

A NEW REGIOSPECIFIC SYNTHESIS OF "BRANCHED" TETRARIBONUCLEOTIDE AND ITS THREE ANALOGUES TO DELINEATE THE CHEMOSPECIFIC ROLE OF THE "BRANCH-POINT" ADENINE NUCLEOTIDE IN SPLICING

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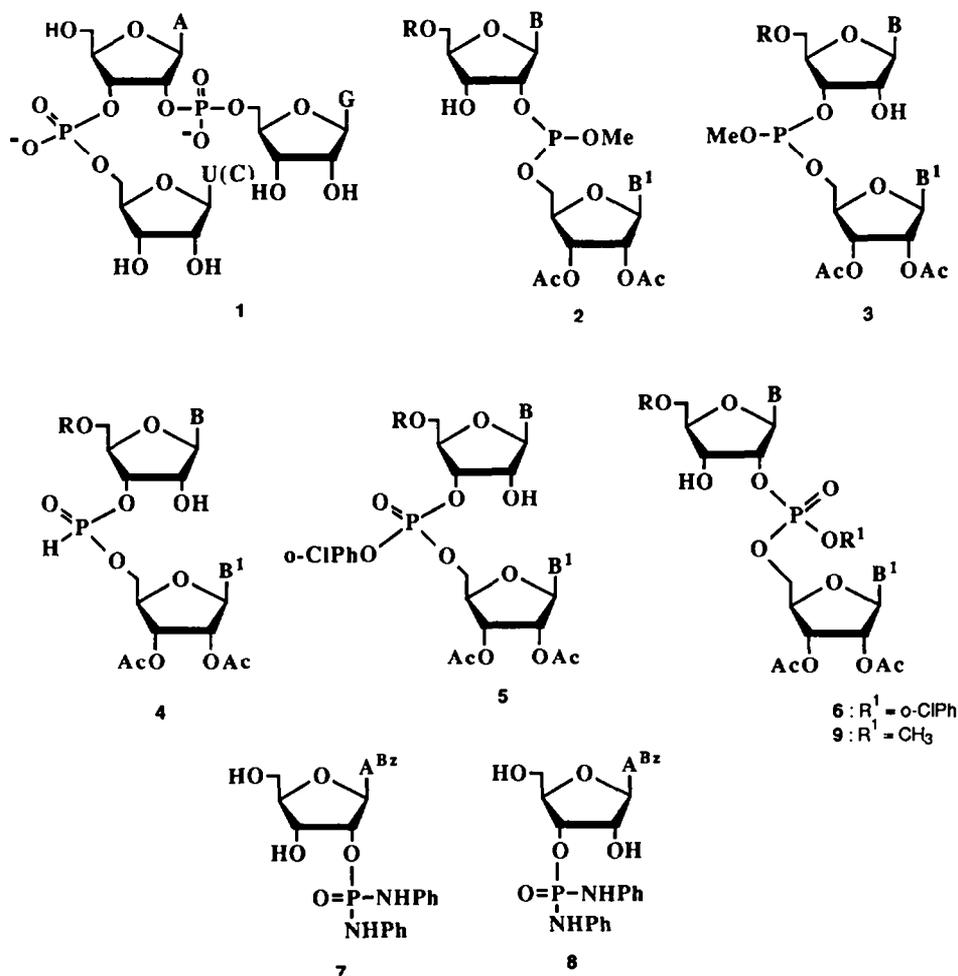
Abstract: The syntheses of four "branched" tetra-ribonucleotides 32 - 35 are reported using the key trimeric building blocks 19 - 22 which have been conveniently prepared by the reactions of 18 with 14 - 17 using the methodologies developed in the H-phosphonate chemistry. An appropriate choice of the complementary 2'-OH protecting groups in the intermediates 19 - 22 has permitted a regioselective removal of one 2'-OH protecting groups (9-phenylxanthen-9-yl-), by a mild acid treatment, at the branch-accepting sugar moiety to give the intermediates 23 - 26 in high yields. These intermediates 23 - 26 have been subsequently converted to the "branched" tetra-ribonucleotides 28 - 31 by reactions with appropriately protected 5'-phosphiteamidite block 27 in the usual manner. The deprotected "branched" tetra-ribonucleotides 32 - 35 have been subsequently characterized by ^1H - & ^{31}P -NMR spectroscopy. Two dimensional ^1H ^{31}P correlation spectroscopy of these branched tetra-ribonucleotides have unequivocally established the regiospecific sites of $3' \rightarrow 5'$ and $2' \rightarrow 5'$ phosphodiester linkages.

