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^{13}C-NMR OF RIBOSYL A-A-A, A-A-G, AND A-U-G

SYNTHESIS AND ASSIGNMENT

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The three RNA trinucleotides; ApApA, ApApG, and ApUpG, have been synthesized in sufficient quantity to obtain natural abundance ^{13}C{^{1}H}-NMR spectra at strand concentrations between 4 and 100 mM. Comparisons between 70°C spectra of the three trimers and their consistuent dimers ApA, ApG, ApU, and UpG allow secure assignments to be made for most of the resonances. This paper describes the syntheses and ^{13}C assignments of the oligomers.

1. Introduction

The ^{1}H-NMR of oligonucleotides has been extensively studied, but much less work on the ^{13}C-NMR of these molecules has beem attempted, primarily because the 1% natural abundance of ^{13}C nuclei requires large samples for one-pulse experiments. However, ^{13}C-NMR spectroscopy may be particularly useful in examining the conformation and dynamics of oligonucleotides.

In oligonucleotides, ^{13}C nuclei with directly attached protons should relax primarily through ^{1}H-^{13}C dipolar coupling [1]. Thus, ^{13}C-NMR studies of the dynamics of DNA and RNA yield more definitive models than ^{31}P- or ^{1}H-NMR studies, since one needs only to consider the motion of fixed-length C-H vectors. ^{31}P and ^{1}H dipolar relaxation occurs primarily through nearby protons which are constantly varying in distance in a flexible molecule such as a trinucleotide. Furthermore, ^{31}P nuclei relax by a mixture of the dipolar and chemical shift anisotropy mechanisms which introduces another level of uncertainty into the calculations.

The three trimers AAA, AAG and AUG (fig. 1) were chosen because they can be synthesized in sufficient quantity, and their sequences differ by one base at a time; this aids the assignment process. In addition, it is widely accepted that the bases in AAA and AAG stack strongly at low

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Abbreviations: ^{13}C{^{1}H}-NMR, broad-band proton-decoupled ^{13}C nuclear magnetic resonance; HPLC, high-pressure liquid chromatography; DSS, 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt; the oligonucleotides discussed in this paper have no terminal phosphates – we usually abbreviate the notation for oligomers by leaving out the phosphodiester linkage; a superscript number on a base indicates its position in the chain, number 1 at the 5'-end.

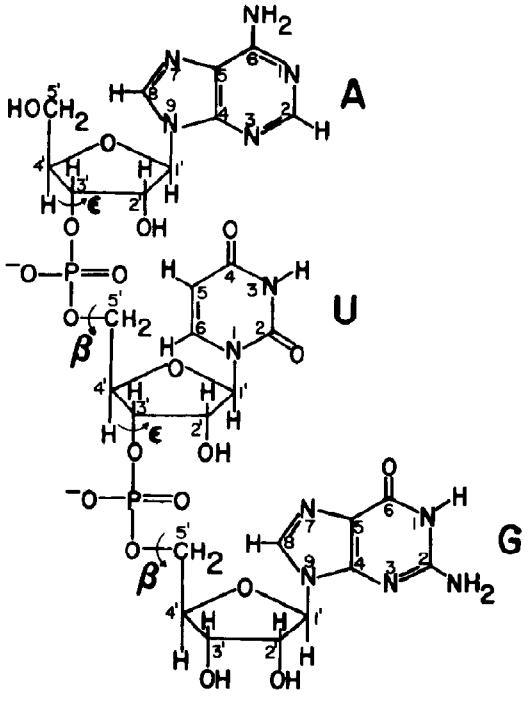


Fig. 1. Covalent structure and nomenclature for ApUpG conforming to the IUPAC-IUB JCBN [20] recommendations.

temperature, whereas it has been proposed that AUG forms a 'bulged base' structure, where the uracil is unstacked and A[1] stacks upon G[3] [2,3]. Such a structure should produce differences in the ^{13}C spectral properties of AUG as compared to AAG and AAA. A comparative analysis of the chemical shifts and coupling constants for the three trimers is the subject of another paper [4]. Analysis of the conformational dynamics using ^{13}C-NMR is in progress.

2. Materials and methods

2.1. Synthesis of oligonucleotides

The synthesis of the three oligonucleotides is described briefly in scheme 1. Further details of the syntheses are described elsewhere [5].

