Volume 11 Number 20 1983 Nucleic Acids Research

Carbon-13 NMR in conformational analysis of nucleic acid fragments. Heteronuclear chemical shift correlation spectroscopy of RNA constitutents

Peter P.Lankhorst, Cornelis Erkelens, Cornelis A.G.Haasnoot* and Cornelis Altona

Gorlaeus Laboratories, State University of Leiden, P.O. Box 9502, 2300 RA Leiden, and
*Department of Biophysical Chemistry, Toernooiveld Faculty of Science, University of Nijmegen, 6525 ED Nijmegen, The Netherlands

Received 25 July 1983; Accepted 20 September 1983

ABSTRACT

The assignment of the non-quaternary ^{13}C resonances by means of two-dimensional heteronuclear chemical shift correlation spectroscopy is presented for several oligoribonucleotides: The dimers m^6_2AU, $m^6_2Am^6_2A$ and $mpUm^6_2A$ and the trimers $m^6_2AUm^6_2A$ and $m^4_2Cm^4_2Cm^6_2A$. The temperature and concentration dependency of the ^{13}C chemical shifts are studied with emphasis on the behaviour of the dimer m^6_2AU.

The present study shows that in the 5-50 mM range the concentration-dependent chemical shift changes of the ribose carbons are negligible compared to chemical shift changes due to intramolecular events. All compounds studied show a surprising correlation between the chemical shifts of the carbon atoms of the ribose ring and the sugar conformational equilibrium as expressed by the percentage N or S conformer. Thus the chemical shift data can be used to obtain the thermodynamical parameters of the two-state N/S equilibrium. Parameters deduced for m^6_2AU are Tm = 306 K and ΔS = -25 cal mol^{-1} K^{-1}, which values are in satisfactory agreement with results obtained earlier from ^1H NMR and from Circular Dichroism.

INTRODUCTION [1,2]

^1H NMR has been shown to be a useful tool in conformational analysis of single stranded oligonucleotides. Vicinal proton-proton coupling constants provide detailed information on the conformation of the sugar ring[3-5] and of the backbone angle γ (C4'-C5')[6].Vicinal proton-phosphorus coupling constants are used to monitor the backbone angles ε (C3'-O3') and β (C5'-O5')[7] and finally thermodynamical parameters of the unstack/stack equilibrium of dinucleoside monophosphates can be derived from chemical shift vs temperature profiles[8,9].

Up till now several ^{13}C NMR studies on nucleosides[10-12] and nucleotides up to the dimer level, [13-18], as well as on poly(rA)[19,20] and poly(rU)[19,21] have appeared. Generally, these spectra are assigned according to Dorman et al.[16] and Jones et al.[10]. However, following Mantsch et al.[17], the original assignment of C2' and C3' resonances in nucleosides and 5'-nucleotides is usually interchanged. 3',5'-Cyclic nucleotides[22] as well as 2',3'-cyclic nucleotides [23] have been studied extensively by ^{13}C NMR. More recently, Uesugi et al.[24] pro-

posed to revise the original assignment[22] of the C3' and C4' signals in 3',5' cAMP. Heteronuclear chemical shift correlation spectroscopy experiments carried out in our laboratory[25] have confirmed the Uesugi assignment. Schleich et al.[19] have examined the ^{13}C spectra of a number of dinucleoside monophosphates, which were assigned on basis of phosphate-deshielding effects and ^{31}P-^{13}C spin-spin coupling constants. These authors studied the concentration dependence of several base carbons and suggested a combination of ring-current shielding effects and electrostatic deshielding effects arising from the phosphates to account for the chemical shift changes observed in the 0.5-0.02 M range. Alderfer and Ts'o[21] examined the pH and temperature dependence of the chemical shifts of several monomers, dimers and polymers. From their results it appeared that the assignment of the C2' and C3' resonances in poly(rA), poly(rU) and in Np-residues of UpU and ApA as given in ref. 13 and 17 should be reversed.

From the above account it is clear that the correct assignment of carbon resonances in nucleic acids has been a matter of much debate in the past. Two resonances in particular, i.e. C2' and C3' of Np- residues, proved difficult to assign unambiguously, because these occur within the same narrow region of the NMR spectrum. Moreover, often both carbons display similar coupling constants to phosphorus. In the present study a different approach is taken. We purport to show that the use of two-dimensional heteronuclear chemical shift correlation spectroscopy allows a simple and unambiguous assignment of each directly bonded carbon-proton pair in a single experiment on condition that relevant proton signals are not accidentally isochronous. This technique was recently applied to solve the ^{13}C spectra of raffinose and its subunits[26], but the present study appears to be the first in the field of nucleic acid constituents.

This investigation deals furthermore with the concentration and temperature dependence of the ^{13}C chemical shifts of several RNA fragments, comprising monomers, dimers and trimers. The ^{13}C chemical shifts of $\bar{A}U$ ($\bar{A} = m^6_2A$, see Figure 1) will be considered in greater detail. This compound thus far represents the most thoroughly studied dinucleoside monophosphate. $\bar{A}U$ is of special interest, because both ^1H NMR[8] and CD studies[27] have shown, that it has a strong tendency to form a right-handed single helix. Both independent techniques yield, within experimental error, the same thermodynamical parameters for the unstack/stack equilibrium. In the present paper the possibility to deduce these parameters from the ^{13}C NMR chemical shift data of $\bar{A}U$ will be discussed. Other compounds studied were chosen for their special conformational characteristics: mpU$\bar{A}$ shows little tendency to adopt a stacked conformation[28]. $\bar{A}U\bar{A}$ occurs as a "bulged-out" structure, in which the interior U-fragment is