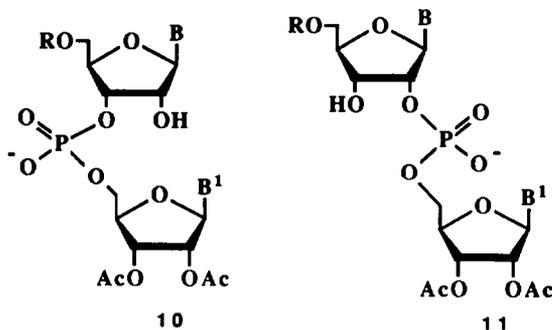
The removal of the introns from the pre-mRNA and ligation of the 3'- and 5'-coding regions (exons) to form a functional RNA for protein biosynthesis is generally called splicing¹⁻⁵. In Group II and nuclear mRNA splicing, first, the pre-mRNA is cleaved at the 5'-splice site and a branched (lariat) intermediate is formed. Then, in the second step, cleavage at the 3'-splice site and ligation of two exons leads to the release of the lariat intron¹⁻¹¹. These lariat structures have adenosine as the "branch-point" residue, linked via a $2' \rightarrow 5'$ phosphodiester bond to a guanosine residue, and a $3' \rightarrow 5'$ phosphodiester bond to a pyrimidine residue¹⁻¹¹. Specific nucleobase requirement of the branch-accepting point for the splicing in eukaryotes have been recently reported¹²⁻¹⁴. Replacement of the central branch-accepting adenosine residue by any other nucleotide in mutants results in a considerable decrease in efficiency in the splicing as compared to the wild type. Mutation experiments have shown that all four nucleotides can serve as branch-acceptors, but it is adenosine and cytidine residues which are preferred to guanosine and uridine residues¹²⁻¹³. Only branches to adenosine or cytidine participate efficiently in the second step of splicing in mutation experiments *in vitro* but the natural splicing in eukaryotes *in vivo* exclusively prefers adenosine as the branch-point for error-free processing of pre-mRNA. We wished to study the structural basis for the evolutionary choice of adenosine as the branch-accepting nucleotide in the splicing reaction by the conformational analysis of the model branched RNA lariats with substituted nucleotides (cytidine, guanosine and uridine) at the branch-point instead of adenosine. We chose a "branched" tetra-ribonucleotide molecule, such as 32, as a representative of the biologically-occurring lariat structure because it has been shown by ^1H -NMR spectroscopy that a simple branched tri-ribonucleotide such as 1 has an unnatural conformation^{36-40,42} and is not recognized as a substrate in enzymatic 5'-phosphorylation reaction^{17b}. On the other hand, a branched tetra-ribonucleotide, such as 32, has the stacking predominantly along the $3' \rightarrow 5'$ axis and resembles a distorted A-RNA helix fragment^{41,42} which is easily recognized as a substrate in the enzymatic 5'-phosphorylation reaction^{17a,b}. We herein report the preparation of all four possible "branched" RNA structures 32, 33, 34 and 35 with identical substituents on the $2' \rightarrow 5'$, $3' \rightarrow 5'$ and $5' \rightarrow 3'$ termini from the "branch-point" which is either naturally-occurring adenosine, or cytidine, guanosine or uridine.

Since the isolation of the "branched" RNA structures, there have been several reports on their synthesis¹⁵⁻²². As a result of these works, three distinctive pieces of ribonucleic acid chemistry have come into our knowledge: (1) internucleotide phosphite-

triester in species such as 2 or 3, which are intermediates in the condensation of N^6 -benzoyl-5'-O-(4-monomethoxytrityl)-adenosine and an appropriately protected 5'-phosphiteamidite block, are not sufficiently electrophilic to undergo an intramolecular nucleophilic attack by the vicinal hydroxyl group, and therefore a second condensation has been feasible to give the branched ribonucleotide¹⁵, (2) a hydrogenphosphonate vicinal to a hydroxyl function such as 4 is strongly electrophilic and is extremely susceptible to intramolecular transesterification forming instantly 2',3'-cyclic H-phosphonate²⁵, (3) species such as 5 or 6 with a phosphotriester vicinal to a hydroxyl function are extremely susceptible to the neighbouring hydroxyl group attack which causes isomerization and degradation^{23,24}. Hata's 2'-phosphoroamidate with a vicinal hydroxyl function such as 7, was also unstable during the fluoride ion promoted deblocking of the 3',5'-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl) group and gave the isomeric 3'-phosphoroamidate 8^{16,21}. Fourrey managed to remove the 2'-O-(*t*-butyldimethylsilyl) group, vicinal to a methyl phosphotriester, at -78 °C in a low yield to give 9²⁰. On the other hand, species such as 10 or 11 can be easily prepared and are reasonably stable for the reactive phosphiteamidite to phosphorylate the hydroxyl function^{17,18,19,22}. Owing to these reasons, all of the successful methods, except Hata's^{16,21} and Fourrey's²⁰ synthesis, first generate a 3' → 5' or 2' → 5' phosphotriester bond with a compatible phosphate protecting group to the vicinal hydroxyl protecting group. The phosphotriester function is then selectively converted to a phosphodiester, and then the 2'- or 3'- protecting group is cleaved in order to generate species such as 10 or 11. The branching 2' → 5' or the 3' → 5' phosphate bond is then generated by reaction of the vicinal hydroxyl function with an appropriately protected phosphiteamidite block^{17,18,19,22}.

We reasoned that if instead of this two-step procedure to generate 10 or 11, one employed the H-phosphonate approach^{26,27} on an appropriately 2'-protected block, such as 14 - 17, one would *directly introduce the 3' → 5' phosphodiester linkage* at the "branch-accepting" nucleotide moiety, as in 19 - 22. This suggests that such a methodology would clearly circumvent the usual problem of the compatibility of the phosphate protecting groups which one comes across in the synthesis of larger



