Temperature-dependent ^{31}P N.M.R. Shifts to assess the Conformations of Branched Tri- and Tetra-nucleotides which are formed as Lariats in the Splicing Reactions

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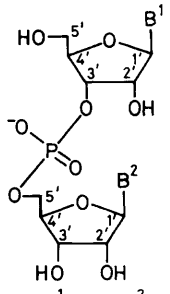
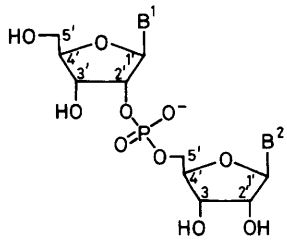
Temperature-dependent changes of ^{31}P n.m.r. chemical shifts ($\Delta\delta$) have been used to estimate the secondary structure of branched tri- and tetra-nucleotides formed in the splicing reaction; these studies have provided independent evidence, confirming our earlier ^{1}H n.m.r. studies, that the 2′ → 5′ stacking is the predominant structural feature that dictates the secondary structure of the branched trinucleotides while the branched tetranucleotides exist in the form of a distorted A-RNA helix without any 2′ → 5′ stacking.

Several factors, such as stacking ⇌ destacking equilibrium, equilibrium of pseudorotamer populations ($N \leftrightharpoons S$), and the –O–P–O– bond and ester torsion angles, effectively contribute to the conformational structure and flexibility of the sugar–phosphate backbone of nucleic acids.[1] Nucleic acids at low temperature exist largely in a base stacked conformation with the phosphate ester predominantly in the *g*,*g* conformation (corresponding to a larger –O–P–O– bond angle and upfield ^{31}P n.m.r. absorptions) while nucleic acids at higher temperatures exist more in unstacked conformations with the phosphate ester in an increased proportion of non-*gauche* or eclipsed conformations which result in a downfield ^{31}P chemical shift.[2–4]

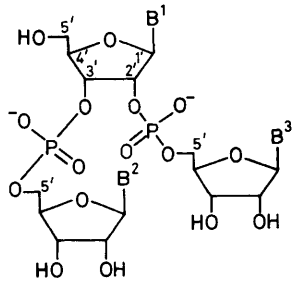
While the temperature-dependent changes of ^{31}P shifts ($\Delta\delta$) of an oligonucleotide cannot provide quantitative thermodynamic information regarding stack ⇌ destack equilibrium,[5] such changes can unambiguously show the relative conformational differences of the phosphate backbone in a qualitative manner under particular measurement conditions. A comparison between two pairs of isomeric adenosine-2′- and 3′-monophosphates has clearly illustrated that although their phosphates have a similar π bond order, –O–P–O–bond angle, and ester torsion angle, the ^{31}P chemical shift of the 2′-nucleoside phosphate nevertheless appears at a higher field than that of the corresponding 3′-phosphate. This is due to the larger strength of the diamagnetic and/or paramagnetic effect of the nucleobase (adenine > guanine > cytosine > uracil) in the 2′-phosphate owing to its closer steric proximity than in the corresponding 3′-phosphate. The ring-current effect of the nucleobase is most efficient for the 2′-phosphate in the *S* conformation (pseudoequatorial to nucleobase) and, conversely, the *N* conformation should favour the ring-current effect for the 3′-phosphate (pseudoaxial to nucleobase). Temperature-dependent 270 MHz ^{1}H n.m.r. studies[10–12] showed that the branched trinucleotides (**5**) and (**6**) which are formed as the *lariat* in the group II and nuclear mRNA mediated splicing reactions[13] exist mainly in the 2′ → 5′ stacked form while the 3′ → 5′ nucleotide moiety is apart and free. Table 1 shows $\Delta\delta$ values for temperature-dependent (10 to 81 °C) ^{31}P chemical shift measurements with several branched tri- and tetra-nucleotides (**5**)—(**14**) and their comparison with values for the constituent dinucleotides (**1**)—(**4**). The downfield ^{31}P shifts

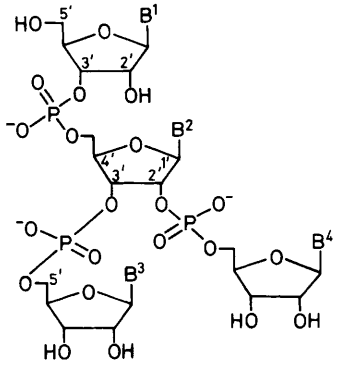


(1) B^1 = A; B^2 = G
(2) B^1 = A; B^2 = U



(3) B^1 = A; B^2 = G
(4) B^1 = A; B^2 = U





(5) B^1 = A; B^2 = U; B^3 = G
(6) B^1 = A; B^2 = C; B^3 = G
(7) B^1 = A; B^2 = G; B^3 = U
(8) B^1 = A; B^2 = G; B^3 = C
(9) B^1 = A; B^2 = B^3 = G
(10) B^1 = B^2 = B^3 = A

