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A 270 MHz ^{1}H-NMR STUDY OF TWO "BRANCHED" RIBONUCLEOTIDES $A^{2'p5'G}_{3'p5'U}$ AND $A^{2'p5'G}_{3'p5'C}$ WHICH ARE PRODUCTS OF PRE-mRNA PROCESSING ("SPLICING") REACTIONS.

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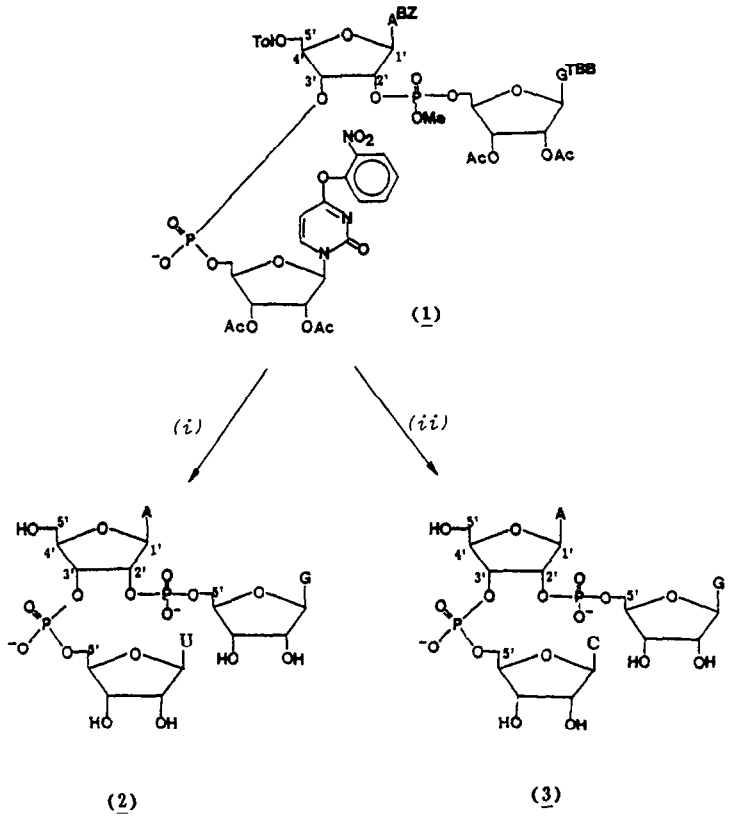
Summary: A 270 MHz ^{1}H-NMR study of two branched trinucleotides 2 and 3 have been carried out by comparing their temperature-dependent chemical shifts with A2'p5'G (4). It has emerged from this study that the adenine and guanine residues in 2 and 3 are quite strongly stacked as in A2'p5'G (4). Appropriate 1D NOE experiments have shown that the pyrimidine residue in 2 and 3 is in an anti conformation with respect to its own sugar moiety while the adenine and guanine residues are in syn conformation. The pyrimidine residue should be coplanar with the adenine residue in order to accomodate the observed deshielding of H8A and H1'A in 2 and 3 as compared to 4. The latter rationale also suggests a spatial proximity of H8A/H1'A and H5C(H5U)/H6C(H6U) in compounds 2 and 3. The proposed conformation of the branched trinucleotides 2 and 3 is thus comprised of a stacked state between adenine and guanine moieties while the pyrimidine residue is apart and should adopt a coplanar state with the adenine part. Finally, the analysis of the conformational states of the branched nucleotides 2 and 3, has led to the development of a suitable rationale for the conformation-driven-energy-pump for the self-catalyzed splicing reactions.

Recently remarkable advances[1-4] have been made about the mechanism and biochemistry of reactions involved in splicing of eukaryotic mRNA precursors (pre-mRNA). Compelling evidences are now available that some of the splicing reaction of pre-mRNA, as found in some nuclear mRNA splicing[4], involving intron excision and exon ligation, requires ATP, $MgCl_2$ and monovalent cations as cofactors. On the other hand, some other self-catalyzed mitochondrial pre-mRNA splicing in group II does not require ATP as an external source of energy but requires only Mg^{+2} and spermidine[4]. Analysis of the splicing reaction products in this group II splicing reactions have shown[1-4] that the excised introns as well as intermediary RNA species have the unusual configuration of a circle containing a branch point which is referred to as "lariats". Chemical and enzymatic analysis of such lariats have shown[3,4] that the branch point within the circular molecule, both in Adenovirus 2 major late transcription unit and in ß-globin RNA, is located at an adenosine residue. The structure of the branch is $A^{2'p5'G}_{3'p5'U}$ (2) in the Adenovirus 2 major late transcription unit and $A^{2'p5'G}_{3'p5'C}$ (3) in ß-globin RNA. It may be pointed out that in both cases the branch is formed by the joining of a guanosine residue to the 2'-OH of adeninyl residue of an ordinary adenylyl(3' → 5')-pyrimidine via a 2' → 5' phosphodiester bond. We have recently reported regiospecific chemical synthesis of both trinucleotides 2 and 3 using a common intermediate 1[8]. We herein report a conformational analysis of branched-trinucleotides 2 and 3 by 270 MHz ^{1}H-NMR spectroscopy, in order to understand the structural make up of introns, in general, that participate in the splicing reaction. It was also envisioned that the elucidation of structures of these branched-trinucleotides may lead us to understand if any conformational constrain or preference in 2 or 3 do act as a



(i) Syn-4-nitrobenzaldoximate ion in dioxane-water for 24 h at 20 °C, aqueous ammonia (d = 0.9) for 5 days at 20 °C and purification on a DEAE Sephdex column.

(ii) Liquid ammonia for 48 h at 20 °C, aqueous ammonia (d = 0.9) for 5 days at 20 °C and purification on a DEAE Sephadex column.