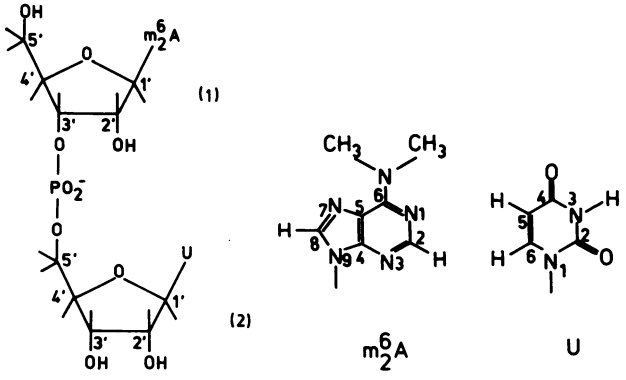

Figure 1: Structure of $\bar{A}U$.

not involved in a nearest-neighbour stacking interaction[29]. Like $\bar{A}U$[8], $\bar{A}\bar{A}$[9] at low temperature prefers to adopt a right-handed helical structure. Finally $\bar{C}\bar{C}\bar{A}$ ($\bar{C}$ = m_2^4C) adopts a regularly stacked $\bar{C}\bar{C}$ part, concomittant with a $\bar{C}\bar{A}$ part which seems to prefer an unusual antiparallel base stack[30]. Moreover, for the sake of completeness, several mononucleotides are included in this study.

MATERIALS AND METHODS.

Sample preparation.

All compounds studied were synthesized via a modified phosphotriester approach[31,32]. NMR samples (10 mm tubes) were prepared in D_2O with nucleotide concentrations ranging from 5-50 mM. A trace of EDTA was added in order to remove paramagnetic contaminations. Tetramethylammonium chloride (TMA) served as an internal reference. Chemical shifts are measured relative to the central peak of TMA and can be converted to the dioxane scale by means of the relationship: $\delta_{dioxane} = \delta_{TMA} - 11.50$.

One-dimensional NMR Spectroscopy.

^{13}C NMR spectra were recorded on a Bruker WM-200 WB spectrometer (operating at 50.3 MHz), and on a Bruker WM-300 spectrometer (operating at 75.3 MHz). One-dimensional ^{13}C NMR spectroscopy was utilized in the present work mainly in order to obtain precise chemical shifts and ^{31}P-^{13}C coupling constants. These coupling constants yield interesting information concerning the conformational behaviour of ribonucleotides along the backbone angles β (C5'-O5') and ε (C3'-O3'). This subject will be treated in a subsequent communication[25].

Heating of the samples dissolved in D_2O ,caused by broadband ^1H decoupl-

ing, poses a serious problem in ^{13}C NMR. Due to this heating the ^{13}C signals of interest may be broadened and measurement of coupling constants will become inaccurate. Therefore special attention was paid to the experimental conditions prevailing during the acquisition of ^{13}C NMR spectra: only a minimal decoupling power (0.5 W) was employed during acquisition (0.3 s). During the 1 s relaxation delay a lower decoupling level (0.1 W) was applied in order to maintain the Nuclear Overhauser Enhancement (NOE). Spectra were recorded on 8K datapoints in order to avoid long acquisition times. The number of transients accumulated varied from 2000 to 30.000, depending on sample concentration. FIDs were multiplied by a Gaussian window and zero filled to 128K datapoints, thus yielding a digital resolution of 0.1 Hz/point in the frequency domain spectrum.

Immediately after completion of each ^{13}C spectrum a ^{1}H spectrum of the same sample was recorded using the decoupler coil in order to determine the exact temperature from the chemical shift difference $\delta_{HDO}-\delta_{TMA}$[8]. This internal temperature calibration was found to be essential: even when the precautions described above are strictly followed, a small temperature increase of the sample (varying between 0-5 °C) may occur, depending on instrumental conditions.

Two-dimensional NMR spectroscopy.

Spectra of $\bar{A}\bar{A}$, $\bar{A}U$, mp$U\bar{A}$, $\bar{A}U\bar{A}$ and $\bar{C}\bar{C}\bar{A}$ were assigned by means of heteronuclear chemical shift correlation spectroscopy[33-35]. The pulse sequence and phase cycling proposed by Bax[35] was used. All two-dimensional spectra were recorded on 2K datapoints in the f_2-dimension and on 128 or 64 datapoints in the f_1-dimension. Each FID consisted of ± 1000 scans. A relaxation delay of 0.8 s was allowed for between transients. Typical acquisition time for a 2048 x 128 data matrix amounted to approximately 30 hours. In the case of $\bar{A}U\bar{A}$ and $\bar{C}\bar{C}\bar{A}$ it was found advantageous to run the 2D spectrum with simultaneous decoupling of ^{31}P (vide infra).

Before Fourier Transformation the two-dimensional time-domain spectra were multiplied either by a phase-shifted ($1/6\pi$) sine square window or by a Gaussian window and zero filled to 4K (f_2) and 512 datapoints (f_1), respectively.

RESULTS AND DISCUSSION.

Assignment.

Fig. 2 shows a contour plot of the 50.3 MHz correlation spectrum of $\bar{A}U$. For reference purposes the 1D carbon spectrum is shown along the horizontal axis (f_2) and the 1D proton spectrum is plotted along the vertical axis (f_1). Cross-peaks, indicating $^{1}J_{CH}$, occur at coordinates determined by the ^{13}C and