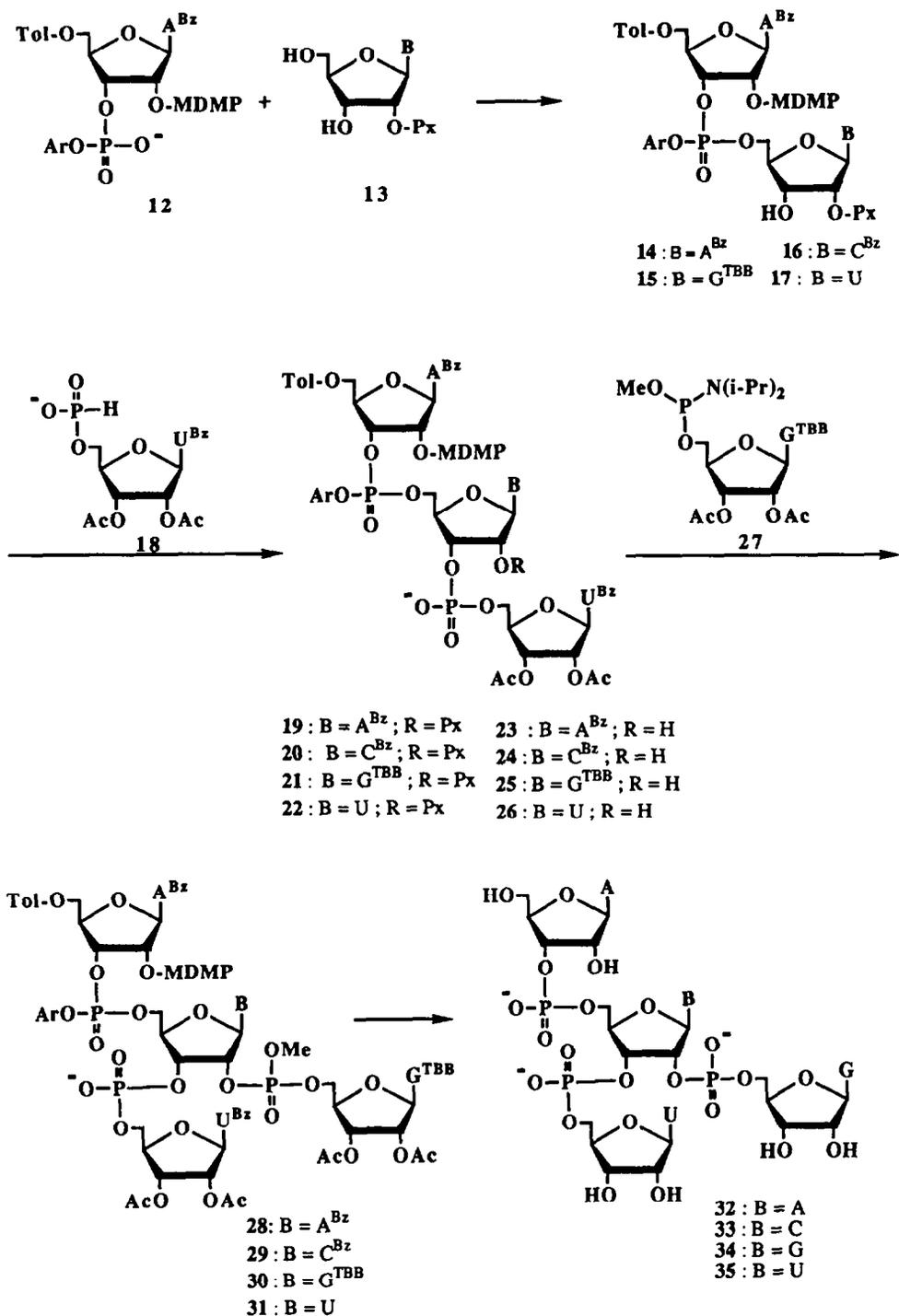


branched RNA sequences than the central branched triribonucleotide 1. It is also clear (*vide infra*) that the above approach has an additional advantage owing to the fact that the 2'-acid-labile group vicinal to the 3' → 5' phosphodiester bond at the "branch-accepting" nucleotide moiety is more easily and selectively cleavable than any other 2'-acid-labile protecting group which is vicinal to the 3' → 5' phosphotriester function in the molecule²⁹⁻³¹.