A^{Bz} = 6-N-Benzoyl-9-adeninyl- ; G^{TBB} = 2-N-(4-t-butylbenzoyl)-9-guaninyl.
A = 9-Adeninyl-
C = 1-Cytosinyl- G = 9-Guaninyl- ; U = 1-Uracilyl-

positive signal for rapid excision and ligation of the intervening sequence in vivo in self-splicing reactions in group II type mitochondrial and/or nuclear pre-mRNA.

The assignments and the study of conformations of these branched trinucleotides 2 and 3 have been based on an incremental analysis starting with the study of the conformational state of the parent dimer - adenylyl (2' → 5') guanosine (A2'p5'G) (4) and then observing the effect of the 3'-substitutent (pU or pC) on 4.

Assignment of resonances.

(a) Adenylyl (2' → 5') adenosine (A2'p5'G) (4).

Fig. 1 shows the 270 MHz 1D ^{1}H-NMR spectrum. The spectra of oligonucleotides can be readily divided in three major parts: (i) the aromatic resonances (7.5 - 8.5 ppm) from H8 and H2 of adenine and H8

of guanine residues; (ii) the anomeric resonances (5.5 - 6.5 ppm) from H1' of pentofuranosylribose moieties; (iii) the high-field absorptions from other sugar protons. The use of 2D NMR[5] (COSY, HOHAHA), 1D NOE and the literature reports[6,7] concerning the oligo-RNA conformations by ^{1}H-NMR spectroscopy have been extensively used in the present work for the assignment of proton resonances of 4. The study of chemical shifts as a function of temperature (0 - 80°C) have been also used for assignment purposes and these studies have provided valuable informations regarding the conformational state of 4. The ^{1}H chemical shifts of 4 are shown in Table 1.

(i) Resonances for aromatic protons in 4.

It is reasonable to assume[15,16,21,24] that the chemical shifts of protons of oligonucleotides at high temperature (ca. 80°C) closely resemble to those found for mononucleotides. It is thus clear from a comparison with mononucleotides that the aromatic singlets from adenine and guanine residues of 4 are shielded in the following order: H8G > H2A > H8A. Furthermore, the intensity of the H8A was found to be reduced, due to its known acidity, during the NMR experiments in ^2H_2O solution, confirming our correct assignment.

(ii) Resonances of sugar protons in 4.

The connectivities of the base and the sugar of each nucleoside residue was based upon 1D NOE experiments. Fig. 2 shows that the irradiation of the H8A proton produces an NOE on the downfield

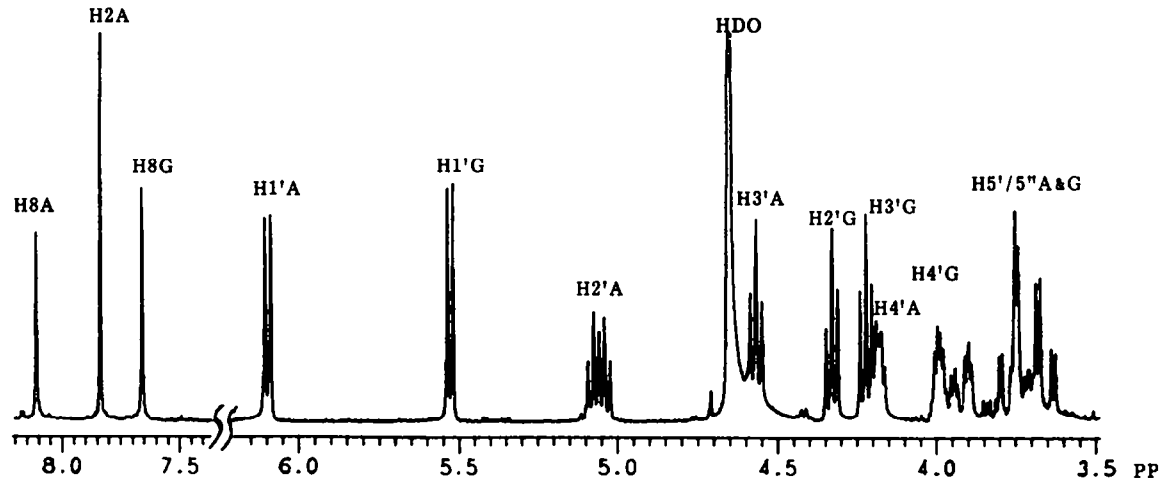


Fig. 1 : 270 MHz ^{1}H-NMR spectrum of A2'p5'G (4) at 30 °C (CH_3CN as reference set at 2.0 ppm)

Table 1: ^{1}H chemical shifts[a] of the dimer A2'p5'G (4).

Nucleotide	H8	H2	H1'	H2'	H3'	H4'	H5'/H5''[b]
Ap	8.11	7.83	6.09	5.06	5.57	4.18	3.71
pG	7.65	-	5.53	4.33	4.22	4.00	3.92

[a]At 30°C from CH_3CN as reference set at 2 ppm.
[b]Average value between 5' and 5''.

H1' and the irradiation of the H8G similarly enhanced the intensity of the upfield H1'. These experiments have led us to conclude that H1'A is more downfield than H1'G; furthermore these NOE experiments establish that the conformation across the glycosidic bond in both nucleoside residues must be syn. The 2D homonuclear correlated spectroscopy (COSY) was used to assign all other protons as shown in Fig. 3.