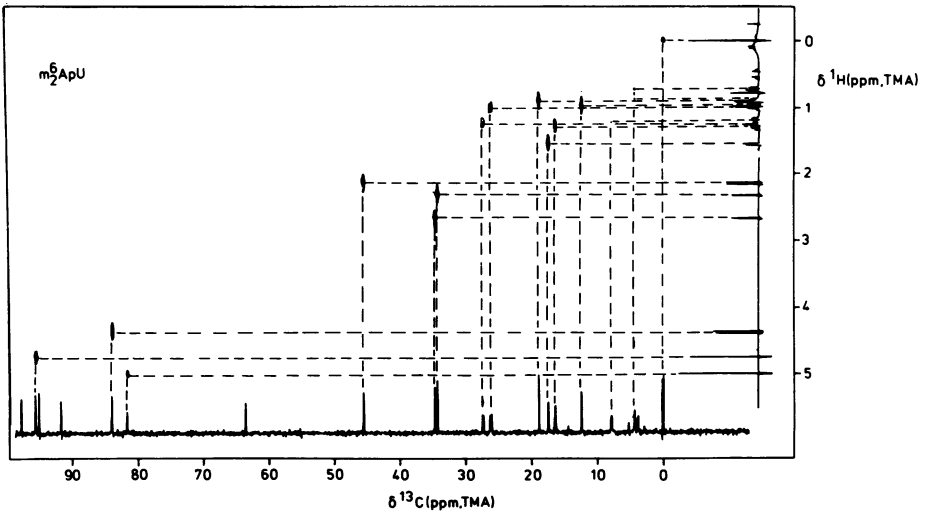

Figure 2: 50 MHz heteronuclear chemical shift correlation map of a 40 mM sample of $\bar{A}U$ at 301 K. The proton spectrum shown along the vertical axis was recorded at 300 MHz. Chemical shifts are relative to TMA.

the ^{1}H chemical shifts involved. In this way, the cross-peaks correlate each ^{13}C resonance to the corresponding ^{1}H resonance, leading to the unequivocal assignment of the ^{13}C spectrum in a single experiment.

Because the heteronuclear shift correlation experiment was optimized to detect J_{CH} between 125-170 Hz, only those carbons, that are directly bonded to one or more hydrogens are detected in this way. The remaining quaternary carbons, i.e. C2 and C4 of uracil and C4, C5, C6 of N6-dimethyladenine are assigned following Jones et al.[10]. It should be pointed out at this point that the assignment of the carbon spectrum of $\bar{A}U$ and of all other compounds studied rests upon the independent assignment of the proton spectra. It goes without saying that knowledge of the carbon spectra can be used in a similar way to assign the proton spectra.

A contour plot of the correlation spectrum of $\bar{A}U\bar{A}$ is shown in Fig. 3. Note, however, that the intensities of the C2', C3', C4' and C5' cross peaks are relatively weak due to ^{31}P-^{13}C splitting. Some of the C5' cross peaks are even lost in the noise. The situation is clearly much better in the experiment depicted in Fig. 4. This 2D spectrum is recorded with simultaneous ^{31}P decoupling which results in a better resolution of the cross-peaks, as well as in higher intensities of the signals in the 5'-region. Unfortunately, H5'(2) and H5'(3), as well as H5"(2) and H5"(3) severly overlap in the ^{1}H spectrum, so that an unam-

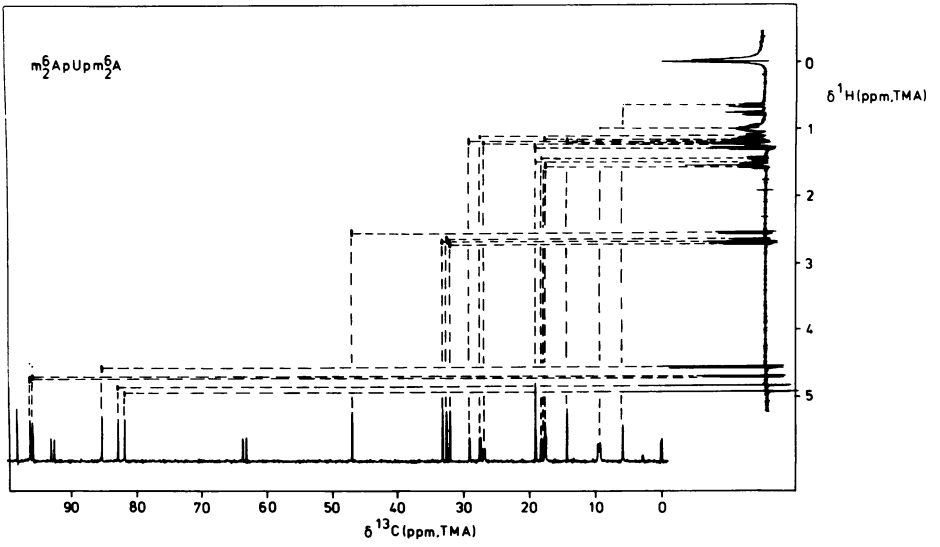

Figure 3: 75 MHz heteronuclear chemical shift correlation map of $\bar{A}U\bar{A}$ (≈ 50 mM, 305 K).

biguous assignment of the C5'(2) and C5'(3) is precluded in the case of the $\bar{A}U\bar{A}$ trimer. The ^{13}C spectra of $\bar{A}\bar{A}$, mpU$\bar{A}$ and $\bar{C}\bar{C}\bar{A}$ were assigned in a similar way. The chemical shifts of the above-mentioned compounds as well as those of $\bar{A}$pm, mpU and $\bar{C}$pm are presented in Tables 1, 2 and 3.

The correct assignment of the C2' and C3' resonances of Np-residues has been a matter of discussion in the past[21] (vide supra). Of course, in some cases the magnitude of the ^{13}C-^{31}P coupling constants can be used to discriminate between these two signals: $^2J_{C3'-P} \approx 4$-6 Hz, whereas $^3J_{C2'-P} \approx 1.5$-6 Hz[25]. Nevertheless, still a rather large range of "overlapping" values exists, in which cases the magnitude of J_{CP} cannot be used for assignment purposes. However, in a following section it is pointed out that a useful rule for the assignment of C2' and C3' resonances in Np-residues can be given.

Stacking thermodynamics from carbon-13 chemical shifts.