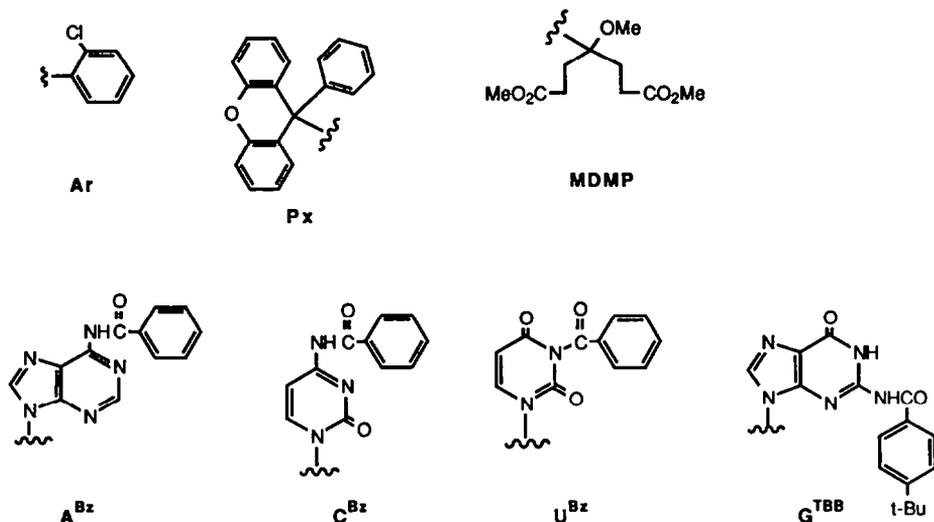
It was first demonstrated by Reese^{28a} that the nature of the 3'-substituent affected the stability of the 2'-acid-labile protecting group. He observed that the 2'-O-tetrahydropyranyl (THP) group was three fold less stable when adjacent to a 3'-*phosphodiester* than when adjacent to a 3'-*hydroxyl* function. In marked contrast, observations from several laboratories have clearly shown that the stability of a 2'-acid-labile group is significantly enhanced when the vicinal 3'-*hydroxyl* group was converted to the 3'-*phosphotriester* function^{29a,b,30,31}. In our laboratory^{30,31}, we have been able to show that the 5'-O-(9-phenylxanthan-9-yl) [Px]³² can be easily and regioselectively removed from mono-, di- and oligonucleotide building blocks, which are protected at 2'-OH with acid-labile group(s) such as 4-methoxytetrahydropyranyl [MTHP]^{28b} or 3-methoxy-1,5-dicarboxymethoxypentanyl [MDMP]³¹ and the vicinal 3'-OH functionalized with the *phosphotriester*, using either ZnBr₂ in anhydrous conditions³⁰ or trichloroacetic acid (10 equiv.) in 2% ethanol-chloroform mixture at ~0 °C³¹. These works have suggested to us that it should be possible to deprotect the 2'-O-Px group, vicinal to the hydroxyl function, quite selectively over the 2'-O-MDMP which is adjacent to the 3'-phosphotriester group in compounds 14 - 17. Furthermore, substitution of the 3'-OH in 14 - 17 with a phosphodiester, as in compounds 19 - 22, should enhance the regioselectivity of the removal of the 2'-O-Px group^{28a,30,31}. We have therefore synthesized compounds 14 - 17 using the standard phosphotriester approach^{33,31,32,34} in good yields (experimental). In our model studies, the 2'-O-Px group from 14 could be *regiospecifically* removed within 150 min at ~0 °C without any degradation of the 2'-O-MDMP group by the treatment of trichloroacetic acid using the above condition³¹. The 2'-O-Px group however could be completely removed only regioselectively from 15, 16 & 17 in ca. 1200, 220 and 180 min, respectively, along with < 5 % degradation of the 2'-O-MDMP group. Subsequently, the trinucleotides 19 - 22, with a 3' → 5' phosphotriester and a 3' → 5' phosphodiester functions, were synthesized in good yields by the reaction of 18 with the dimeric blocks 14 - 17 using the H-phosphonate approach^{26,27} (experimental). This one-step synthetic transformation [14 - 17 → 19 - 22] clearly circumvented the previously encountered problems of having to use two compatible phosphate protecting groups for the synthesis of a branched tetraribonucleotide or a bigger molecule. The 2'-O-Px group now from these blocks 19 - 22 could be removed *regiospecifically* within 2 - 4 min at ~0 °C under the above conditions to give the 2'-hydroxy blocks, as in 23 - 26, in ca. 60 - 70 % isolated yields (experimental). This 40 - 75 fold increase in the relative rates of hydrolysis of the 2'-O-Px group is clearly due to an enhanced stabilization of the transient 2'-O-Px carbenium ion by the 3'-phosphodiester group³⁰. These "branch-accepting" building blocks 23 - 26 were then reacted with the appropriately protected guanosine-5'-phosphite- amidite 27 to yield the partially protected "branched" tetramers 28 - 31 which were not isolated, they were directly deprotected in the usual manner^{18,22} to give the pure "branched" tetramers 32 - 35 in 12 - 40% overall yields [23 - 26 → 28 - 31 → 32 - 35] (see experimental section for details).

Structural characterization of 32 - 35 by NMR spectroscopy

The 1D ¹H-NMR spectrum of 32 was identical to the one reported earlier by us²². Its ¹H and ³¹P-NMR resonances were completely assigned^{41,42}. The analogous branched tetramers 33 - 35 possess ¹H resonances (Figs. 1 - 3) in the aromatic and anomeric regions which are well enough resolved to delineate their exact structures. Two dimensional NMR spectra show the correlation between H-6 and H-5 and H-1' and H-2'. In addition, ³¹P/¹H correlation spectra show the exact location of 2' → 5', 3' → 5' and 5' → 3' phosphodiester bonds from the "branch-point". For such assignments, we chose 2D NMR experiments which do not require large matrix and memory space. Thus in Fig. 4, a DOSE-COSY (Double quantum spin-echo-



COSY) spectrum for 33 is displayed. Fig. 5 shows that the 2' → 5' phosphate moiety of the "branch-point" cytidine is the most upfield. The double quantum filtered SECSY (DFQSECSY) spectrum for 34 is represented in Fig. 6. The H-2' and H-3' of the "branch-point" guanosine are clearly separated leading to an unambiguous assignment of ³¹P resonances (Fig. 7). Finally, compound 35 was characterized by DOSE-SECSY (Double quantum spin-echo-SECSY) experiment (Fig. 8). Fig. 9 shows the 2D ³¹P/¹H correlation spectrum depicting the exact location of 2' → 5', 3' → 5' and 5' → 3' phosphodiester bonds from the "branch-point" uridine. Further reasons for the above assignments along with the conformational studies of branched tetranucleotides 32 - 35 will be reported shortly.



A = 9-adeninyl- ; C = 1-cytosinyl- ;
 G = 9-guaninyl- ; U = 1-uracilyl- ;

EXPERIMENTAL

¹H-NMR spectra were recorded (in δ scale) with Jeol FX 90 Q and JNM GX 270 spectrometer at 90 and 270 MHz respectively, using TMS (0.0 ppm) or residual HOD peak (set at 4.55 ppm at 30 °C) as the internal standards. ³¹P-NMR spectra were recorded (in δ scale) at 36 MHz and 109 MHz in the same solvent using 85 % phosphoric acid as the external standard. TLC was carried out using Merck pre-coated silica gel F₂₅₄ plates in the following solvent systems: (A) methanol-dichloromethane (9:1, v/v), (B) methanol-dichloromethane (7:3, v/v). The column chromatographic separations were carried out using Merck G60 silica gel. DEAE-Sephadex A-25 from Pharmacia was used for the ion exchange chromatography.