(11) B^1 = U; B^2 = A; B^3 = U; B^4 = G
(12) B^1 = U; B^2 = A; B^3 = C; B^4 = G
(13) B^1 = A; B^2 = A; B^3 = U; B^4 = G
(14) B^1 = A; B^2 = A; B^3 = C; B^4 = G

A = adenin-9-yl; U = uracil-1-yl; C = cytosin-1-yl; G = guanine-1-yl.

shown for different branched tri-[6] and tetra-nucleotides[7] and for their constituent dinucleotides with increasing temperature (Table 1) are interpreted as being due to transition from base stacked to unstacked and also due to changes of sugar conformation ($N \leftrightharpoons S$) and phosphodiester torsion angle. Such downfield shifts at elevated temperature for various larger single stranded DNA or RNA molecules have been similarly interpreted.[14] What is noteworthy is that the flexibilities of the two phosphodiester backbones (2′ → 5′ *vs.* 3′ → 5′) in the branched trinucleotides (**5**)—(**10**) are clearly different as seen in their relative ^{31}P Δδ values. The larger Δδ for a particular ^{31}P shift over a range of temperatures can be qualitatively interpreted as being due to its less flexible conformational state. Clearly, this alteration of physical state of conformation will be observed more amongst the interacting parts of the branched tri- and tetra-nucleotides. The following conclusions can, therefore, be drawn from the observation of temperature-dependent chemical shifts and Δδ values shown in Table 1: (i) a comparison of ^{31}P Δδ values for 3′ → 5′ and 2′ → 5′ dinucleoside phosphates cannot be attributed to their actual degree of stackings since it has been shown[15] that a noticeable amount of stacking is present in A2′p5′U and A2′p5′C even at high temperature. The ^{31}P Δδ values can, however, be compared amongst the 2′ → 5′ dinucleoside phosphates or amongst 3′ → 5′ dinucleoside phosphates as a measure of constraint due to $N \leftrightharpoons S$ and/or stack ⇆ destack equilibrium. (ii) In branched trinucleotides (**5**)—(**10**), the 2′ → 5′ stacking is overwhelmingly more dominant than the 3′ → 5′ stacking as seen through their respective ^{31}P Δδ values, consistent with our previous ^1H n.m.r. work.[10—12] The strength of the stacking is, however, determined by the nature of the 2′ → 5′ linked nucleobase (cytosine ≥ uracil > guanine). (iii) In branched trimers, the 3′ → 5′ linked nucleobase is free and apart from the 2′ → 5′ stacked nucleobases. This may suggest that 3′ → 5′ phosphate –O–P–O– torsion angle should be similar in all branched nucleotides. Therefore, the observed Δδ values presumably represent the influence of the branch-point sugar conformation on the 3′ → 5′ phosphate –O–P–O– torsion angle. Thus we have observed that a higher proportion of *S* pseudorotamer in blocked conformation in the branch-point sugar moiety normally results in a larger Δδ value for its 3′ → 5′ phosphate. For example, branch-point sugars with ~90% *S* conformations, as in $A^{2'p5'U}_{3'p5'G}$ (**7**)[10] and $A^{2'p5'C}_{3'p5'G}$ (**8**),[10] produce ^{31}P Δδ values of 0.50 and 0.48 p.p.m., respectively, while the branch-point sugars with ~75% S as in $A^{2'p5'A}_{3'p5'A}$ (**10**),[12] and ~65% *S*, as in $A^{2'p5'G}_{3'p5'U}$ (**5**)[11] and $A^{2'p5'G}_{3'p5'C}$ (**6**),[11] produce ^{31}P Δδ values of 0.40, 0.31, and 0.32 p.p.m.

Table 1. ^{31}P Chemical shifts[a] at 20 °C, δ(^{31}P), for different internucleotide phosphates, and Δδ(^{31}P)[b] values.