^1H chemical shifts have been shown to be a suitable probe to quantitatively monitor the stack/destack equilibrium of dinucleotides[8,9]. In the present section we wish to explore the potential usefulness of ^{13}C shift vs. temperature profiles for the same purpose. A total of 27 spectra of $\bar{A}U$ were recorded at temperatures between 0 °C and 100 °C at four different concentrations (5, 10, 20 and 40 mM). In this way, it is possible to correct the chemical shifts for the phenomenon of self-association, which is known to be strongly present in nucleotides containing

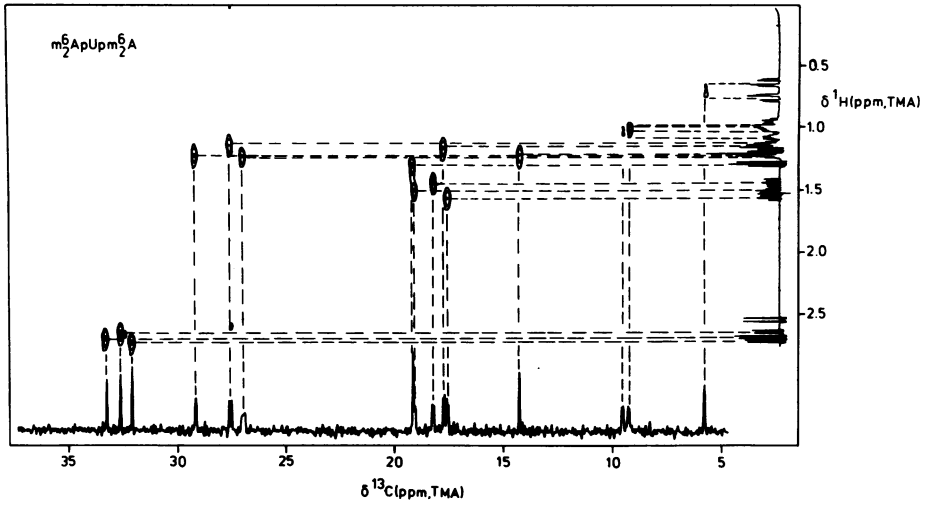

Figure 4: 75 MHz heteronuclear chemical shift correlation map of $\bar{A}U\bar{A}$ (≈ 50 mM, 305 K) with simultaneous 31P-decoupling. Only the ribose region of the spectrum is shown. The 1D carbon spectrum, shown along the horizontal axis is 31P-coupled.

purine bases.

In a recent paper from this laboratory[8] the thermodynamics of stacking and of self-association of $\bar{A}U$ have been determined from ^{1}H chemical shifts with the aid of the Differential Concentration Temperature Profile method (DCTP). In this method the existence of two independent two-state equilibria is assumed, i.e.: (a) the stack/destack equilibrium, and (b) an association equilibrium between free species and a bimolecular complex with two $\bar{A}$p-moieties stacking on

Table 1: Chemical shifts (± 0.003 ppm) of the adenine ring of several oligoribonucleotides at ≈ 300 K.

residue	C2	C4	C5	C6	C8	N(CH3)
$\bar{A}$pm	96.357	93.383	64.223	98.923	+	-16.375
$\bar{A}U$(1)	96.035	92.303	63.813	98.293	82.064	-16.363
$\bar{A}\bar{A}$(1)	96.329•	92.720•	63.451•	98.247•	82.045•	-16.533
$\bar{A}\bar{A}$(2)	95.720*	92.179*	63.207*	97.940*	81.125*	-16.533
mp$U\bar{A}$(2)	96.840	94.010	63.844	99.004	82.262	-16.433
$\bar{A}U\bar{A}$(1)	95.962	93.180•	63.802	98.348	82.961	-16.513
$\bar{A}U\bar{A}$(3)	96.347	92.664*	63.247	98.348	81.958	-16.604
$\bar{C}C\bar{A}$(3)	96.754	93.606	63.647	98.807	81.806	-16.268

+ signal could not be detected.
assignments marked with • and * are arbitrary.

Table 2: Chemical shifts (± 0.003 ppm) of the pyrimidine rings of several oligoribonucleotides at ≈ 300 K.

residue	C2	C4	C5	C6
$\bar{A}U(2)$	95.486	110.045	45.991	84.525
mpU$\bar{A}$(1)	96.262	110.785	47.148	85.691
$\bar{A}U\bar{A}(2)$	96.066	110.255	47.116	85.417
$\bar{C}C\bar{A}(1)$	101.189•	107.683•	37.952•	84.113•
$\bar{C}C\bar{A}(2)$	100.863✲	107.404✲	37.713✲	83.337✲
mpU	96.519	110.922	47.252	86.196

For footnotes see Table 1.

top of each other. The various chemical shifts monitored, δ_{obs}, can be written as:

$$\delta_{obs} = \delta_{FU} + P_A\Delta_A + P_X\Delta_X$$

where δ_{FU} represents the chemical shift of non-associated, unstacked molecules, P_X the fraction of stacked molecules, and Δ_X the effect of stacking on each chosen chemical shift. P_X is related to the equilibrium constant K_X by

$$P_X = K_X/(1+K_X)$$

and K_X is related to Tm_X and ΔS_X in the usual way. The parameters that describe the association equilibrium, i.e. p_A, Δ_A, Tm_A and ΔS_A are defined in an analogous way. Thus, the observed chemical shifts can be written as a function of 7 parameters, i.e. δ_{FU}, Δ_A, Δ_X, Tm_A, ΔS_A, Tm_X and ΔS_X, which can be determined in a least-squares approach (ASSTAK, for further details see ref. 8), provided that a large quantity of data at different temperatures and concentrations are available. Moreover the method requires that the chemical shift mon-

Table 3: Chemical shifts (± 0.003 ppm) of the ribose carbons of several oligoribonucleotides at ≈ 300 K.