After purification on DEAE-Sephadex column, the ammonium counterions of 32, 33, 34, 35 were replaced with Na⁺ by passing the compounds through a Dowex (Na⁺ form) column. Samples were dried from ²H₂O and then were dissolved in 0.5 ml of ²H₂O "100 atom % D" (Aldrich). The pH was found to be about 7.5. The 1D ¹H-NMR spectra reveal that the purity of 32 - 35 exceed 95%.

The 2D NMR experiments such as DQFSECSY and ³¹P/¹H correlation have been earlier used by other groups of workers^{43,44}. The double quantum coherence experiments such as DOSE-COSY and DOSE-SECSY were also described as ¹H-¹H INADEQUATE⁴⁵ and DECSY⁴⁶ respectively. For a full quadrature detection in both F₁ and F₂ dimensions, a phase shift of 45° is needed which was not available in our spectrometer. The quadrature detection is, therefore, not achieved in the F₁ direction. As a result some artefacts (zero quantum coherence and P-type peaks) are folded back in the area of interest when a small frequency range is used in F₁. With DOSE-SECSY, they are removed easily by symmetrization while with DOSE-COSY, a simple symmetrization is not possible. The reading pulse was 135° or 155° to avoid the appearance of remote connectivities. When pure absorption (phase sensitive) spectra are not needed such pulse sequences are very convenient for assignment purposes because of short acquisition time and small memory area.

Preparation of dimer blocks 14 - 17³⁴

The 5'-protected phosphodiester block 12 (0.5 mmol) was added to a solution of the 2'-O-Px block 13 (0.5 mmol) in dry pyridine (20 ml) and the volume reduced to ~6 ml on the rotavapor. To this solution was added 1-mesitylenesulfonyl chloride (0.75 mmol) and 1-methylimidazole (1.5 mmol). The reaction was stirred for 30 min, poured into satd. NaHCO₃ solution (100 ml) and extracted with CH₂Cl₂ (3 x 100 ml). The combined extracts were then pooled, coevaporated with toluene, and the pyridine-free residue purified on a short silica gel column. The desired fractions were collected, evaporated and the yield measured as a glass: 14 [R_f (A) 0.62, yield 78 %, ³¹P-NMR: -7.49 & -7.59]; 15 [R_f (A) 0.66, yield 80 %, ³¹P-NMR: -8.79 & -8.91]; 16 [R_f (A) 0.58, yield 92 %, ³¹P-NMR: -7.01 & -7.59]; 17 [R_f (A) 0.56, yield 90 %, ³¹P-NMR: -7.02 & -7.57]:

Preparation of N³-benzoyl-2',3'-di-O-acetyl-5'-hydrogenphosphonate 18³⁵

To a suspension of 1,2,4-triazole (16 mmol) in CH₂Cl₂ (50 ml) were added phosphorous trichloride (5 mmol) and 1-methylimidazole (16 mmol), and the clear solution stirred for 20 min at RT and then 20 min in an ice bath. To this chilled solution was added dropwise a solution of N³-benzoyl-2',3'-di-O-acetyl uridine (1 mmol) in CH₂Cl₂ (10 ml) and the reaction stirred for 20

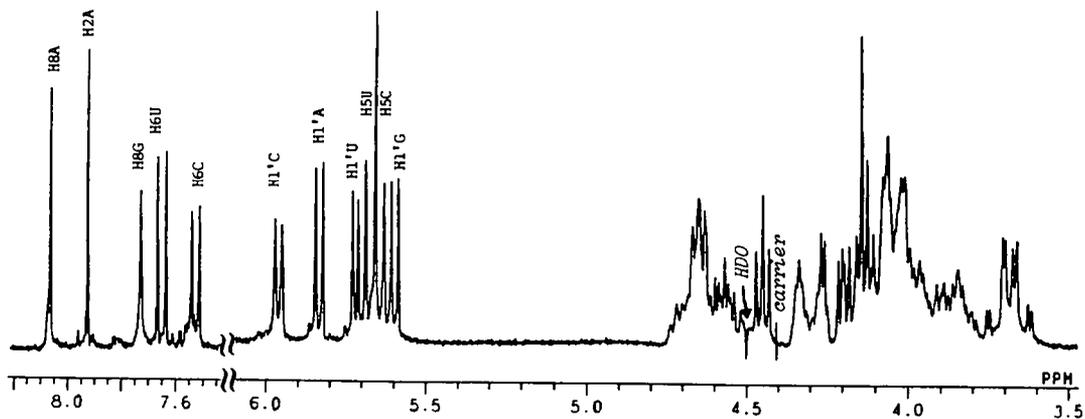


Figure 1 : 270 MHz ^1H -NMR spectrum of $\text{A}3'\text{p}5'\text{C}^*2'\text{p}5'\text{G}$ (33) in $^2\text{H}_2\text{O}$ at 308 K using WEFT.