ApApA: Poly(A) was prepared from 5'-ADP using polynucleotide phosphorylase (PNPase), hydrolyzed in base, neutralized, and terminal phosphates removed with bacterial alkaline phosphatase (BAP) to produce $(AP)_nA$ oligomers.

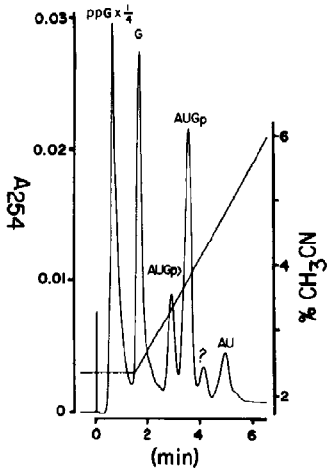


Fig. 2. HPLC elution profile for the ApU + ppG → ApUpGp + Gp + P_i reaction. Reverse-phase separation on radial compression column (microbondapak C_{18}, Waters Corp.). 7.5 ml/min, 5 min gradient 2.5–6% CH_3CN/1% $NH_4C_2H_3O_2$ in H_2O.

ApApG: Poly(A,G) random copolymer was prepared from ADP and GDP with PNPase, hydrolyzed with RNase T_1, and treated with bacterial alkaline phosphatase to produce $(Ap)_nG$ oligomers.

ApUpGp: ApUpGp was prepared using primer-dependent polynucleotide phosphorylase (PNPase T), ApU and GDP; the action of ribonuclease T_1 ensured a single addition to the ApU primer; the reaction mixture was boiled to inactivate PNPase and treated with bacterial alkaline phosphatase to yield ApUpG. PNPase T was prepared according to the method of Klee and Singer [6].

$$\text{ppA} \xrightarrow{\text{PNPase}} \text{poly(A)} \xrightarrow{\text{KOH}} (\text{Ap})_n\text{Ap} \xrightarrow{\text{BAP}}$$

$$(\text{Ap})_n\text{A} \xrightarrow{\text{DEAE}} \text{ApApA}$$

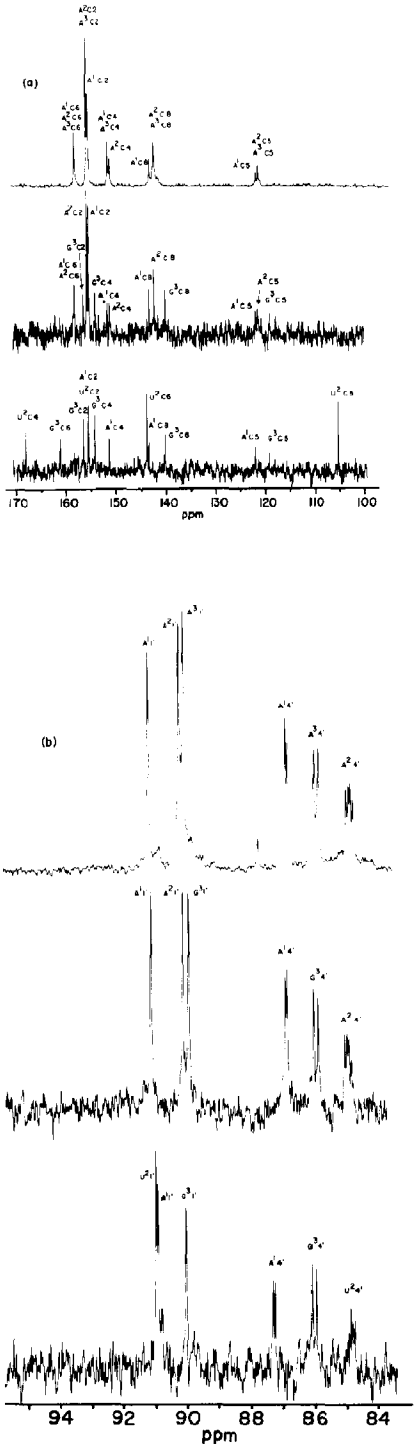
$$\text{ppA} + \text{ppG} \xrightarrow{\text{PNPase}} \text{poly(A,G)} \xrightarrow{\text{RNase T}_1}$$

$$(\text{Ap})_n\text{Gp} \xrightarrow{\text{BAP}} (\text{Ap})_n\text{G} \xrightarrow{\text{DEAE}} \text{ApApG}$$

$$\text{ApU} + \text{ppG} \xrightarrow{\text{PNPase T}} \text{ApUpGp} \xrightarrow{\text{BAP}}$$

$$\text{ApUpG} \xrightarrow{\text{DEAE}} \text{ApUpG}$$

Scheme 1.