residue	C1'	C2'	C3'	C4'	C5'
$\bar{A}pm$	32.632	17.599	19.448	29.943	6.175
$\bar{A}U(1)$	34.331	17.514	17.061	27.754	4.687
$\bar{A}U(2)$	34.135	18.813	12.774	26.511	8.132
$\bar{A}\bar{A}(1)$	34.359	17.484	17.356	27.415	5.057
$\bar{A}\bar{A}(2)$	32.712	19.095	13.254	26.729	8.585
mpU$\bar{A}$(1)	32.365	17.884	19.046	26.423	9.241
mpU$\bar{A}$(2)	31.992	18.527	14.753	27.782	10.055
$\bar{A}U\bar{A}(1)$	33.302	17.622	19.146	29.238	5.814
$\bar{A}U\bar{A}(2)$	32.709	17.735	18.278	27.023	9.277
$\bar{A}U\bar{A}(3)$	32.168	19.190	14.300	27.626	9.523
$\bar{C}C\bar{A}(1)$	35.104	17.937	16.278	26.639	4.130
$\bar{C}C\bar{A}(2)$	34.401	18.260	16.345	24.830	9.279•
$\bar{C}C\bar{A}(3)$	31.852	20.118	14.784	28.251	7.865✲
mpU	33.403	18.439	14.319	27.824	9.162

For footnotes see Table 1.

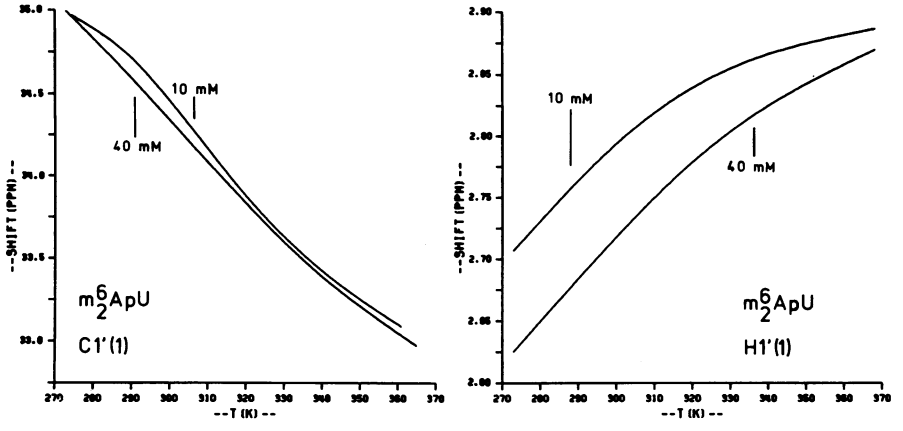

Figure 5: Chemical shift vs. temperature profiles for A: C1'(1) and B: H1'(1) of $\overline{A}U$. Two different concentrations are shown: 10 mM and 40 mM.

itored is sensitive to at least one of the two equilibria.

In the present case a slightly different approach is used. The ^{13}C chemical shifts (except for the base carbons C6, C4 and C2 of $\overline{A}$) are only slightly dependent on sample concentration. This is clear from Fig. 5, where the chemical shift profiles of H1'(1) and C1'(1) at 10 and 40 mM are compared. Of course, the relative insensitivity inherent to ^{13}C NMR precludes the compilation of a large set of spectra, especially in the low concentration range. For this reason the values of Tm_A and ΔS_A were not determined from the ^{13}C shifts but taken from reference 8. In this way a correction for the concentration dependence of the carbon shifts was taken into consideration. The remaining five parameters were refined. Results are listed in Table 4 for all ribose carbons and for C5 and C6 of Uracil. The other base carbons are omitted from this table because the limited number of data available (due to long T1's), especially at low concentrations, precludes a meaningful least-squares fit of the parameters. We note here, that the base carbons of adenine display a significant shielding effect (in the order of 0.5 ppm), when the sample concentration is increased. The latter effect may be explained in terms of ring current shieldings due to association. The ribose carbons of the $\overline{A}(1)$ moiety, however, behave in a completely different manner: the concentration dependent effects Δ_A are negligible, i.e. they are typically a factor 10-20 smaller than the concentration independent effects (Δ_X). This stands in remarkable contrast with the ^1H chemical shifts of $\overline{A}U$, where the concentration dependency of the proton shifts, particularly of those of the $\overline{A}(1)$ moiety sometimes even predominates the intramolecular effect. Surprisingly, the

Table 4: Thermodynamical parameters (Tm and ΔS), association shifts (Δ_A), stacking shifts (Δ_X) and intrinsic shifts ($\delta_{F,U}$), obtained from a two-state analysis of several observables of $\bar{A}U$, i.e. carbon-13 chemical shifts, proton chemical shifts and CD. Estimated accuracies: Tm (± 2-5 K), ΔS (± 2-5 cal mol^{-1} deg^{-1}), Δ_A (± 0.05-0.1), Δ_X (± 0.1-0.3), $\delta_{F,U}$ (± 0.04-0.1).

probe	Δ_A	Δ_X	Tm_X	ΔS_X	$\delta_{F,U}$
C1'(1)	-0.32	3.2	314.2	-22.0	32.46
C3'(1)	-0.13	-3.0	301.5	-26.3	18.63
C4'(1)	0.10	-1.7	307.1	-28.1	28.67
C5'(1)	-0.16	-2.3	294.7	-23.6	5.76
mean(1)			307.5	-24.6	
C6(2)	-0.46	-1.5	310.7	-27.0	85.66
C5(2)	-0.36	-1.2	302.3	-28.3	46.78
C1'(2)	-0.31	1.3	289.7	-29.8	33.78
C2'(2)	-0.29	1.9	324.2	-17.0	17.65
C3'(2)	-0.39	-2.0	306.0	-23.9	14.03
C4'(2)	-0.38	-1.3	308.6	-25.1	27.39
C5'(2)	-0.21	-2.1	306.8	-22.1	9.37
mean(2)			305.8	-25.2	
mean(1+2)			306.5	-25.0	
mean 1H shift			308.6	-22.3	
CD			309.7	-20.9	

association shifts Δ_A displayed by the ribose carbons of residue U(2) are slightly larger than those of $\bar{A}$(1), although the proton NMR experiments indicate that the $\bar{A}$ bases of $\bar{A}U$ interact with each other and not with the U base. Nevertheless the Δ_A values of U(2) are still relatively small compared to Δ_X values of the carbons of this residue.