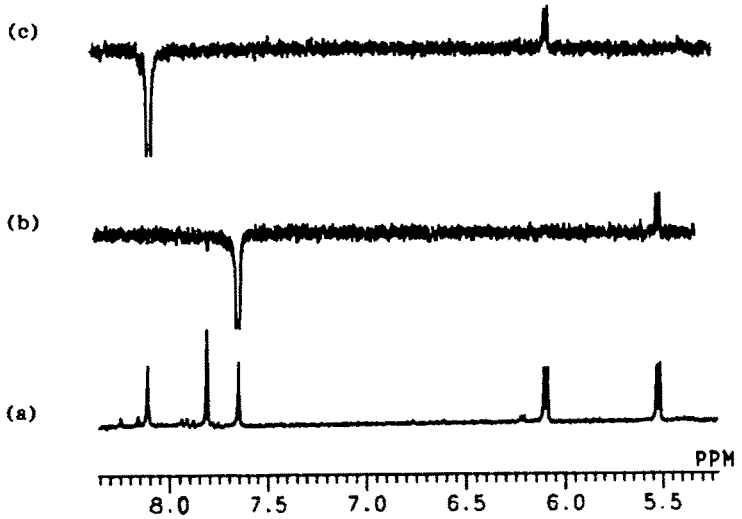


Fig. 2 : NOE difference spectra of 4 at 20 °C; (a) 270 MHz 1D spectrum, (b) irradiation of H8G showing the intensity enhancement of H1'G, (c) irradiation of H8A showing the intensity enhancement of H1'A

(b) Branched trinucleotides: $A^{2'p5'G}_{3'p5'U}$ (2) and $A^{2'p5'G}_{3'p5'C}$ (3).

A comparison of 1D ^{1}H-NMR of 2 and 3 with that of 4 clearly showed additional signals in the aromatic and anomeric parts due to the presence of H6 and H5/H1', respectively, from the 3'→ 5' phosphodiester linked pyrimidine residues (U or C). However, H6 and H5 of the pyridimidine residue was easily identified due to their spin-spin coupling of ca. 8 Hz for U and ca. 7.5 Hz for C which were independent of variation of temperature. The H1'A is the most downfield of signals in the anomeric region which has been confirmed by COSY experiments[8]. Similarly, a comparison of anomeric proton chemical shifts between 5'-GMP and 5'-UMP or 5'-CMP clearly showed that the H1'G was more shielded than H1'U or H1'C[9,10]. Furthermore, an NOE between H6C/H6U with the respective H2' was used to establish the presence of anti conformation in these pyrimidine nucleoside moieties. Hartmann-Hahn NMR spectroscopy[11] was conveniently employed for sugar-proton assignment purposes since 270 MHz COSY spectra of 2 and 3 failed to assign all proton resonances in high field region because of severe spectral overlap.

We have previously shown[8] the usefulness of 1D subspectra of each sugar residues in the assignments of all sugar protons of 2 and 3 using Hartmann-Hahn spin-locked spectroscopic techniques[12]. The 2D spectra of 2 and 3 using the HOHAHA pulse sequence[13] are shown in Fig. 4 and 5 respectively. The main advantage of HOHAHA pulse sequence is that the intensity of cross peak does not depend on the J couplings as in Relay COSY[5]; furthermore, it is possible to obtain a full correlation from the starting point of the J network until the end depending upon appropriate propagation delay.

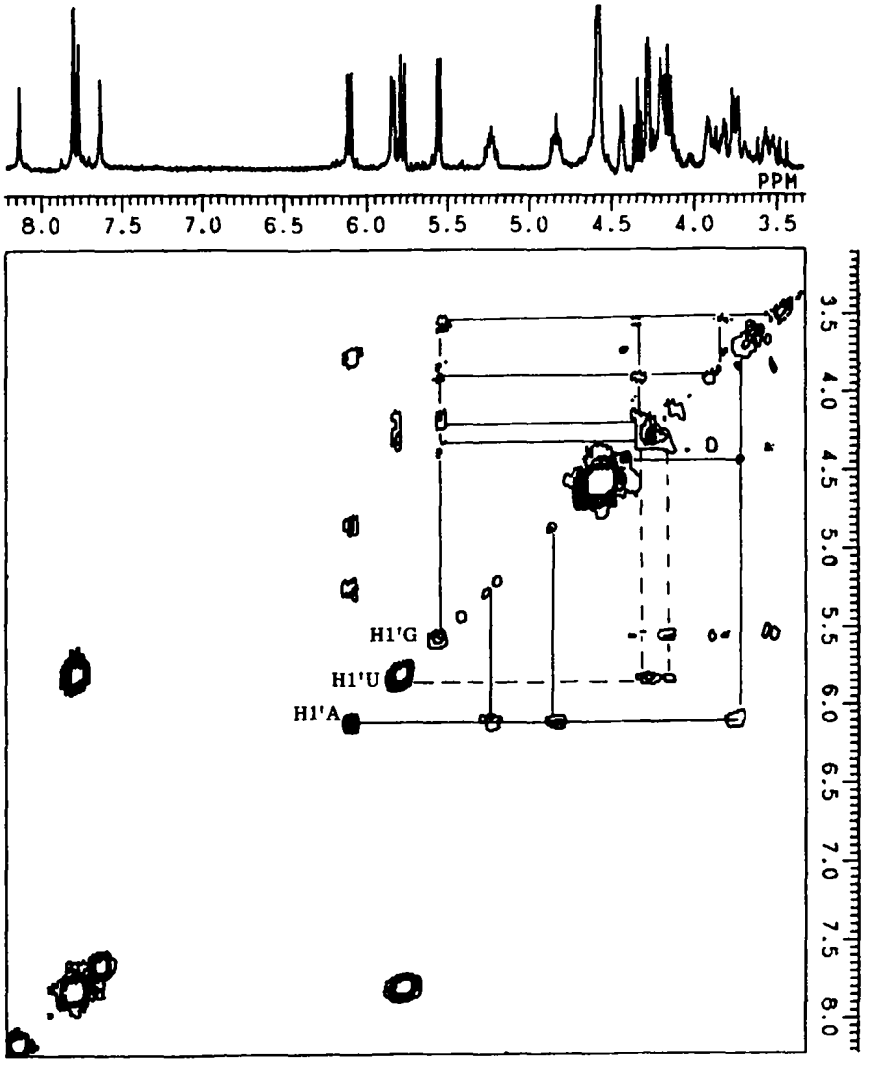


Fig. 4 : 270 MHz HOHAHA spectrum of 2 at 35 °C. The connection of J network from each sugar moiety is shown from each H1'.