Compound[c]	Type of phosphate	δ(^{31}P)	Δδ
(**1**)	3′ → 5′	−0.86	0.56
(**2**)	3′ → 5′	−0.96	0.64
(**3**)	2′ → 5′	−1.30	0.48
(**4**)	2′ → 5′	−1.58	0.54
(**5**)	3′ → 5′	−0.90	0.31
	2′ → 5′	−1.65	0.59
(**6**)	3′ → 5′	−0.86	0.32
	2′ → 5′	−1.55	0.50
(**7**)	3′ → 5′	−1.06	0.50
	2′ → 5′	−1.94	0.65
(**8**)	3′ → 5′	−1.05	0.48
	2′ → 5′	−2.0	0.66
(**9**)	3′ → 5′	−0.96	0.39
	2′ → 5′	−1.61	0.55
(**10**)	3′ → 5′	−0.96	0.40
	2′ → 5′	−1.56	0.44
(**11**)	2′ → 5′	−1.46	0.50
	U(3′ → 5′)A	−1.16	0.62
	A(3′ → 5′)U	−1.01	0.31
(**12**)	2′ → 5′	−1.34	0.41
	U(3′ → 5′)A	−1.12	0.63
	A(3′ → 5′)C	−1.0	0.35
(**13**)	2′ → 5′	−1.49	0.51
	A(3′ → 5′)A	−1.16	0.70
	A(3′ → 5′)U	−1.0	0.32
(**14**)	2′ → 5′	−1.41	0.42
	A(3′ → 5′)A	−1.17	0.66
	A(3′ → 5′)C	−0.99	0.33

[a] In p.p.m. relative to 10% H_3PO_4 in D_2O in a capillary. [b] Difference between δ (^{31}P) values, at 10 and at 81 °C with 3′,5′-cAMP [δ (^{31}P) −1.74 p.p.m., referenced to 10% H_3PO_4 in D_2O] as reference. [c] All compounds were purified on a Dowex column (Na^+) and then on a Chelex 100 (Na^+) column buffered at pH 6.5 with $MeCO_2^-$ Na^+. After lyophilisation, the sample was dissolved in H_2O to give a concentration *below* 5 mM. The final volume of the solution was always the same in order to minimize the relative bulk susceptibilities among the samples. The pH of the sample solutions was between 6.5 and 7.5. The ^{31}P chemical shifts are referenced to an external solution of 10% H_3PO_4 in D_2O and the solvation-induced changes of chemical shifts can be corrected using 3′,5′-cAMP as reference.[5] The accuracy of the ^{31}P chemical shifts is estimated to be ±0.01 p.p.m. The spectra were recorded at 109.4 MHz using a Jeol GX270 spectrometer and 10 mm o.d. tubes. The decoupling mode was used to avoid overheating of the sample by bi-level decoupling (the full power of the decoupler, 9 W, was used during the acquisition, otherwise 4 W). Temperatures (±1 °C) were calibrated using a thermocouple under the above conditions.

respectively. These observations suggest that when the branch-point sugar is blocked in the *S* conformation the 3′ → 5′ phosphate backbone is less flexible. (iv) In branched tetramers,[16] ^{1}H n.m.r. studies have shown that the stacking between the branch-point A and the 2′ → 5′G residue is absent because of their stackings with the 5′-terminal nucleobase and also because of stackings along the axis of 3′ → 5′ linked nucleotide residues (reminiscent of a distorted A-RNA helix) as compared with the branched trimers. The ^{31}P Δδ values in these branched tetramers (**11**)—(**14**) suggest that (a) the branch-point A (A*) has a stronger tendency to stack with 5′-terminal A (Δδ ~ 0.68 p.p.m.) than with 5′-terminal U (Δδ 0.62 p.p.m.), (b) A* has a stronger tendency to stack with its 3′ → 5′ terminal C (Δδ ~ 0.34 p.p.m.) than with 3′ → 5′ terminal U (Δδ 0.31 p.p.m.), (c) the phosphate backbone of A*2′ → 5′G is more constrained with 3′ → 5′U than with 3′ → 5′C [compare Δδ 0.50 and 0.51 p.p.m. in (**11**) and (**13**) with Δδ 0.41 and 0.42 p.p.m. in (**12**) and (**14**), respectively], consistent with our ^{1}H n.m.r. studies.[16] Further studies are in progress to explore how the change of the branch-point nucleobase (A*) to either G, C, or U can control the 2′ → 5′ stacking ⇆ destacking and *N* ⇆ *S* equilibria and the –O–P–O– bond and ester torsion angles in other analogous branched trinucleotides.

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