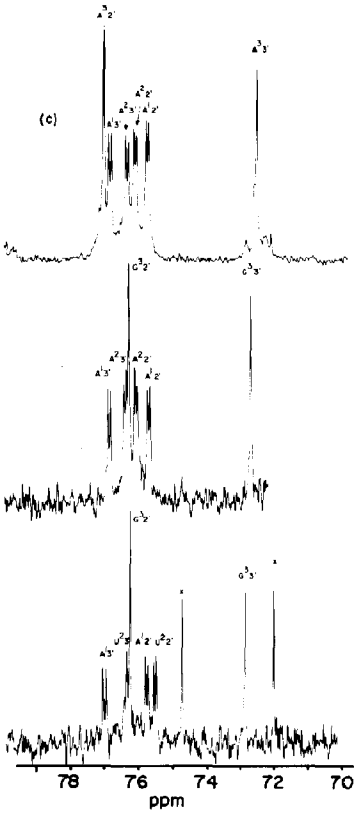


Fig. 3. ^{13}C-NMR spectra of AAA (100 mM, top), AAG (10 mM, middle), and AUG (10 mM, bottom) at 70°C, 10000–20000 transients (ppm from DSS); (a) aromatic carbons, (b) ribose 1′ and 4′ carbons, and (c) ribose 2′ and 3′ carbons. In panel a the weak signals from $G^3$6 of AAG and $A^1$6 of AUG were not observed in these particular spectra.

2.2. Purification

Each of the trinucleotides was purified preparatively by anion exchange on DEAE-Sephadex A25 (Pharmacia). Development of appropriate reaction

Table 1

Yield of trimers, starting quantities, and estimated trimer extinction coefficients (A_{260} units/μmol strand)

Trimer	Starting materials		ε
AAA (180 mg)	ADP (4 g)		39
AAG (300 mg)	ADP (5.2 g),	GDP (1.7 g)	37
AUG (30 mg)	ApU (50 mg),	GDP (240 mg)	34

Table 2

Chemical shifts of carbon resonances in AAA, AAG, AUG and their constituent dinucleotides at 70°C (ppm from DSS)

t, tentative assignment; o, assignment unambiguous, but two signals overlap; ?, peak not observed due to low signal-to-noise ratio.

Carbon	Position	AA	AG	AAA	AAG	AUG	UG	AU
6	1	158.14 ^{o}	158.31	158.02 ^{t}	158.04 ^{o}	158.43	144.10	158.43
	2	158.14 ^{o}	161.18	157.95 ^{t,o}	158.04 ^{o}	143.66	?	143.65
	3	–	–	157.95 ^{t,o}	161.06	161.08	–	–
2	1	155.18 ^{t}	155.28	155.06 ^{t}	155.11 ^{t}	155.26 ^{o}	?	155.35 ^{t}
	2	155.55 ^{t}	156.37	155.26 ^{t}	155.47 ^{t}	155.26 ^{o}	156.51	155.40 ^{t}
	3	–	–	155.40 ^{t}	156.27	156.30	–	–
4	1	151.06 ^{o}	151.13	151.30 ^{t,o}	151.47 ^{t}	151.20	168.40	151.33
	2	151.06 ^{o}	153.99	150.89 ^{t}	151.01 ^{t}	167.97	154.16	168.23
	3	–	–	151.30 ^{t,o}	153.87	154.02	–	–
8	1	143.03	143.10	143.00	143.10	143.18	–	143.13
	2	142.23	140.02	142.01 ^{t}	142.08	–	140.24	–
	3	–	–	142.13 ^{t}	140.02	140.03	–	–
5	1	121.36 ^{o}	?	121.63	121.80	121.82	?	?
	2	121.36 ^{o}	119.03	121.14 ^{t}	121.31	105.13	?	104.89
	3	–	–	121.24 ^{t}	119.08	119.03	–	–
1′	1	91.23	90.16	91.21	91.18	91.04 ^{o}	91.81	91.21 ^{t}
	2	90.26	90.22	90.26 ^{t}	90.21 ^{t}	91.04 ^{o}	90.19	91.81 ^{t}
	3	–	–	90.12 ^{t}	90.00 ^{t}	90.09	–	–
4′	1	86.76	86.98	86.85	86.91	87.33	86.01 ^{o}	86.93
	2	85.85	85.89	84.84	84.97	84.90	86.01 ^{o}	85.43
	3	–	–	85.90	85.98	86.09	–	–
3′	1	76.67	76.82	76.77	76.83	76.99	75.91	76.63
	2	72.46	72.62	76.28 ^{t}	76.35 ^{t}	76.26	72.77	71.93
	3	–	–	72.46	72.62	72.82	–	–
2′	1	75.69	75.75	75.69	75.69	75.73	75.52	75.73
	2	76.70	76.22	76.05 ^{t}	76.05 ^{t}	75.50	76.14	76.28
	3	–	–	76.97	76.22	76.22	–	–
5′	1	63.70	63.79	63.77	63.79	63.92	63.31	63.71
	2	67.48	67.64	67.38 ^{t}	67.45	67.40	67.74	67.18
	3	–	–	67.47 ^{t}	67.66	67.75	–	–