The stacking shifts, Δ_X, are listed in the second entry of Table 4. It is interesting to note that Δ_X values of the $\bar{A}$p-moiety are of the same order of magnitude (or even larger) compared to the Δ_X values of the -pU moiety. A completely different situation was found for the comparable values from ^{1}H NMR: Δ_X values of the -pU moiety tend to be a factor 2-5 larger than those of the $\bar{A}$p-moiety due to the stronger ring-current of the adenine base. Apparently, the temperature induced changes of the ^{13}C shifts of the ribose ring are not dominated by ring current effects arising from changes in the base-base stacking equilibrium. Instead, these shifts appear to be sensitive to changes in accompanying conformational equilibria, such as e.g. a shift of the N/S equilibrium or of the backbone torsional angles with temperature. The fact that C1'(2) and C2'(2), in contradistinction to H1'(2) and H2'(2), are deshielded upon lowering the temperature also accords with this conclusion.

In the third and fourth entry of Table 4 ΔS_X and Tm_X are listed for all ribose carbons and C5(2) and C6(2). The values obtained for the individual ^{13}C

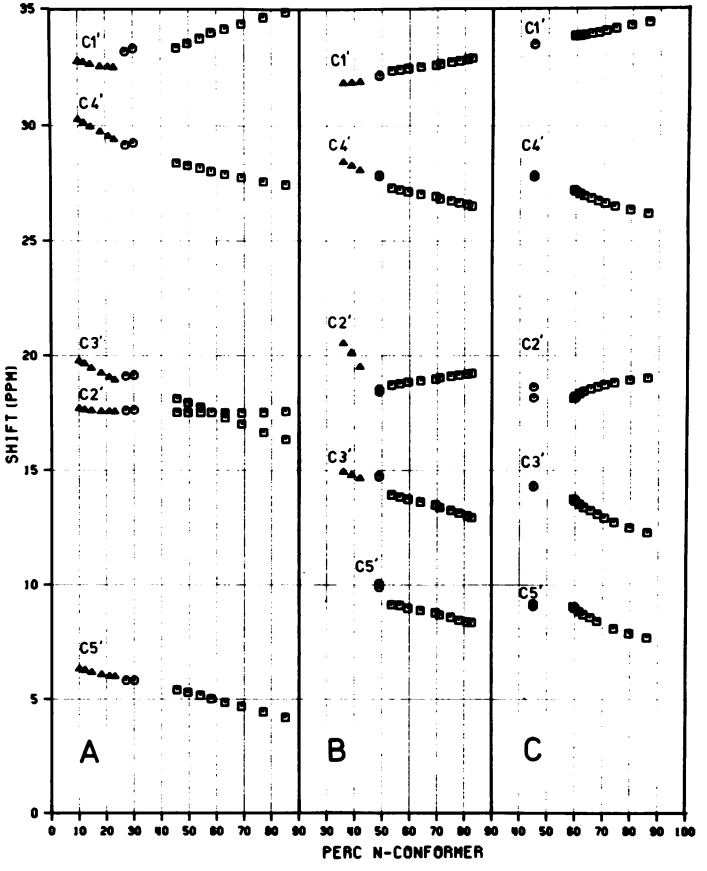

Figure 6: Chemical shift vs % N-conformer of all ribose carbons of
A: $\bar{A}p$- residues, △ = $\bar{A}pm$, ⊙ = $\bar{A}U\bar{A}$, □ = $\bar{A}U$.
B: -p$\bar{A}$ residues, △ = $\bar{C}\bar{C}\bar{A}$, ⊙ = mpU$\bar{A}$, □ = $\bar{A}\bar{A}$.
C: -pU residues, ⊙ = mpU, □ = $\bar{A}U$.

atoms differ significantly from each other. However, the weighted average of the 12 carbons monitored yields approximately the same parameters (i.e. Tm_X = 306.5 K and ΔS_X = -25 cal mol^{-1} K^{-1}) as were found in our ^{1}H NMR and CD studies.

Carbon-13 shifts and sugar conformation.

At this point it is well to realize that a given stacked state of an RNA fragment allows little conformational freedom with respect to the constituent backbone angles or the sugar ring[7]. Unstacking results in a blend of microstates characterized by a certain amount of freedom along each of the backbone

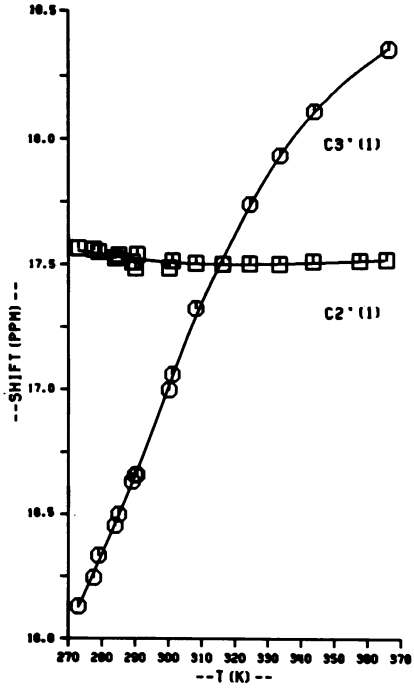

Figure 7: Chemical shift vs temperature profile of C3'(1) and C2'(1) of $\bar{A}U$ at 40 mM.