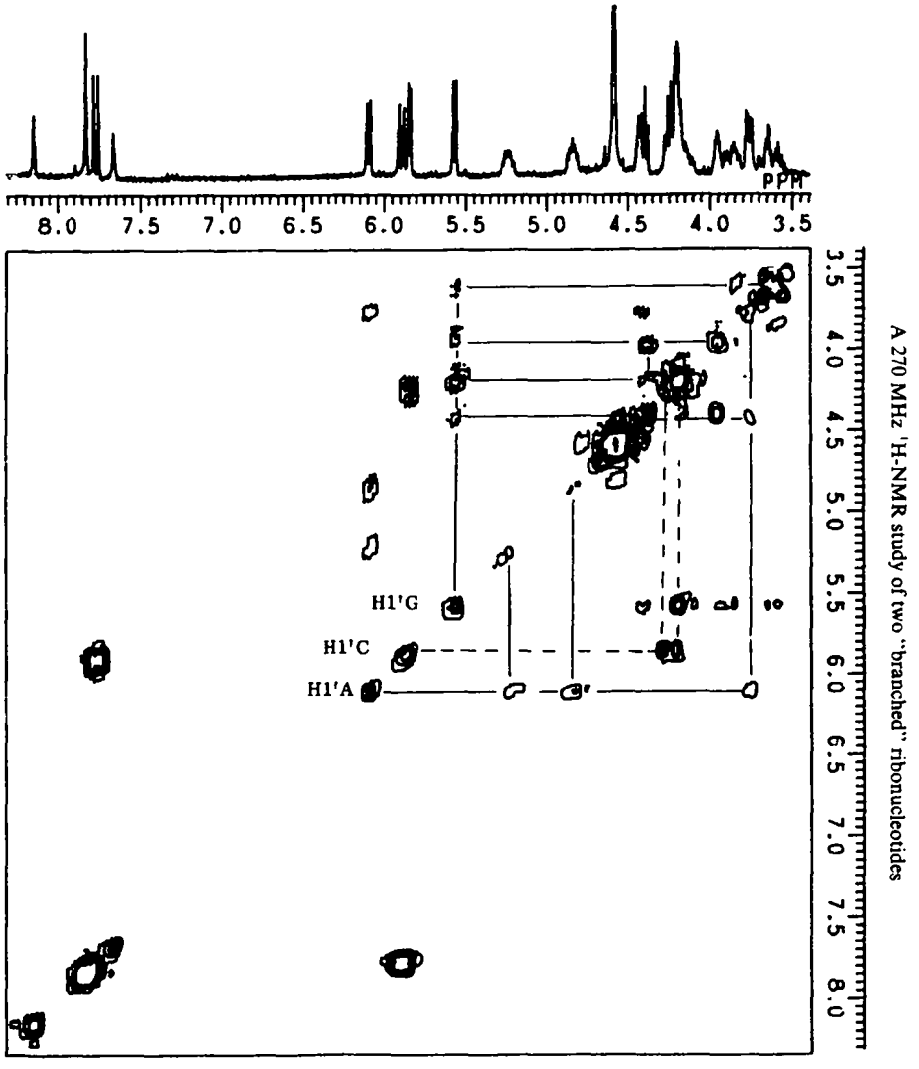


Fig. 5 : 270 MHz HOHAHA spectrum of 3 at 35 °C. The connection of J network from each sugar moiety is shown from each H1'.