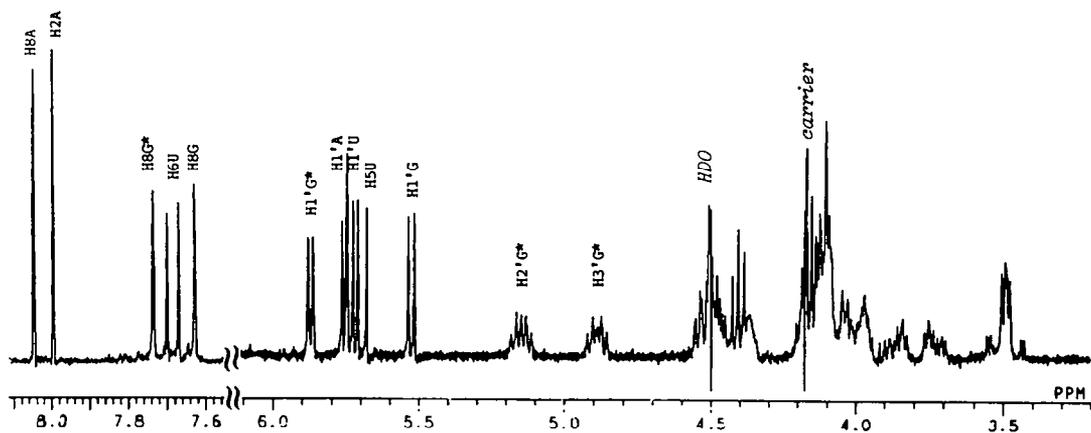


Figure 2 : 270 MHz ^1H -NMR spectrum of $\text{A}3'\text{p}5'\text{G}^*2'\text{p}5'\text{U}$ (34) in $^2\text{H}_2\text{O}$ at 308 K using WEFT.

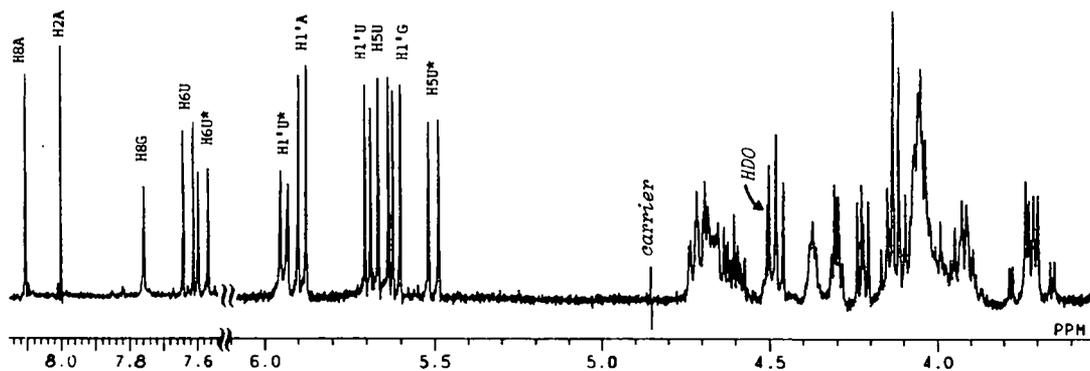


Figure 3 : 270 MHz ^1H -NMR spectrum of $\text{A}3'\text{p}5'\text{U}^*2'\text{p}5'\text{G}$ (35) in $^2\text{H}_2\text{O}$ at 308 K using WEFT.

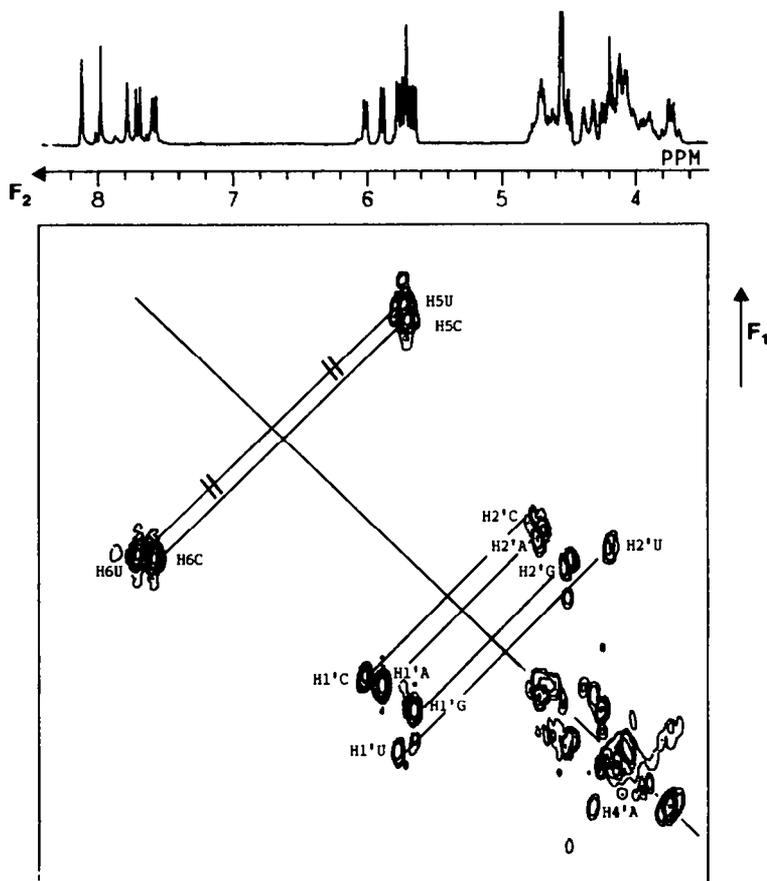


Figure 4 : Two dimensional DOSE-COSY experiment for $A3'p5'C^*2'p5'G/3'p5'U$ (33) recorded at 308 K [Experimental conditions : reading pulse 135° , $\Delta t_1 = 0.233$ ms, $F_1 = F_2 = 2150$ Hz, 160 FIDs were accumulated for each 256 experiments. The resolution was 4.2 Hz / point in each dimension after zero filling to $512 \times 1K$. A sine-bell window was applied. The spectrum was not symmetrized. The correlation was obtained with respect to the skew diagonal.]

