away, on a time-average basis, from the shielding cones of the neighbouring adenine bases, both in the trimer as well as in the pentamer. This means that the U- $\bar{A}$ and $\bar{A}$ -U stacking interactions in these oligomers are considerably reduced compared to the stacking observed in the corresponding dimers. Nevertheless, the two U-residues in the pentamer appear on the average to reside in slightly different environments judging from the mutual differences in chemical shifts. The chemical shift profiles of U(2) are almost superimposable upon those of the trimer, whereas the protons of U(4) are more shielded. We conclude that the overall time-average geometry in the first three residues of $\bar{A}U\bar{A}U\bar{A}$ is not significantly different from that in $\bar{A}U\bar{A}$, whereas U(4) appears to be somewhat more involved in stacking interactions. The

Fig 8: Cross sections of the 2D J-resolved spectrum (Fig. 3) taken parallel to F_1 .

difference in behaviour between U(2) and U(4) is also clear from Tables 2 and 3, where differential shieldings, i.e. $\Delta\delta = \delta_{\text{monomer}} - \delta_{\text{oligomer}}$ are collected for the uridine and adenosine residues, respectively. The data from Table 2 show that the U(2) protons of $\bar{A}U\bar{A}U\bar{A}$ are shielded by the same small amount as the U(2)

Fig 9: Chemical shift vs. temperature profiles of H6(A), H5(B) and H1'(C) of internal U-residues. $\square$: meUme, $\circ$: $\bar{A}U\bar{A}(2)$, $\triangle$: $\bar{A}U\bar{A}U\bar{A}(2)$, $+$: $\bar{A}U\bar{A}U\bar{A}(4)$, $\times$: $\bar{A}U(2)$.

protons of $\bar{A}U\bar{A}$. These shieldings are much smaller than those characteristic of a regularly stacked compound e.g. $\bar{A}U^3$. Further comparison shows that the $\Delta\delta$ values are even much smaller than those measured for the $\bar{A}(3)$ residue. In fact, the $\Delta\delta$ values displayed by the U(2) protons are comparable to those observed for $\bar{A}(1)$ and $\bar{A}(5)$. This is a remarkable finding, because the uridyl residues possess two *nearest-neighbour* adenosyl residues whereas the adenosyl residues possess either one or two *next-nearest-neighbour* adenosyl residues. Therefore, one would expect the $\Delta\delta$ of the U(2)-protons to be much larger than those of the $\bar{A}$ -protons, when the compound stacks regularly to a significant amount. Consequently, it is concluded that the pentamer preferentially adopts a time-average conformational state in which the adenine bases exert mutual shielding effects, but hardly affect the uridyl(2) residue. This conclusion accords with the "bulge out" model referred to in the Introduction.

Table 2. Differential shieldings (10^{-2} ppm) of the uridine fragments of several oligomers relative to iprU meU or meUme, 20°C, 5 mM, pH 7.

proton	$\bar{A}U$	$U\bar{A}$	$\bar{A}U\bar{A}$	$\bar{A}U\bar{A}U$	$\bar{A}U\bar{A}U\bar{A}$	$\bar{A}U\bar{A}U\bar{A}U$
1'	40	39	14	16	14	24
5	55	16	16	19	13	25
6	35	17	10	12	10	17

Table 3. Differential shieldings (10^{-2} ppm) of the adenosine fragments of several oligomers relative to $\bar{A}me$ or $me\bar{A}$, 20 °C, 5 mM, pH 7.

proton	$\bar{A}U$	$\bar{A}U\bar{A}$	$\bar{A}U\bar{A}U$	$\bar{A}U\bar{A}U\bar{A}$	$\bar{A}U\bar{A}U\bar{A}$	$U\bar{A}$	$\bar{A}U\bar{A}$	$\bar{A}U\bar{A}U\bar{A}$
1'	6	14	15	15	33	-4	13	11
2	-1	18	14	25	22	0	13	10
8	-7	18	15	22	33	0	24	17

Obviously the "bulge out" model does apply to the 5'-terminal triplet of residues of $\bar{A}U\bar{A}U\bar{A}$ in the same way as it applies to $\bar{A}U\bar{A}$, and also to the first three residues of $\bar{A}U\bar{A}U$. The 3'-terminal triplet of residues of the pentamer seems to behave in a slightly different manner (last column of Table 2). The observed shieldings point to the existence of a relatively larger, but still small, percentage of nearest-neighbour stacking in this part of the molecule.