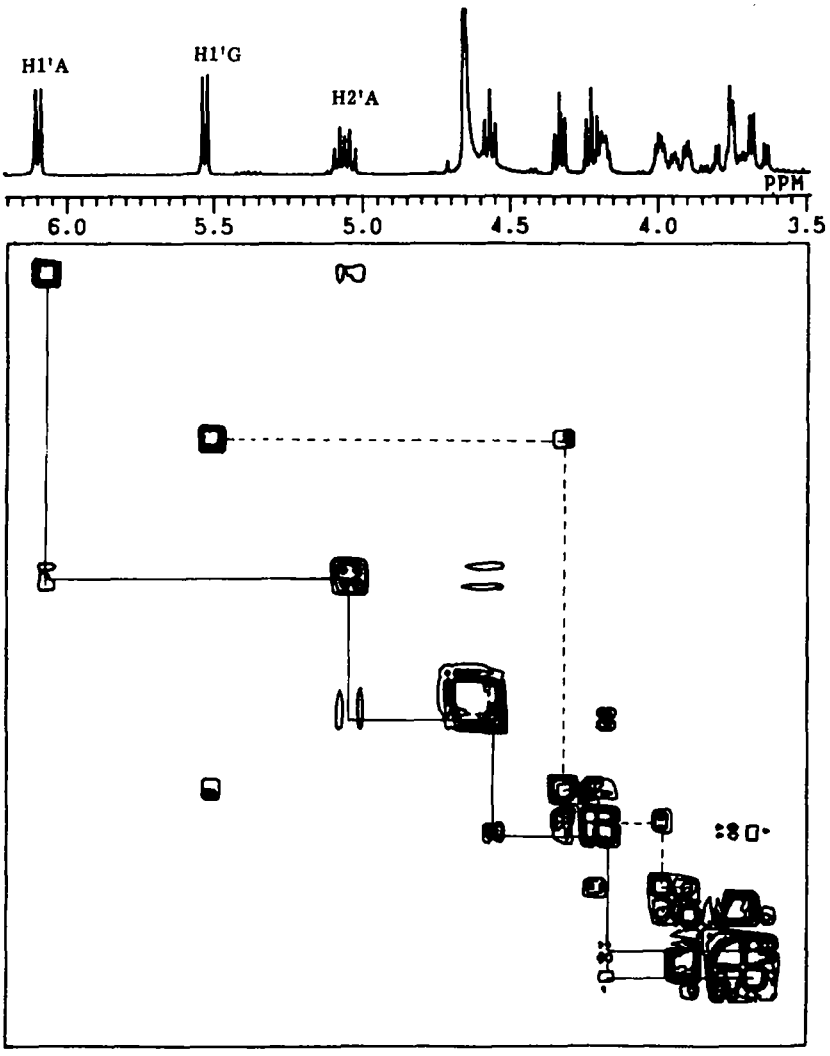


Fig.3 : 270 MHz COSY spectrum of 4 at 30 °C where aromatic protons are not shown. (———) adenosine part; (------) guanosine part.

Finally, the $^{31}P/^{1}H$ two dimensional shift correlation NMR spectroscopy[14] confirmed that the 2' → 5' phosphodiester phosphate is more shielded and experience spin-spin couplings with H5'G and H5''G. The Fig. 6 shows the $^{31}P/^{1}H$ correlated 2D spectrum of 3 as an example; it should be noted that the compound 2 also gave a similar correlation.

RESULTS AND DISCUSSIONS

Temperature dependent chemical shifts of aromatic and anomeric protons from different nucleoside residues of trinucleotides 2 and 3 were primarily used to assess their conformations which have been summarized in Fig. 7. It has been earlier shown by different groups of workers[15,16,17] that there are three major factors that determine the chemical shifts in oligonucleotides: (i) the ring-current effect of the base; (ii) the diamagnetic anisotropy of the electron-rich groups in the molecule like C=O or P=O bonds; (iii) electric-field effects.

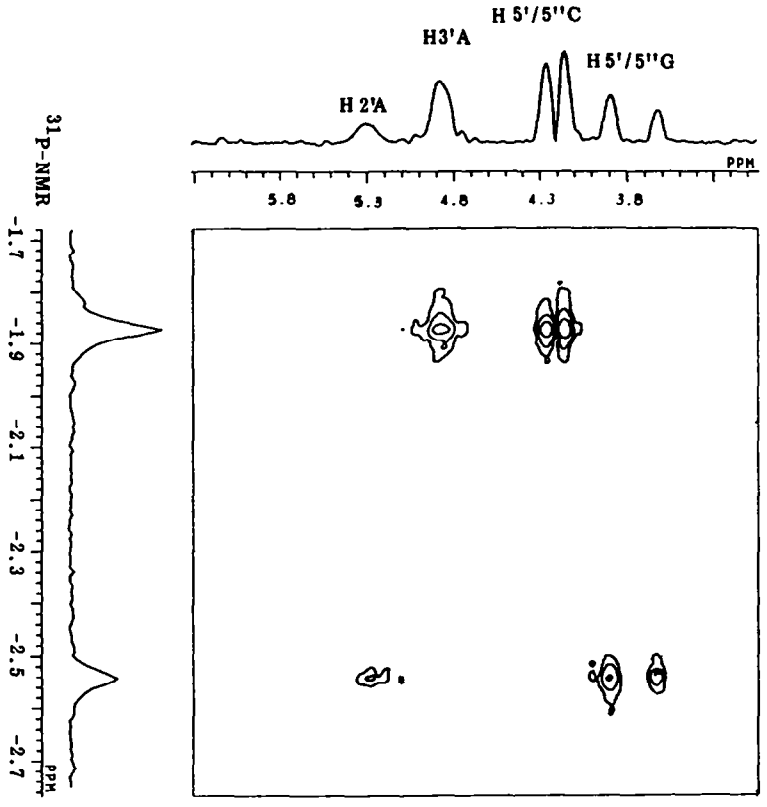


Fig. 6 : $^{31}P/\ ^{1}H$ correlation spectrum of compound 3 at 30 °C. The projections are represented in each direction.

In the stacked form, the aromatic and anomeric protons experience an upfield shift due to the ring-current effect of the neighbouring base. It is known that in the stacked oligonucleotides H2A and H5C or H5U go more downfield upon an increase of temperature, showing a weak dependence on the glycosidic torsion angle. It has been also noted that the conformation across the glycosidic bond can induce shielding or deshielding of H8A, H8G, H6U or H6C due to the anisotropy of the ribose-ring-oxygen and the 5'-phosphate group.

Conformational analysis of A2'p5'G (4).

H2A of 4 moves upfield by ca. 0.2 ppm upon a decrease of temperature from 80°C to 5°C while H8A and H8G are shielded by ca. 0.04 ppm (Table 2 and Fig. 7) suggesting a stacking between adenine and guanine residues. An approximate population of pseudorotamers[18] of two sugar residues in 4 at various temperatures have been subsequently estimated from the $J_{1',2'}$ coupling constants using the procedures described by Altona and his coworkers[19]. The following equation has been employed in order to calculate the percentage of N pseudorotamers at a temperature under consideration:

$$^{3}J^{Exp}_{1',2'} = X_N {}^{3}J^{N}_{1',2'} + (1-X_N) {}^{3}J^{S}_{1',2'}$$

Where X_N is the mole fraction of the N-type conformers, $^{3}J^{N}_{1',2'}$, $^{3}J^{S}_{1',2'}$ and $^{3}J^{Exp}_{1',2'}$ represent the couplings between H1' and H2' for a pure N-type, S-type and for the compound under consideration, respectively. Using the general conditions for pseudorotational analysis of ribose[20]

($P = 9°$, $\psi = 38°$, $J^{N}_{1',2'} = 1$ Hz and $J^{S}_{1',2'} = 7.8$ Hz), we estimated the percentage of N conformers in adenosine and guanosine residues which are shown in Table 3. It has thus been clear that both purine riboside moieties adopt 50% of N conformation at low temperature. It should be noted that these experimental results are in good agreement with the work described by Doornbos et al.[21] who, showed that the 2' → 5' linked dinucleoside phopshates have a stronger tendency to stack than the corresponding 3' → 5' linked dimers. These workers have subsequently concluded that various modes of stacked forms with an equal population of N and S conformers are possible in purinylyl(2' → 5')-purine-riboside which also seems to be the case with our adenylyl(2' → 5')guanosine (4).