conditions was greatly facilitated by reverse-phase HPLC [7] using a radial compression column (Waters Associates, C18). Such an analytical separation is illustrated in fig. 2. The desired product, ApUpGp, is separated in less than 5 min from starting materials (ApU and ppG), Gp, and the reaction intermediate ApUpGp > (bearing a 2′, 3′-cyclic phosphate). Table 1 lists the yield and extinction coefficient for each trinucleotide.

2.3. NMR

^{13}C-NMR spectra were obtained at 62.9, 50.3 and 45.3 MHz. Samples were dissolved to various concentrations between 2 and 100 mM in the minimum amount of buffer necessary to obtain a spectrum (usually 1.5 ml in a 10 mm NMR tube). The buffer solution contained 10 mM phosphate or cacodylate buffer (pH 7.2), 0.1 mM EDTA,

Table 3

Chemical shifts of carbon resonances in AAA, AAG, AUG and their constituent dinucleotides at 25°C (ppm from DSS)

t, tentative assignment; o, assignment unambiguous, but two signals overlap.

Carbon	Position	AAA	AAG	AUG
6	1	157.63 [t]	157.72 [t]	158.11
	2	157.43 [t]	157.64 [t]	142.99 [o]
	3	158.23 [t]	160.96	161.21
2	1	154.03 [t]	154.55	154.89 [o]
	2	154.63 [t]	155.23	154.89 [o]
	3	154.93 [t]	156.24	156.34
4	1	150.43 [t]	150.52 [t]	150.56
	2	150.73 [t]	150.28 [t]	168.26
	3	150.43 [t]	153.54	153.91
8	1	142.51	142.69	142.99 [o]
	2	141.13 [t]	141.06	140.14
	3	141.40 [t]	139.73	–
5	1	121.43	121.48	121.72
	2	120.93 [t,o]	120.99	104.84
	3	120.93 [t,o]	118.66	118.74
1′	1	92.03	92.01	91.68 [t]
	2	91.98 [t]	90.82 [t]	90.93 [t]
	3	90.93 [t]	90.36 [t]	89.93
4′	1	86.22	86.66	86.93
	2	83.73	83.98	84.53
	3	85.48	85.81	86.18
3′	1	76.03	76.51	76.68
	2	75.03 [t]	75.60 [t]	75.73
	3	72.43	72.41	72.93
2′	1	75.53	75.68	75.73 [o]
	2	75.03 [t]	76.10 [t]	75.73 [o]
	3	77.43	76.51	76.43
5′	1	63.53	63.40	63.53
	2	66.53 [t]	66.57	66.73
	3	67.03 [t]	67.21	67.73

$^{2}H_2O$ for spectrometer lock, and 5 μl t-butanol as an internal reference. Chemical shifts are referenced to the DSS scale where t-butanol = 32.35 ppm. Typical acquisition times were 15–20 h (10000–20000 scans) at 10 mM, and 100 h (50000–100000) scans at 4 mM; pulse repetition rates were typically 3 s. Spectra were obtained using a sweep width of 180 ppm, 8K real data points zero-filled to 16K, and pulse widths between 30 and 90°.