Ribose Conformations

The furanose ring in ribonucleotides exists in an equilibrium between two rapidly interconverting conformers, which are denoted N and S^{18,19}. The geometry of these conformers can be expressed by two parameters, a phase angle of pseudorotation P and a puckering amplitude Φ_m . The mol fraction of N-conformer can be calculated from experimental coupling constants ($J_{1',2'}$, $J_{2',3'}$ and $J_{3',4'}$) by means of an iterative least-squares program (PSEUROT)²⁰. In this program the generalized Karplus equation^{22,23} (Eqn.1), which takes into account the electronegativity $\Delta\chi_i$ and the orientation "sign" ξ_i of a substituent S_i , is used to calculate $^1H - ^1H$ torsion angles. The $^1H - ^1H$ torsion angles are related to the pseudorotation parameters P and Φ_m by equations 2 a,b,c which were derived from 178 X-ray structures²¹.

$$^3J_{HH} = 13.24 \cos^2\phi - 0.91 \cos\phi + \sum \Delta\chi_i \{0.53 - 2.41\cos^2(\xi_i\phi + 15.5|\Delta\chi_i|)\} \quad (1)$$

$$\phi_{1',2'} = 123.3^\circ + 1.102 \Phi_m \cos(P-144^\circ) \quad (2a)$$

$$\phi_{2',3'} = 0.2^\circ + 1.090 \Phi_m \cos(P) \quad (2b)$$

$$\phi_{3',4'} = -124.9^\circ + 1.095 \Phi_m \cos(P+144^\circ) \quad (2c)$$

As only three experimental coupling constants are available in the case of a ribose ring, at best three conformational parameters can be derived from the data at hand. For this reason Φ_N and Φ_S were constrained to adopt predetermined values within the range known from X-ray crystallography. P_N , P_S and x_N (mol fraction of N-conformer) were then calculated from the couplings extracted from the 2D J-resolved NMR spectrum (Table 4). In our experience x_N hardly depends on the chosen values of Φ_N and Φ_S and therefore the x_N value can be considered quite reliable ($\pm 2\%$). P_N and P_S , however, are known to be strongly correlated

Table 4. Pseudorotational parameters of the riboserings of the title compound and mol fractions of N conformer at 44 °C. An asterisk indicates a constrained value.

	$\Phi(N)^*$	P(N)	$\Phi(S)^*$	P(S)	x(N)	rms(Hz)
$\bar{A}(1)$	36	16	36	160	.29	.007
U(2)	36	20	36	154	.25	.009
$\bar{A}(3)$	36	-1	36	152	.38	.000
U(4)	38	-3	38	149	.42	.000
$\bar{A}(5)$	38	19	38	152	.50	.004

with Φ_N and Φ_S and these values are subject to possible systematic errors ($\pm 20^\circ$ for the minor conformation and $\pm 5^\circ$ for the predominant one)⁹.

2D experiments are rather time consuming and therefore only a single complete set of coupling constants was obtained (at 44 °C). However, over the temperature range covered by our 1D NMR experiments (0-86 °C), the H1' protons, except H1'(4), are separated well enough to permit the measurement of $J_{1'2'}$, even at 300 MHz. It is of course not possible to perform a detailed pseudorotational analysis based on a single coupling, but nevertheless relevant information can be obtained if we make the usual assumption that the geometrical

Table 5. Mol fractions of N conformer for various compounds as a function of temperature. Temperatures are rounded off to the nearest 5 °C.