Table 2: ^{1}H chemical shifts[a] of 4 versus temperature.

T °C	H8A	H2A	H8G	H1'A	H1'G
5	8.113	7.770	7.650	6.109	5.519
20	8.110	7.809	7.653	6.101	5.523
40	8.107	7.857	7.660	6.090	5.533
80	8.111	7.958	7.689	6.083	5.571

[a]Shifts (±0.003) are given relative to CH_3CN set at 2 ppm.

Table 3: $J_{1',2'}$ coupling constant[a] of 4 and the percentage[b] of N conformer versus temperature.

T °C	$AJ_{1'-2'}$	%N	$GJ_{1'-2'}$	%N
5	4.3	50	4.3	50
20	4.5	48	4.6	47
40	4.9	42	4.7	45
80	5.3	35	5.0	40

[a]Accuracy estimated at ±0.3 Hz.
[b]See discussion part.

Conformational analysis of $A^{2'p5'G}_{3'p5'U}$ (2) and $A^{2'p5'G}_{3'p5'C}$ (3).

A comparison between temperature-dependent ^{1}H chemical shifts in 2 or 3 with that of 4 (Fig. 7) clearly shows that the magnitude of the shielding of H2A and H8G in 2 or 3 is very similar to that of H2A and H8G in 4 (ca. 0.2 and 0.05 ppm, respectively) upon a decrease of temperature from 80° to 5°C (Table 4). On the other hand, a comparison of magnitude of temperature-dependent chemical shifts of the pyrimidine residues (U or C) in A3'p5'U[15] or A3'p5'C[15] with those of deshielding of H6U (ca. 0.05 ppm) and shielding of H5U (ca. 0.02 ppm), H5C (0.008 ppm) and H6C (0.006 ppm) in 2 and 3 upon a temperature change from 80°C to 5°C clearly show that U or C residues in 2 and 3 do not participate in any form of stacking interaction. It is however possible to rationalize some of these small shieldings or deshielding by either invoking the ring-current effect from the neighbouring bases and/or the effect of the 5'-phosphate. A deshielding of ca. 0.06 ppm for H8A in compounds 2 and 3 as compared to 4 indicates a paramagnetic effect of the pyrimidine ring on the H8A in 2 and 3. This can be achieved if one conceives of a model in which the adenine and the pyrimidine residues are coplanar in order to promote the paramagnetic effect of the pyrimidine ring. Such deshielding of H8A by the pyrimidine residue is particularly feasible when the adenosine residue adopts a syn conformation as determined by a 1D NOE experiment (not shown).

A ^{1}H-NMR study of compounds 2 and 3 at higher concentration (15-20 μmole) as a function of temperature reveals the effects of both intermolecular and intramolecular interactions as exhibited by the upfield chemical shifts of all protons in general (Table 4). It is likely that at higher concentration, additional intermolecular associations[22] (vertical stacking between A and G, for example) exist explaining the further shielding of aromatic protons of A and G residues.

The pseudorotational analysis of $J_{1',2'}$ coupling constants of sugar residues indicates that the adenosine part adopts more S conformation (ca. 65% S at 20°C) in 2 and 3 as compared to the adenosine sugar part (ca. 52% S at 20°C) in 4 while the sugar part of guanosine in 2 and 3 still shows almost an equal population of S and N conformers as in the parent compound 4. On the other hand, it should be noted that the conformational states of sugar parts of pyrimidine residues (U or

Table 4: ^1H chemical shifts[a] for 2 and 3 at low and high concentrations as a function of temperature.

Compound	Fragment	Proton	concentration	5°C	20°C	40°C	80°C
$A^{2'p5'G}_{3'p5'U}$		H8	b	8.174	8.171	8.167	8.170
			c	8.136	8.147	8.157	8.164
	pAp	H2	b	7.762	7.798	7.858	7.973
			c	7.685	7.733	7.832	7.960
		H1'	b	6.136	6.133	6.130	6.133
			c	6.098	6.107	6.116	6.126
		H6	b	7.824	7.814	7.800	7.773
			c	7.820	7.810	7.793	7.766
	pU	H5	b	5.765	5.782	5.797	5.812
			c	5.782	5.787	5.794	5.808
		H1'	b	5.859	5.861	5.858	5.849
			c	5.862	5.856	5.854	5.846
		H8	b	7.657	7.662	7.674	7.704
	pG		c	7.585	7.611	7.655	7.697
		H1'	b	5.564	5.570	5.581	5.613
			c	5.520	5.540	5.571	5.611
$A^{2'p5'G}_{3'p5'C}$		H8	b	8.161	8.161	8.158	8.162
			d	8.154	8.155	8.157	8.164
	pAp	H2	b	7.768	7.807	7.863	7.975
			d	7.740	7.792	7.855	7.973
		H1'	b	6.106	6.111	6.116	6.126
			d	6.087	6.103	6.112	6.127
		H6	b	7.777	7.782	7.784	7.783
			d	7.769	7.779	7.781	7.782
	pC	H5	b	5.863	5.889	5.916	5.947
			d	5.860	5.889	5.915	5.948
		H1'	b	5.843	5.853	5.861	5.869
			d	5.840	5.852	5.860	5.870
		H8	b	7.665	7.673	7.684	7.711
	pG		d	7.638	7.660	7.679	7.713
		H1'	b	5.571	5.577	5.588	5.617
			d	5.557	5.570	5.585	5.619

[a]From CH_3CN set at 2 ppm; [b]4 μmole in 0.6 ml of ^2H_2O; [c]20 μmole in 0.6 ml of ^2H_2O; [d]15 μmole in 0.6 ml of ^2H_2O.