3. Results

Fig. 3 presents the natural abundance ^{13}C-NMR spectra of the three trinucleotides at 70°C. The general characteristics of the spectra are as follows. The base carbons resonate from 100 to 170 ppm downfield from TMS, and are singlets. The protonated carbons have shorter T_1 relaxation times, and are usually more intense as a result. The ribose carbons resonate between 60 and 100 ppm. Some of these nuclei are coupled to neighboring phosphorus nuclei in the backbone of the molecules, and appear as doublets. The pNp 4′ carbons are coupled to two phosphorus nuclei, and exhibit two coupling constants. The terminal Np 5′ and pN 2′ and 3′ carbons are singlets. Earlier work on RNA and DNA oligomers [9] established the relative order of the carbon resonances in the spectra. Some assignments for the dimer ApA were completed by Alderfer and Ts'o [10]. Table 2 lists the chemical shifts of the carbons in the seven oligomers at 70°C; the assignments are briefly discussed in section 4. We have included the chemical shifts of the trinucleotides at 25°C (table 3) for the sake of completeness.

4. Discussion

4.1. Assignments

The method of comparative assignment at ≥ 70°C has been applied with considerable success to the ^{1}H-NMR spectra of oligonucleotides [11–13]. In assigning the ^{13}C spectra of AAA, AAG, and AUG we make comparisons between the trimers, and of the trimers with their constituent dimers; this was particularly successful for AUG, where there is the least overlap of resonances at 70°C.

The temperature dependence is also useful in making some assignments. At 70°C the oligomers are quite disordered though some base stacking remains. Many of the resonances shift by large amounts as temperature decreases. The base carbons usually become more shielded, as do most of the sugar carbons although the 1′ signals are deshielded [4]. In distinguishing the overlapping 2′

and 3′ doublets we note the general trend that the 2′ signals shift very little with temperature whereas the 3′ resonances become substantially more shielded with decreasing temperature [10].

In some cases the methods discussed above do not lead to unique assignments. At low temperatures AAA and AAG are known to stack strongly from optical spectroscopy [14] and ^{1}H-NMR [2] in a manner consistent with the A-RNA model from X-ray diffraction [15]. At 70°C, only a fraction of the bases are stacked, but ^{1}H-NMR spectra are consistent with the residual stacking being similar to that at lower temperature. Therefore, when no other procedure yields a unique assignment, we consider the relative effects of ring current shielding of the bases of a Kendrew model built with the A-RNA geometry. Ring current effects decrease in the order A, G, C, U [16]. We note, however, that for ^{13}C nuclei, consideration of ring current shifts alone probably cannot explain the observed shieldings [4].

After our measurements had been completed [5], several multiple-pulse methods were developed that afford unequivocal assignments of most C-H carbons in oligonucleotides. Standard carbon-proton shift correlation experiments on some di- and trinucleotides require approx. 30 h accumulations at 50 mM, and are best performed with simultaneous ^{31}P decoupling [17]. More, recently, a method requiring much smaller amounts of material was developed which uses polarization transfer and observation of ^{13}C nuclei through the proton channel [18,19]. This provides a theoretical enhancement of 63-fold in sensitivity over standard one-pulse detection in the carbon channel, offering the possibility of proton-carbon shift correlation at 1–10 mM. Both methods rely on prior assignment of the proton spectra, usually by a combination of two-dimensional COSY and NOESY spectra, and may not be useful for assigning carbons with no attached protons. On the other hand, the comparative method is equally useful for quaternary and protonated carbons, and gives a completely independent method for assignment. Every assignment procedure short of isotopic substitution has the potential to introduce ambiguities, so it is important to have independent methods for checking assignments. The following paragraph gives a brief overview of our assignments; full details are described in the appendix.

Straightforward comparison allows about half of the assignments to be made without question. This is a consequence of the large range of chemical shift that characterizes ^{13}C spectra. For example, there can be no question about the G^{6} base carbon assignment, which is 3 ppm from the nearest signal, and correlates closely for every G-containing oligomer (see table 2). On the other hand, the 2′,3′ region presents a substantial challenge and the details of these, and other complex assignments, are given in the appendix. Tables 2 and 3 contain the results and indicate whether the assignment is tentative. AUG has the least overlap of signals and all 29 of its signals are securely assigned, whereas 8 of the AAG and 19 of the AAA resonances must be regarded as tentative. Fortunately, the tentative assignments usually concern resonances of a single type, e.g., the three A^{6} signals of AAA. These usually exhibit a very similar temperature dependence of chemical shift and nearly identical relaxation parameters, so the conformational and dynamic arguments are not affected by the uncertain assignment.