5' terminal $\bar{A}$ residues:

T(°C)	$\bar{A}me$	$\bar{A}U$	$\bar{A}U\bar{A}$	$\bar{A}U\bar{A}U$	$\bar{A}U\bar{A}U\bar{A}$
0	11	91	26	32	30
20	15	74	27	30	30
45	18	57	31	28	30
60	21	49	32	30	30

Internal U residues:

T(°C)	iprU	$\bar{A}U$	meUme	$\bar{A}U\bar{A}$	$\bar{A}U\bar{A}U$	$\bar{A}U\bar{A}U\bar{A}$	$\bar{A}U\bar{A}U\bar{A}U$
0	45	90	24	30	34	25	40
20	43	76	25	30	32	25	--
45	--	64	25	32	32	25	41
60	--	25	33	32	25	--	--

Internal $\bar{A}$ -residues:

T(°C)	me $\bar{A}me$	$\bar{A}U\bar{A}U$	U $\bar{A}U$	$\bar{A}U\bar{A}U\bar{A}$
0	14	86	100	43
20	16	81	86	41
45	--	68	68	39
60	22	51	--	38

3'-terminal $\bar{A}$ -residues:

T(°C)	me $\bar{A}$	U $\bar{A}$	$\bar{A}U\bar{A}U\bar{A}$
0	34	59	56
20	34	56	51
45	--	53	46
60	--	53	46

parameters of the sugar rings do not vary significantly with temperature. Therefore P_N , P_S , $\bar{f}_N$ and $\bar{f}_S$ are constrained to adopt the values deduced from the complete analysis carried out on the 44 °C data (Table 4). In this way the population x_N can be estimated at various temperatures (Table 5).

Interestingly, the first two ribose rings at the 5' end of the three compounds $\bar{A}\bar{U}\bar{A}$, $\bar{A}\bar{U}\bar{A}\bar{U}$ and $\bar{A}\bar{U}\bar{A}\bar{U}\bar{A}$ closely resemble each other; these residues display approximately a 25-30% population of N conformer, which population hardly varies with the temperature. It should be pointed out that the $\bar{A}(3)$ residue in the tetramer is involved in a regular stacking interaction with the U(4) residue. For this reason the $\bar{A}(3)$ ribose ring in the tetramer strongly prefers to adopt the N conformer. No such phenomenon is observed in the case of the trimer or the pentamer.

The pentamer $\bar{A}\bar{U}\bar{A}\bar{U}\bar{A}$ can be thought of as consisting of two $\bar{A}\bar{U}\bar{A}$ triplets sharing a common $\bar{A}(3)$ residue. In the absence of specific conformational transmission or terminal phosphate effects one might expect that both parts of the oligonucleotide would display similar behaviour. This is only true to a certain extent. All three ribose rings at the 3' terminal part of the pentamer show relatively low percentages of N conformer ($\bar{A}(3)$ 43%, U(4) 40%, $\bar{A}(5)$ 50% at 0°C) when compared to the stacking dimer $\bar{A}\bar{U}$ ($\bar{A}(1)$ 91%, U(2) 90% at 0°C), but especially U(4) shows significantly more preference for the N-type sugar than U(2) does (25%). At the same time the population of N conformer of residues $\bar{A}(3)$ and $\bar{A}(5)$ decreases slightly upon raising the temperature from 0-60°C (Table 5). RNA oligomers in general prefer an A-type stacking pattern with N-type ribose conformations, (c.f. $\bar{A}\bar{U}$, $\bar{U}\bar{A}\bar{U}$, Table 5), whereas monomers prefer S-type sugar conformation. Therefore, a decrease of the population of N-type sugars can be regarded as an indication for 'destacking'. Thus coupling constant data of the ribose rings corroborate our interpretation of the chemical shift data in terms of the presence of a small amount of regular stack in the $\bar{A}(3)$ -U(4)- $\bar{A}(5)$ terminal triplet, particularly at lower temperatures, and the (almost) total absence of such regular stack in the $\bar{A}(1)$ -U(2)- $\bar{A}(3)$ triplet.