C) hardly show any change with the change of temperature. This suggests that the spatial environment of the 3'-pyrimidine substituent of A2'p5'G, as in compounds 2 and 3, is free enough from any kind of intramolecular interactions to induce displacement in the equilibrium between N and S conformation. It is also clear from above results that the conformational state of adenosine-sugar part in compounds 2 and 3 is quite different from that of 2',3'-cAMP[23] in which the population of N and S conformations are almost equal as those found in 4 while in these branched trinucleotides 2 and 3, there is a clear preference for the S conformation.

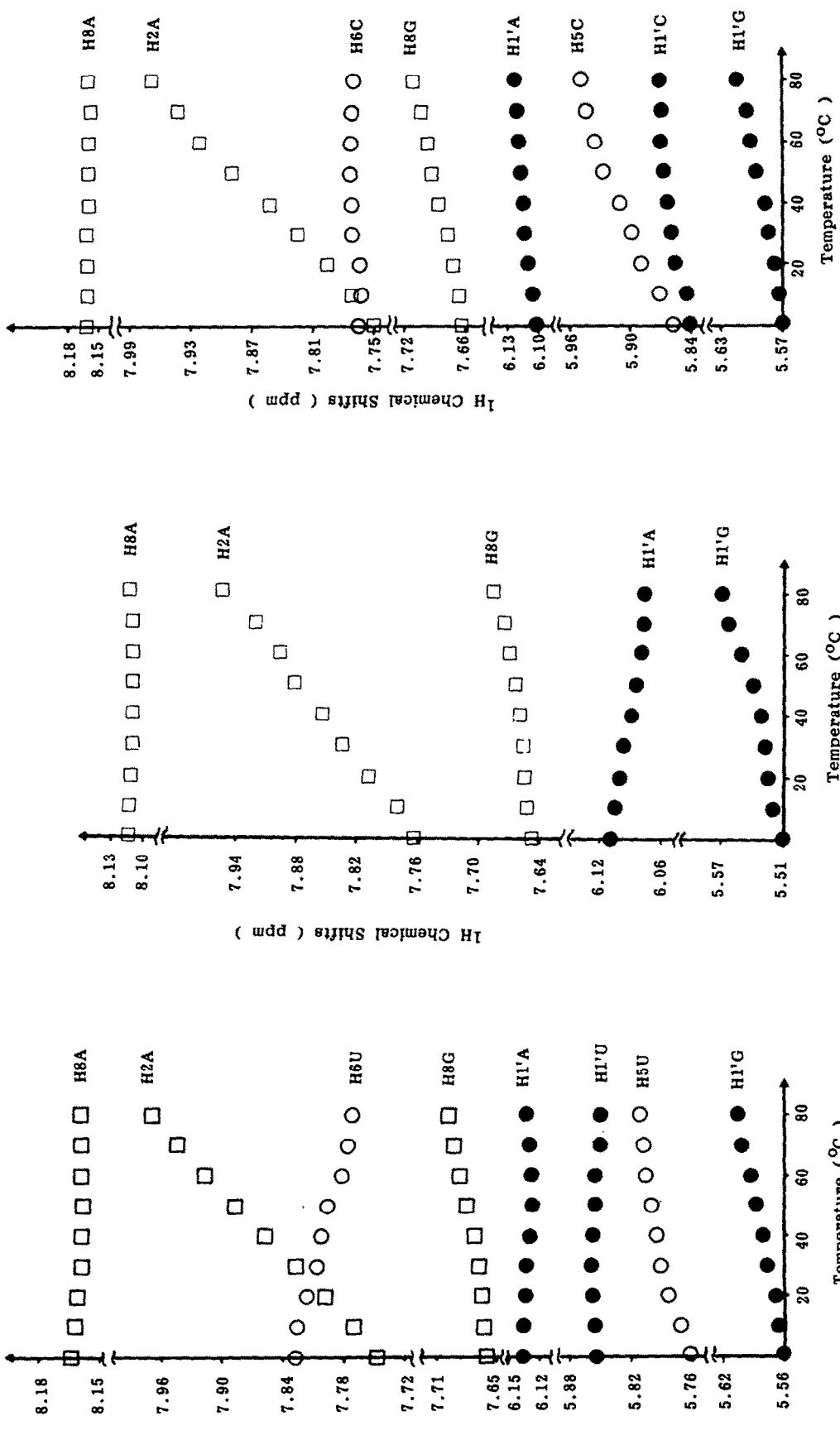


Fig. 7 : Temperature dependent variation of ^1H chemical shifts of aromatic and anomeric protons of (A) compound 2, (B) compound 4 and (C) compound 3

Table 5: Variation of $J_{1',2'}$ coupling constant[a] of 2 and 3 and the percentage[b] of N conformer as a function of temperature[c].

Compound	T °C	$A_{J1'-2'}$	%N	$G_{J1'-2'}$	%N	Pyrimidine	%N
	5	5.1	30	4.7	45	4.4	49
2	20	5.4	35	4.8	44	4.1	54
	40	6.0	25	5.1	40	4.5	48
	80	6.0	25	5.5	34	4.5	48
	5	5.4	35	4.5	48	3.9	57
3	20	5.5	34	4.7	45	4.1	54
	40	5.6	32	5.0	40	4.0	56
	80	5.9	27	5.4	35	4.2	53

[a]Accuracy estimated at ±0.3 Hz; [b]See discussion part; [c]4μmole in 0.6 ml of ^2H_2O.