Appendix: Detailed assignments of carbon resonances

A.1. Assignment of base carbons

Fig. 4 is a correlation diagram for the base carbons in the seven oligomers; the chemical shifts are collected in table 2. Examination of fig. 4 leads to a straightforward assignment of the U4, G6, G2, G4, G8, G5 and U5 signals in AUG and AAG. A6 is simply assigned in AUG and AAG; A^{1}6 and A^{2}6 overlap in AAG. In AAA, the least shielded resonance is thought to be A^{1}6 based on consideration of the Kendrew model.

A2 and U2 overlap in AUG and are very similar to the corresponding peaks in AU. In AA we assign the most shielded resonance as A^{1}2 since it is slightly more shielded than the A2 of AG and a G should shield less than an A in a similar geometry. G^{3}2 in AAG has no attached proton and as a result its T_1 relaxation time is nearly

twice that of $A^1$2 and $A^2$2 (L. Levy and P. Borer, unpublished data). This can also be seen in the dramatically reduced intensity of the $G^3$2 signal compared to $A^1$2 and $A^2$2 in fig. 3a (middle panel at approx. 115 ppm). The distinction between $A^1$2 and $A^2$2 in AAG is difficult so we regard the current assignment as tentative. At 25°C the signal we assign as $A^2$2 has a slightly longer T_1 and lower nuclear Overhauser enhancement than $A^1$2. This indicates more motional freedom at $A^1$2; the base at the end of the chain should move more freely than $A^2$2. The assignments of the AAA 2-carbons are less secure. Each of these AAA resonances is shielded slightly more than the corresponding AAG signal as expected upon replacing G^3 with A^3. The difference in chemical shift between $A^1$2 and $A^3$2 for AAA in the current assignment is close to the difference for $A^1$2 and $A^2$2 in AA; this is consistent with these carbons residing at the ends of the trimer and dimer chains, respectively.

A4 in AU, AG and AUG is easily assigned. In AAG and AAA we assign $A^2$4 as the most shielded resonance, since it is in the middle of the chain. This is consistent with its resonance position in relation to the A4 in AA and AG. The $A^1$4 in AAG and the overlapping A4 signals in AAA appear to be less shielded than in the corresponding dimers. The effect is small but we have no ready explanation for this unexpected result. Therefore we must regard the A4 assignments in AAA and AAG as tentative.

In the A8, U6 region of fig. 4, $A^1$8 has a very similar position in all six of the oligomers containing this carbon so this assignment is secure. The U6 shifts are also internally consistent. $A^2$8 in AAG is easily assigned and as seen by comparison with AA is shielded mostly by A^1. This is also expected from the Kendrew model. In AAA the $A^2$8 and $A^3$8 signals are difficult to distinguish. We assign the most shielded carbon as $A^2$8 because it should be shielded by both neighbouring adenines.