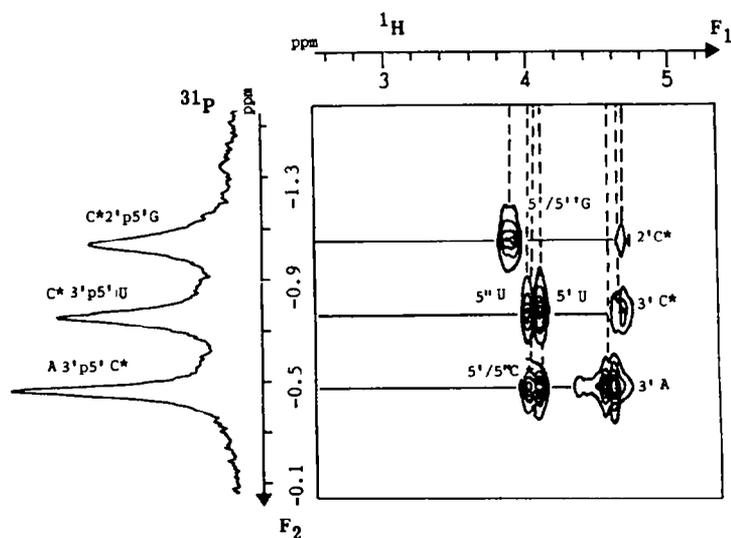


Figure 5 : Two dimensional $^{31}P/^1H$ correlation experiment for $A3'p5'C^*2'p5'G/3'p5'U$ (33) at 109.25 MHz [$T = 308$ K in 2H_2O]. The two delays were at 51 ms and 30 ms.

min. This solution was then poured into 1M triethylammonium bicarbonate (TEAB) solution (100 ml) and extracted with 10 % ethanol-CH₂Cl₂ mixture (4 x 100 ml). The combined extracts were evaporated, and the residue purified by silica gel chromatography using CH₂Cl₂ and Et₃N (98:2,v/v) mixture and an ethanol gradient (0 - 10 %). The desired fractions were collected, evaporated and the quantitative yield measured as a glass. ³¹P-NMR: +3.49 (¹J_{H,P} = 621.0 Hz & ³J_{H,P} = 6.2 Hz)

Preparation of partially-protected trimers 19 - 22

A solution of the dimer (14 - 17) (0.3 mmol) and the H-phosphonate block 18 (0.5 mmol) in dry pyridine (20 ml) was concentrated to ~6 ml on the rotavapor, and freshly distilled pivaloyl chloride (3 mmol) was added and the reaction stirred for 30 min. A freshly prepared solution of iodine in THF-water (4:1, v/v) was then added and the solution stirred for 20 min. It was then poured into a 5% solution of NaHSO₃ (200 ml) and extracted with CH₂Cl₂ (3 x 100 ml). The combined extracts were washed with TEAB buffer and evaporated, the residue was subsequently purified by silica gel chromatography using CH₂Cl₂ and Et₃N (98:2,v/v) mixture and an ethanol gradient (0 - 4 %). The desired fractions were collected, evaporated and the yield measured as a glass: 20 [R_f (A) 0.84, yield 65 %, ³¹P-NMR: -1.34, -6.9 & -8.31]; 21 [R_f (A) 0.75, yield 70 %, ³¹P-NMR: -0.92, -8.86 & -9.22]; 22 [R_f (A) 0.8, yield 70 %, ³¹P-NMR: -1.32, -8.15 & -8.50]; 19 was not isolated, it was directly deprotected.

Removal of the 2'-O-Px group from the partially-protected trimers 19 - 22 to give 23 - 26

To a solution of the trimer 19 - 22 (0.1 mmol) in CHCl₃ (5 ml) in an ice-bath (~0 °C) was added a solution of 0.1 M Cl₃CCO₂H in 2 % ethanol-CHCl₃ mixture (5 ml) and the reaction was found to be complete within 2 min for 24 & 26, it however took 4 min for 23 & 25. The reaction mixture was poured into a saturated solution of NaHCO₃ (50 ml) and extracted with CH₂Cl₂ (4 x 100 ml). The combined extracts were evaporated, and the residue purified by silica gel chromatography. The desired fractions were collected, evaporated and the yield measured as a glass: 23 [R_f (B) 0.84, yield 44 % (overall yield in two steps: condensation & removal of the 2'-O-Px), ³¹P-NMR: -0.51, -7.37 & -7.52]; 24 [R_f (B) 0.5, yield 66 %, ³¹P-NMR: -1.34 & -7.51]; 25 [R_f (B) 0.35, yield 79 %, ³¹P-NMR: 0.05, -7.32 & -7.99]; 26 [R_f (B) 0.3, yield 60 %, ³¹P-NMR: -1.83 & -7.66].

Preparation of fully-deprotected tetramers 32 - 35.

To a solution of the trimer 23 - 26 (65 μmol) and the 5'-phosphiteamidite 27 (260 μmol) in dry THF (4 ml) was added tetrazole (1 mmol) and the reaction stirred for 1 h at ~20 °C. A solution of iodine in THF-pyridine-water (8:1:1, v/v/v) was added to the reaction mixture and stirred for 20 min. It was then poured into a 5% solution of NaHSO₃ (50 ml) and extracted with CH₂Cl₂ (4 x 100 ml). The combined extracts were evaporated with toluene, and the residue was then directly deprotected in the following manner: (i) *syn*-4-nitrobenzaldehyde and 1,1,3,3-tetramethylguanidine (0.65 mmol each) in dioxane-water (5 ml each) for overnight, (ii) aq. ammonia (d 0.8, 20 ml) at 50 °C overnight, (iii) the volatile matters were evaporated and the residue dissolved in 80 % aq. acetic acid and stirred for 5 h at ~20 °C and then concentrated. The residue was then dissolved in water (50 ml) and washed with CH₂Cl₂ (4 x 50 ml). The aqueous phase was then concentrated and applied to a DEAE Sephadex column and eluted with triethylammonium bicarbonate buffer (pH 7.3) by linear gradients of 500 ml each: 0 - 0.25 M, 0.25 M - 0.5 M, 0.5 M - 0.75 M. The desired fractions were collected, evaporated and the yield, measured after exchange to the Na⁺ form, as the freeze-dried powder: 32 [yield 13 %, ³¹P-NMR: -0.88, -0.95 & -1.34]; 33 [yield 34 %, ³¹P-NMR: -0.51, -0.78 & -1.10]; 34 [yield 16 %, ³¹P-NMR: -0.54, 0.68 & -1.07]; 35 [yield 40 %, ³¹P-NMR: -0.51, 0.81 & -0.93].