angles α, β, γ, δ, ε and ζ. Now one may ask which of these degrees of freedom is primarily responsible for the chemical shift changes displayed by the ribose carbons when the molecule reverts from a virtually complete stacked state towards the unstacked situation. It is known that the classical right-handed stacked state of a ribo-oligomer is characterized, among other properties, by an outspoken preference for N-type (^3_2T or ^3E) ribose rings[3,7]. This preference reverts into an N/S equilibrium that approximates the behaviour of the constituent monomeric units upon destacking. In other words, the ribose ring gains a significant amount of conformational freedom in the unstacked state and it stands to reason that this particular behaviour is reflected in the ^{13}C shifts of the sugar carbons. This idea can be tested in a straightforward manner, because previous proton NMR work on the compounds under study under various conditions has yielded accurate N/S equilibrium data[7,8,28,29,30]. For each compound the mole fraction of N-conformer has been calculated, using a generalized Karplus equation[36] and empirical relations between ^1H-^1H torsion angles and the pseudorotation parameters P and Φ[37]. A detailed description of this method is

Table 5: Temperature dependency of C2' and C3' resonances of some monomers and dimers. $\Delta\delta = \delta(70\ ^{\circ}C) - \delta(0\ ^{\circ}C)$.

	$\Delta\delta$C2'(1)	$\Delta\delta$C3'(1)
$\bar{A}$U	-0.04	1.78
$\bar{A}\bar{A}$	0.04	1.56
$\bar{A}$pm	-0.13	-0.82
mpU$\bar{A}$	-0.38	-0.23

given elsewhere[38].

In Figure 6a a plot of ^{13}C chemical shifts vs % N-conformer is shown for the $\bar{A}$p- parts of all compounds studied (for the sake of clarity data of $\bar{A}\bar{A}$ are not shown in Figure 6a; the behaviour of $\bar{A}\bar{A}$ is completely analogous to the behaviour of $\bar{A}$U). In these ribose rings the percentage of N-conformer covers the fairly large range of 10%-85%. Similar plots are shown for -p$\bar{A}$ parts and -pU parts (Figure 6b and 6c). However, in the latter two cases a smaller range of percentage N-conformer is covered (35%-85% and 45%-85% respectively). It is apparent from Fig. 6, that the δ ^{13}C vs %N curves are reasonably smooth. Small deviations from the main trends are, of course, observed. They may arise from other conformational properties, such as variations of χ between the various compounds and/or subtle differences between the backbone angles. However, these factors seem to be of minor importance. The fact, that several oligonucleotides, in spite of their different conformational properties, follow grosso modo the same trend can be taken as an indication for the strong dependence of the ribose ^{13}C chemical shifts (2-4 ppm) on the position of the N/S equilibrium.

The chemical shift of C2' of the -p$\bar{A}$ residue in $\bar{C}\bar{C}\bar{A}$ displays anomalous behaviour compared to the remaining δC2' of -p$\bar{A}$ residues (Fig. 6b). Instead of the normally observed small downfield shift with increasing N content, a pronounced upfield shift is seen. Presumably, this behaviour is related to the existence of an antiparallel stack between $\bar{C}(2)$ and $\bar{A}(3)$ which has been postulated recently by Doornbos[38] on the ground of CD, ^{1}H NMR and ^{31}P NMR measurements.

Assignment of C2' and C3' in Np- residues.

In all oligoribonucleotides studied thus far, the C2' chemical shift of Np-residues is virtually temperature independent, whereas the C3' resonance often strongly varies with temperature (see Fig. 7 and Table 5). A similar observation on UpU, ApA and the corresponding polymers was made by Alderfer and Ts'o[21]. This property appears useful for the assignment of C2' and C3' signals. Note, that these resonances tend to cross over at a certain temperature and therefore it is not possible to discriminate between these signals, when only their relative

positions at a single temperature are known. It should be mentioned, that as the chemical shift changes of the C3' originate from a shift of the N/S equilibrium of the ribose ring (vide supra), this rule is not applicable in those cases where the N and S populations remain constant over the entire accessible temperature range. A case in point is the behaviour of mpUĀ, where application of the temperature-dependency criterion would have led to an erroneous assignment of C2' and C3' signals (Table 5). Only two-dimensional NMR provides reliable assignments in these cases.

CONCLUSIONS.

Heteronuclear chemical shift correlation spectroscopy is shown to be a useful tool for the assignment of ^{13}C spectra of oligonucleotides, under the condition that the assignment of the ^{1}H spectrum is known. Especially in the more crowded spectra of trinucleoside diphosphates, it is found highly advantageous to remove ^{13}C-^{31}P splittings through ^{31}P-decoupling. This results in a better resolution and higher intensities of the cross peaks. With this technique it is possible to unequivocally assign all ribose carbons, including the C2' and C3' resonances of Np-residues, which have caused problems in the past. In those cases, where the population of N-conformer decreases with increasing temperature these two resonances can be discriminated from the temperature dependent behaviour of their chemical shifts: C3' is deshielded upon rising the temperature, whereas the chemical shift of C2' remains constant.

From ^{13}C shift vs temperature profiles thermodynamical parameters are derived for the stack/destack equilibrium of ĀU. It is shown that the ^{13}C shifts of the ribose ring in a number of RNA fragments are correlated to the position of the N/S ribose conformational equilibrium.

ACKNOWLEDGEMENTS.

This research was supported by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

Spectra were recorded at the Dutch National 500-200 MHz hf NMR Facility at Nijmegen and on the 300 MHz spectrometer in the Department of Chemistry at Leiden. We wish to thank Ing. P.A.W. van Dael and Ing. W. Guijt for technical assistance. We are indebted to Gerry Wille and Yvonne van den Hoogen for synthesis of the compounds studied.