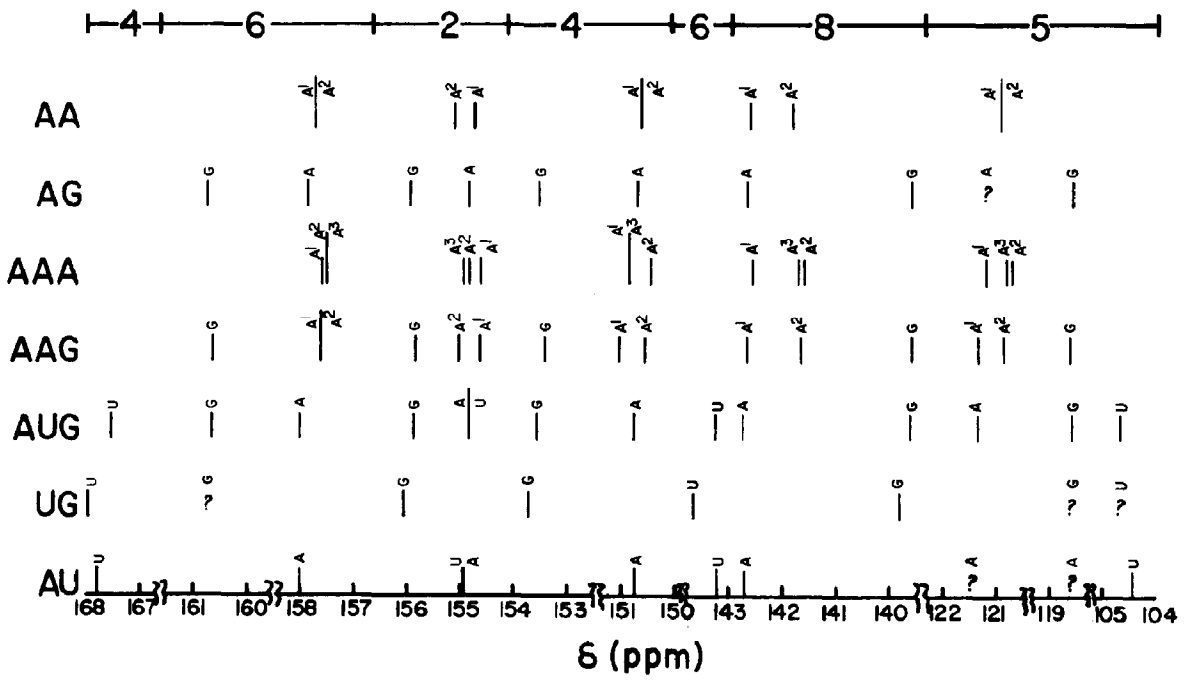


Fig. 4. Correlation diagram for the ^{13}C chemical shifts of the base carbons of AA, AG, AAA, AAG, AUG, UG, and AU at 70°C (all resonate as singlets). A question mark signifies a resonance not observed because of low signal-to-noise ratio.

A5 in AUG is easily assigned, and comparison with AAG and AAA allows a secure assignment in the other trimers. Because of its low intensity we could not determine its position in AG and AU. Comparison of the three trimers establishes that $A^2$5 should be most shielded in AAG and AAA. It is reasonable that $A^1$5 should be least shielded. The Kendrew model also supports this suggestion. The $A^2$5 and $A^3$5 assignments are tentative for AAA.

A.2. Assignments of ribose carbons

Fig. 5 is a correlation diagram for the ribose carbons; again the numerical values are collected in table 2. Alderfer and Ts'o [10] assigned the AA peaks which provides the basis of our other assignments. Examination of the signals for the four dimers shows the suprising result that they are very sensitive to composition effects even at 70°C.

We start assigning the 1′ singlets by comparing AU and UG which establishes resonance positions for G1′ in AG and AUG. Knowing G1′ in AG and UG allows assignment of A1′ in AG and U1′ in UG. AA should have a similar geometry to AG so we have assigned $A^1$1′ and $A^2$1′ as in fig. 5. Comparing the top five spectra suggests that $A^1$1′ should be least shielded in the trimers. U1′ overlaps A1′ in AUG and is much more shielded than in UG. The $A^2$1′, $G^3$1′ and $A^3$1′ assignments in AAG and AAA are uncertain. We tentatively assign the most shielded signal in AAG as $G^3$1′ by analogy with AG, though it could well be $A^2$1′. Since AAG and AAA probably have similar conformations it is likely that the two most shielded resonances correspond, as do the two at slightly lower field.