Backbone angles

Torsion angle β (P-O5'-C5'-C4'): The conformational preference about β is monitored by means of the sum $\Sigma' \equiv J_{5,P} + J_{5,P}$. The populations of trans conformer about β ($p\beta^t$) are calculated by means of a newly parametrized⁷ sum rule, eq 3.

$$p\beta^t = (23.9 - \Sigma')/18.9, \quad (3)$$

Table 6 lists the $p\beta^t$ values obtained at 44 °C for the title compound and for the smaller fragments $\bar{A}\bar{U}\bar{A}\bar{U}$, $\bar{A}\bar{U}\bar{A}$ and $\bar{A}\bar{U}$. It is seen that the β^t populations occur within the range normally encountered in oligonucleotides, i.e. 70-85%. The

conclusion must be that the "bulged out" situation is not associated with special geometrical requirements involving angle β : β^+ or β^- for example. The fully stacked state requires a pure β^t geometry⁷.

Torsion angle γ (O5'-C5'-C4'-C3'): The conformational behaviour along γ is monitored by means of the couplings $J_{4,5'}$ and $J_{4,5''}$. It is well known that the population of γ^+ conformer can be deduced from the sum $\Sigma J_{4,5'} + J_{4,5''}$ by means of an appropriate sum rule, independently of the correct stereochemical assignment of the H5' and H5'' resonances. Matters are different for the population distribution between the γ^t and γ^- rotamers. In the present work we adhere to the usual H5', H5'' assignment according to Remin and Shugar²⁴. Very few exceptions from this assignment have been recorded⁷. The situation about γ is of some importance because a right-handed stack requires pure γ^+ , whereas in Z-DNA the left-handed structure demands γ^t in -pdG residues²⁵.

The γ^+ , γ^t and γ^- population distributions shown in Table 7 were calculated according to Haasnoot et al²⁶, i.e. corrected for electronegativity effects exerted by substituent oxygen and carbon atoms at C4' and C5'. A glance at Table 7 reveals that the γ^- rotamer occurs, as usual, in very minor amounts. The γ^+ rotamer predominates along the phosphodiester linkages of all compounds studied in the A-U series and also at the free 5'-OH end of $\bar{A}(1)$. Again there seems nothing unusual in these distributions except, perhaps, the increase noted for γ^t (33%) in the central $\bar{A}(3)$ residue of the pentamer at the expense of the γ^+ species. The significance of this observation remains unclear for the time being.

Torsion angle ϵ (C4'-C3'-O3'-P): In the present study the conformational situation about ϵ is mainly monitored by means of $^3J_{3,P}$. This approach suffers from the drawback that only a single three-bond coupling is available. Two rotamers about ϵ are usually considered, ϵ^t and ϵ^- , and these have a more or less symmetrical disposition with respect to the H3'-C3'-O3'-P angle. In other words, $^3J_{3,P}$ remains rather insensitive to the position of the possible ϵ^t/ϵ^- equilibrium. A clue to the presence of the ϵ^- rotamer is afforded by the detection of a long-range $^4J_{2,P}$ coupling, as this coupling requires a W-path: i.e. a planar

Table 6. Percentage of trans-conformer for the backbone angles β in various compounds at 44 °C.

	$\bar{A}U\bar{A}U\bar{A}$ %t	$\bar{A}U\bar{A}U$ %t	$\bar{A}U\bar{A}$ %t	$\bar{A}U$ %t
U(2)	74	79	79	83
$\bar{A}(3)$	72	73	71	--
U(4)	82	83	--	--
$\bar{A}(5)$	71	--	--	--

Table 7. Population distribution (%) for the backbone angle γ in various compounds at 44 °C.