REFERENCES

1. This paper is part 34 in the series "Nucleic Acid Constituents" from this laboratory. For part 33 see: Mellema, J.-R., Haasnoot, C.A.G., van der Marel, G.A., Wille, G., van Boekel, C.A.A., van Boom, J.H. and Altona, C. (1983), *Nucleic Acids Res.* 11, 5717-5738
2. Abbreviations used: NMR, Nuclear Magnetic Resonance; NOE, Nuclear Overhauser Effect; CD, Circular Dichroism; TMA, tetramethylammonium chloride; EDTA, ethylenediaminotetraacetic acid; $\bar{A}$, 6-N-(dimethyl)adenine; $\bar{C}$, 4-N-(dimethyl)cytosine. Mononucleotides mentioned in this study bear a terminal methyl phosphate group, these are abbreviated as follows: $\bar{A}$pm, mpU and $\bar{C}$pm.
3. Altona, C. and Sundaralingam, M. (1972) *J. Am. Chem. Soc. 94,* 8205-8212.
4. Altona, C. and Sundaralingam, M. (1973) *J. Am. Chem. Soc. 95,* 2333-2344
5. Haasnoot, C.A.G., de Leeuw, F.A.A.M., de Leeuw, H.P.M. and Altona,C. (1981) *Org. Magn. Reson. 15,* 43-51.
6. Haasnoot, C.A.G., de Leeuw, F.A.A.M., de Leeuw, H.P.M. and Altona, C.(1979) *Recl. Trav. Chim. Pays-Bas 98,* 576-577.
7. Altona, C. (1982) *Recl. Trav. Chim. Pays-Bas 101,* 413-433.
8. Hartel, A.J., Lankhorst, P.P. and Altona, C. (1982) *Eur. J. Biochem. 129,* 343-357. Note that the conversion factor "a" for the 0-52 °C temperature range should read a = 177.6.
9. Olsthoorn, C.S.M., Doornbos, J., de Leeuw, H.P.M. and Altona,C.(1982) *Eur. J. Biochem. 125,* 367-382.
10. Jones, A.M., Winkley, M., Grant, D.M. and Robins, R.K. (1970) *Proc. Natl. Acad. Sci. (U.S.) 65,* 19-26.
11. Uesugi, S., Miki, H. and Ikehara, M. (1981) *Chem. Pharm. Bull., 29* 2199-2204
12. Hruska, F.E. and Blonski, W.J.P. (1982) *Can. J. Chem., 60* 3026-3032.
13. Smith, I.C.P., Mantsch, H.H., Lapper, R.D., Deslauriers R. and Schleich, T. (1973) *Conformations of Biological Molecules and Polymers,* Bergmann, E. and Pullmann, B. Ed., New York, N.Y. Academic Press, 381-401.
14. Schleich, T. Cross, B. and Smith, I.C.P. (1976) *Nucleic Acids Res. 3,* 355-370.
15. Niemczura, W.P. and Hruska, F.E. (1980) *Can. J. Chem. 58,* 472-478.
16. Dorman, D.E. and Roberts, J.D. (1970) *Proc. Natl. Acad. Sci. (U.S.) 65,* 19-26
17. Mantsch, H.H. and Smith, I.C.P. (1972) *Biochem. Biophys. Res. Commun. 46,* 808-815.
18. Blonski, W.J.P., Hruska, F.E., Sadana, K.L. and Loewen, P.C. (1983) *Biopolymers 22,* 605-616.
19. Schleich, T., Cross, B.P., Blackburn, B.J. and Smith I.C.P. (1975) in *Structure and Conformation of Nucleic Acids and Protein-Nucleic Acid* Interactions. Sundaralingam, M. and Rao, S.T. Ed., University Park Press, Baltimore
20. Govil,G., Fisk, C.L., Howard, F.B. and Miles, H.T. (1981) *Biopolymers 20,* 573-603
21. Alderfer, J.L. and Ts'o P.O.P. (1977) *Biochemistry 16,* 2410-2416.
22. Lapper, R.D. and Smith, I.C.P. (1973) *J. Am. Chem. Soc. 95,* 2878-2880.
23. Lapper, R.D. and Smith, I.C.P. (1973) *J. Am. Chem. Soc. 95,* 2880-2884.
24. Uesugi, S, Tanaka, S. and Ikehara, M. (1979) *Org. Magn. Res. 12,* 143-145
25. Lankhorst, P.P. to be published.
26. Morris, G.A. and Hall, L.D. (1981) *J. Am. Chem. Soc. 103,* 4703-4711.

27. Olsthoorn, C.S.M., Haasnoot, C.A.G. and Altona, C. (1980) *Eur. J. Biochem. 106,* 85-95.
28. Lankhorst, P.P., van den Hoogen, Y. and Altona, C. to be published.
29. Lankhorst, P.P., Groeneveld, C. Wille, G. van Boom, J.H. and Altona, C. (1982) *Recl. Trav. Chim. Pays-Bas 101,* 253-263.
30. Doornbos, J, thesis Leiden (1983).
31. Van Boom, J.H. (1977) *Heterocycles 7,* 1197-1226.
32. Van Boom, J.H. and Burgers, P.M.J. (1977), *Recl. Trav. Chim. Pays-Bas 97,* 73-80
33. Maudsley, A.A.. Müller, L., Ernst, R.R. (1977) *J. Magn. Reson. 28,* 463-469.
34. Bodenhausen, G. and Freeman, R. (1977) *J. Magn. Reson. 28,* 471-476.
35. Bax, A. thesis Delft (1982).
36. Haasnoot, C.A.G., de Leeuw, F.A.A.M.,, de Leeuw, H.P.M. and Altona,C. (1980) *Tetrahedron 36,* 2783-2792.
37. de Leeuw, H.P.M., Haasnoot, C.A.G. and Altona, C. (1980) *Isr. J. Chem. 20,* 108-126.
38. de Leeuw, F.A.A.M. and Altona, C. (1983) *J. Comp. Chem.* in the press.