The 4′ multiplets are easily assigned by examin-

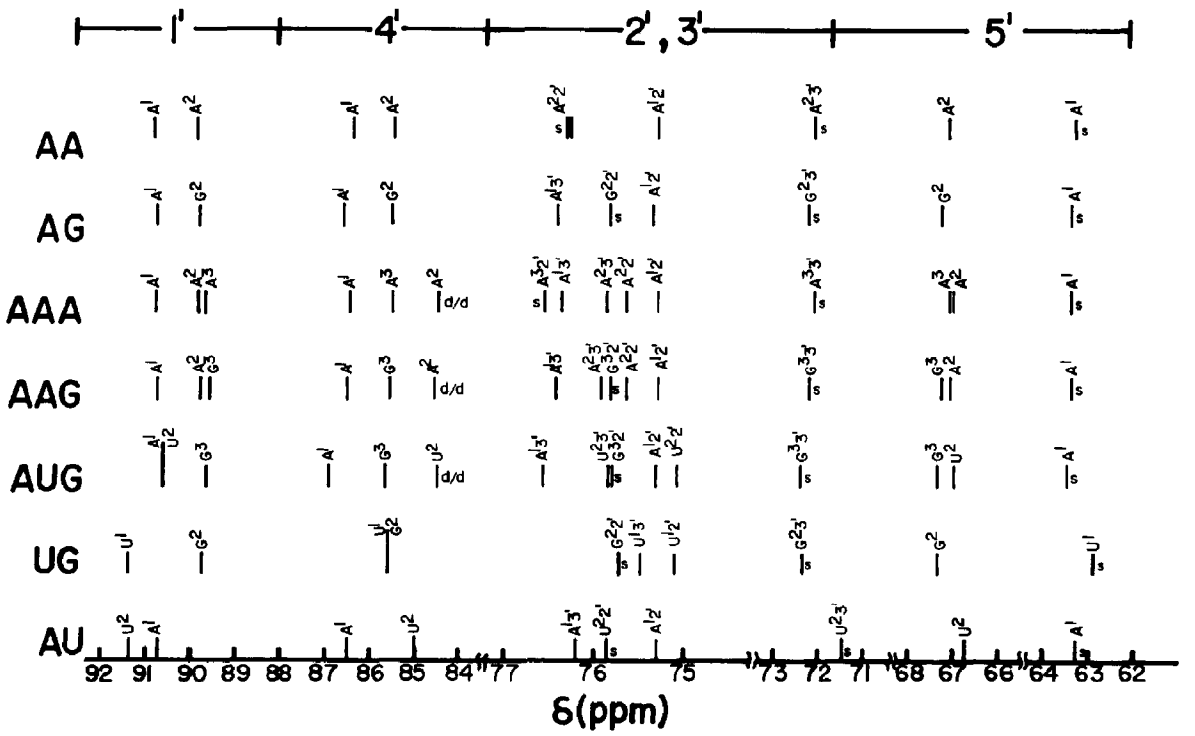


Fig. 5. Correlation diagram for the ^{13}C chemical shifts of the ribose carbons of AA, AG, AAA, AAG, AUG, UG, and AU at 70°C [the 1′ are all singlets, the others are doublets except those marked s (singlet) or d/d (doublet of doublets)].

ing fig. 5 and their ^{13}C-^{31}P coupling constants. In the trimers the doublet of doublets (d/d) corresponds to A^24' in AAG and AAA, and to U^24' in AUG. In each of the G-containing oligomers there is a resonance near 85.9 ppm with a coupling constant of about 8 Hz which we assign to G4'. A similar chemical shift and coupling constant is observed for the signals we assign as A^34' of AAA and A^24' of AA. The least shielded signal in each molecule has a coupling constant of about 3 Hz and is assigned to A^14'. The 4' doublets in UG overlap.

In the 2' and 3' region the singlet resonances are easily assigned. The most shielded resonance is assigned to the 3' carbon with the free 3' hydroxyl. This signal consistently occurs at about 72.4 ppm. The other singlet must be the adjacent 2' carbon. Its chemical shift is strongly influenced by effects of composition and sequence; G2' and U2' are always near 76.2 ppm, whereas A^22' in AA is at 76.70 ppm and A^32' in AAA is at 76.97 ppm.

The doublets in AG and AU are assigned by analogy with AA; in UG we assume U3' is less shielded than U2', again by analogy with the other dimers. A^12' in AAG and AAA is very similar to the corresponding resonance in AA and AG, and closely matches the signal identified as A2' in AUG. This latter signal might be confused with that assigned as U2' but the present assignment nicely matches the U2' signals in AUG and UG. The signals discussed in this paragraph shift very little with temperature, which suggests that they all belong to the 2' group (see paragraph 2 in section 4).

The A^13' doublets in AAA and AAG correspond with the analogous A^13' signals in AA and AG. We assign the least shielded 3' doublet in AUG as A3' because of its similarity to the A^13' signals just discussed; also in AU, A3' is the least shielded of the 2' and 3' carbons. That leaves only the resonance assigned as U3' among the AUG signals; it shifts upfield more than 0.4 ppm upon lowering the temperature to 30°C as expected for a 3' signal. In AAA and AAG the doublets near 76 ppm are identified as A^22' by their temperature-invariant chemical shifts. The doublets near 76.3 ppm are assigned as A^23'; these become more shielded by about 1 ppm at 30°C. We consider the A^22' and 3' assignments in AAG and AAA to be tentative because they are based upon the temperature dependence of their chemical shifts and coupling constants. The other 2' and 3' assignments are secure, a rather remarkable outcome for such a crowded spectral region.

In the 5' region the upfield singlets correspond to the 5' terminal residue. G5' in AUG corresponds with that in UG as does U5' with that in AU. G5' in AAG matches nicely with that in AG; it is also the least shielded of these trimer signals in agreement with AUG. The A^35' assignment in AAA should similarly be the least shielded by analogy with the other two trimers; further it corresponds with A^25' in AA. We assign A^25' in AAG and AAA as the remaining 5' doublet.

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