	$\overline{AUAUA}$			$\overline{AUAU}$			$\overline{AUA}$			$\overline{AU}$		
	g	t	g	g	t	g	g	t	g	g	t	g
$\overline{A}(1)$	76	21	3	70	29	1	76	21	3	76	19	5
U(2)	78	20	2	81	18	1	86	14	0	82	17	1
$\overline{A}(3)$	63	33	4	70	29	1	72	25	3	--	--	--
U(4)	77	19	5	87	13	0	--	--	--	--	--	--
$\overline{A}(5)$	71	24	6	--	--	--	--	--	--	--	--	--

H2'-C2'-C3'-O3'-P geometry. Model building indicates that such a geometry can be realized only at the simultaneous occurrence of S-type ribose and ϵ^- backbone rotamer. A right-handed stack requires pure ϵ^t and a left-handed stack (as in Z-DNA) displays both ϵ^t and ϵ^{-25} .

The values found for $^3J_{3,P}$ in $\overline{AUAUA}$ range from 7.8-8.0 Hz (Table 1), and this range appears characteristic of a ϵ^t/ϵ^- blend. In a pure ϵ^t state of a ribose 3' phosphate one expects $^3J_{3,P} = 9.0-9.7$ Hz²⁷. The detection of a long-range $^4J_{2,P}$ couplings in all pertinent residues A(1), U(2), A(3) and U(4) also leads to the tentative conclusion that an ϵ^t/ϵ^- equilibrium exists in the bulged out pentamer.

CONCLUSIONS

From a previous study it is known¹⁰ that the alternating purine-pyrimidine RNA tetramer $\overline{AUAU}$ does not assume a continuous single-helical form. Instead, the underlined part of the sequence displays conformational freedom of the sugar-phosphate backbone and the U(2) base spends most, if not all, of its time outside the shielding cones of the neighbouring A bases. At the 3' terminal of this tetramer normal right-handed $\overline{A}(3)$ -U(4) stacking occurs. The present study shows that the addition of another $\overline{A}$ residue at the 3'-end to form the pentamer $\overline{AUAUA}$ disrupts the $\overline{A}(3)$ -U(4) stack to a significant extent. A "double bulge-out" blend of conformational states is apparently preferred by this molecule over a continuous right-handed stack. Both pyrimidine bases take up an extrahelical conformational blend of states with concomitant formation of purine-purine (1)--(3)--(5) stacking interactions.

The existence of extrahelical bases in double stranded nucleic acids has long been postulated²⁸. Experimental studies^{29,30} on RNA duplexes indicate that the hybridization of complementary polyribonucleotides with extra

non-Watson-Crick bases inserted into one strand can result in a double helix in which the extra bases are expelled from the regular stack. Evidence for the existence of single extrahelical bases in duplex DNA has been reported recently³¹. Our studies show that these phenomena are not limited to the duplex structure but can occur on the RNA single-stranded blend as well, although a purine-pyrimidine-purine sequence seems to be required. Thus far, studies undertaken in this laboratory on single-stranded DNA's containing similar pu-py-pu sequences indicate the existence of normal right-handed single helices of the B-DNA type³². This is in accordance with the suggestion³¹ that extrahelical bases are energetically less favourable in duplex DNA than they are in duplex RNA.

The next phase of our researches will be directed towards non-methylated self-complementary (A-U)_n sequences in order to supply an answer to the question whether or not the formation of Watson-Crick base pairs can induce a reversal of the bulge-out and force the molecule into a normal helical stacking pattern.

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2. All oligonucleotides mentioned in this study have modified A-bases: 6-N-(dimethyl)adenine, the only exception being AACC. Modified A-bases will in the remainder of this work be abbreviated as $\bar{A}$. Thus, for example, 6-N-(dimethyl)adenylyl-(3'-5')-uridine is abbreviated as $\bar{A}U$. Mononucleotides mentioned in this study bear terminal methyl phosphate groups or an isopropyl group. These are abbreviated as follows: $\bar{A}me$, $me\bar{A}me$, $me\bar{A}$, Ume , $meUme$ and $iprU$.
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