It therefore appears that the conformation of these two branched trinucleotides are quite unique in that their secondary structures are primarily controlled by strong stacking between adenine and guanine residues as in a purine(2'→ 5')purine dinucleotides while the pyrimidine residue adopts a coplanar state with the adenine moiety.

It seems to be clear from the literature[15,16,21,24] that purine(3'→ 5')purine dinucleotides are more stacked than the corresponding purine(3'→ 5')pyrimidine and pyrimidine(3'→ 5')purine dinucleotides. It is also well known[24] that the stacking of bases of purine(2'→ 5')purine dinucleotides are more pronounced than the corresponding purine(3'→ 5')pyrimidine dinucleotides. Since, our present study with the branched trinucleotides 2 and 3 has clearly shown that the thermodynamically stable intramolecular stacking in purine(2'→ 5')purine is overwhemingly more preferred than the alternative intramolecular purine(3'→ 5')pyrimidine stacking, it is likely that the formation of the additional 2'→ 5' linkage to give these branched trinucleotides 2 and 3 may act as a switch for the natural conformation-driven-energy-pump for the splicing reaction. In both enzyme-mediated splicing in nuclear mRNA and in self-catalyzed mitochondrial splicing reactions (group II), it seems to be logical that the phase one consists of folding of pre-mRNA (enzyme-mediated stabilization of the duplex or in self-catalyzed splicing, it should depend upon the strength of Watson-Crick hydrogen-bonded duplex!) and then an intramolecular transesterification reaction allowing the formation of an additional sterically hindered 2'→ 5' linked circular RNA on adenosine as the branched point, as well as the release of the 5' exon by the cleavage at the 5' splicing site. The resulting system, with the 3' exon attached, then starts the thermodynamically preferred conformation-driven (stacking-driven) energy equilibriation process to prepare for the final stage of the splicing reaction. It is conceivable that at this stage, the conformational transition from the energetically less stable state (3'→ 5') to a higher stacked state (2'→ 5') (free energy minimum) should liberate an extra energy; this energy could then be used in the scission of a 3'→ 5' phosphodiester bond at the 3' splice site resulting in the release of the 3' exon. Finally, Mg^{+2} ion could act as a cofactor in ligating the 5' and 3' exons together giving the processed mRNA which is then ready to be transcribed by tRNA-ribosome complex to protein.

It should also be noted that in group 1 splicing[4], it is the ~3'-adeninyl(5'→ 3')pyrimidine-5'~ internucleotide bond at the splice junction which is always cleaved by an intermolecular transesterification reaction by the 3'-hydroxyl of guanosine or 5'-GMP to give the ~3'-adeninyl-(5'→ 3')Guanine-5'~ junction which should be thermodynamically more stable as in the case of normal dinucleotides[24]. Thus, it seems to be clear that the force of thermodynamic stabilization is the ultimate factor for the participation of a guanosine residue in all types of splicing reactions.

Further work is in progress to understand (i) the actual thermodynamic parameters in the stacking of the branched nucleotide by pseudorotational studies and (ii) to probe if a similar preferred 2'→ 5' stacking exists in a larger synthetic oligoribonucleotide comprising the features of the splice-point.

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EXPERIMENTAL

NMR samples.

The compounds 2, 3 and 4 were synthesized according to reported procedures[8]. The samples were treated with Dowex Na^+ exchange resin and lyophilized two times from 99.8% ^2H_2O and then co-evaporated in 99.9% ^2H_2O. Finally the samples were dissolved in 0.6 ml of 99.9% ^2H_2O. 4 µmole (ca. 3.7 mg) of 2 or 3 for the low concentration study and 15 µmole (ca. 15 mg) of 3, 20 µmole (ca. 20 mg) of 2 for the high concentration study and 6 µmole (ca. 3.6 mg) of the dimer 4 were used. The pH was found to be ~6.5. A trace of dry acetonitrile was added to the samples as an internal reference (set at 2 ppm).

NMR spectroscopy.

Spectra were obtained on a Jeol GX-270 spectrometer. 1D spectra were recorded on 16 K datapoints. Resolution was enhanced by aplication of Gaussian window and FIDs were zero-filled to 32 K datapoints before Fourier transformation. With a spectral range of 2000 Hz, the digital resolution was 0.12 Hz.

The NOE measurements were made by NOE difference method where a peaK under consideration was slightly saturated by a continous irradiation. Although a magnetization transfer by spin diffusion cannot be excluded, therefore the NOE information must be used only qualitatively but it is usefull for assignment purposes and for the determination of the connections between the sugar part and its base.

The COSY spectrum was recorded by the basic pulse sequence[5] where the last pulse was a 45° pulse. 256 FIDs consisting of 1 K datapoints were recorded, a sine bell window was applied in both directions. Before Fourier transformation the spectrum was zero-filled to 512 points in the F_1 direction in order to get the same resolution in both axes.

The $^{31}P/^1H$ 2D shift correlation was recorded using the method described by A. Otter and coworkers[14]. The spectral range was 200 Hz for ^{31}P direction (F_2) and 2000 Hz for ^1H direction (F_1). The delay 1/2J and 1/4J were set at 58 ms and at 29 ms respectively. A 512 x 512 data set was collected and zero-filled to 512x1024 complex points.

The 2D Hartmann-Hahn spectra were recorded using the pulse sequence MLEV17 as proposed by Bax and coworkers[25]. The power of the observation channel was adjusted to get a 90° pulse of 80 µs (16 µs for the full power). The spin-locked time was 2 ms with an appropriate repetition time to reach a propagation delay of 150 ms. Recently pratical details were described by Bax and coworkers[26]. A total of 128 FIDs consisting of 2 K datapoints were recorded, a Gaussian window with a broadening factor of 8 was applied in both directions. The matrix was zero-filled to 256 x 2048 complex points.

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