Figs. 1 - 9 show their 270 MHz ¹H-NMR, 2D ¹H,¹H correlation and ¹H,³¹P correlation spectra in support of their structures.

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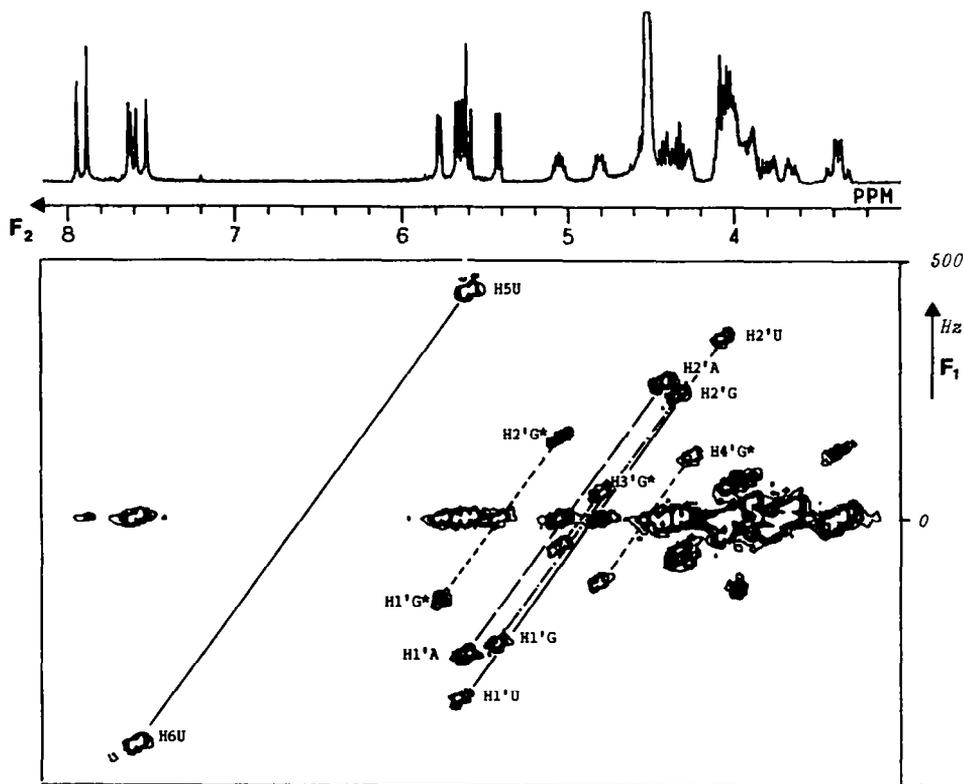


Figure 6 : Double quantum filtered SECSY 2D spectrum for $A3'p5'G^*2'p5'G$ (34) recorded at 270 MHz in 2H_2O at 308 K [Experimental conditions : $\Delta t_1 = 0.5$ ms, $F_1 = 1000$ Hz, $F_2 = 2200$ Hz, 80 FIDs were accumulated for each 256 experiments. The resolution was 1.9 Hz / point in F_1 and 3.8 Hz / point in F_2 after zero filling to 512 x 1K. A sine-bell apodization was used prior to Fourier transformation and the spectrum was symmetrized].

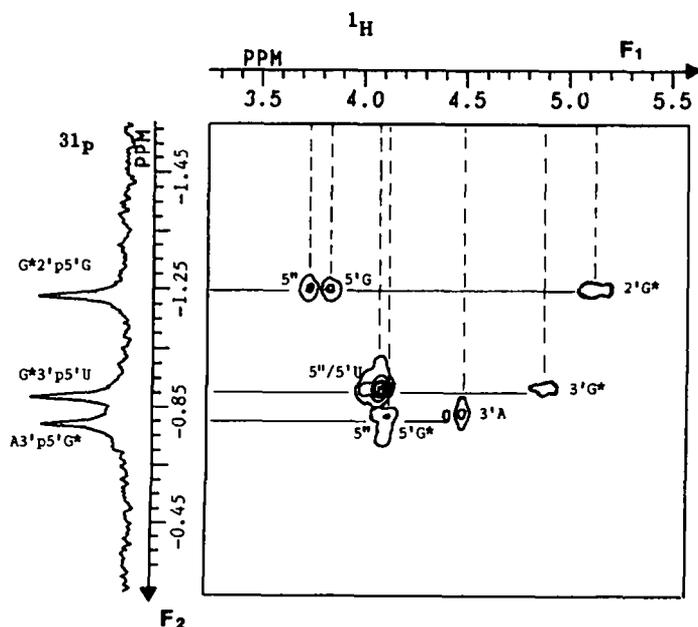


Figure 7 : Two dimensional $^{31}P/^1H$ correlation experiment for $A3'p5'G^*2'p5'G$ (34) at 109.25 MHz [$T = 303$ K in 2H_2O]. The two delays were at 58 ms and 29 ms.

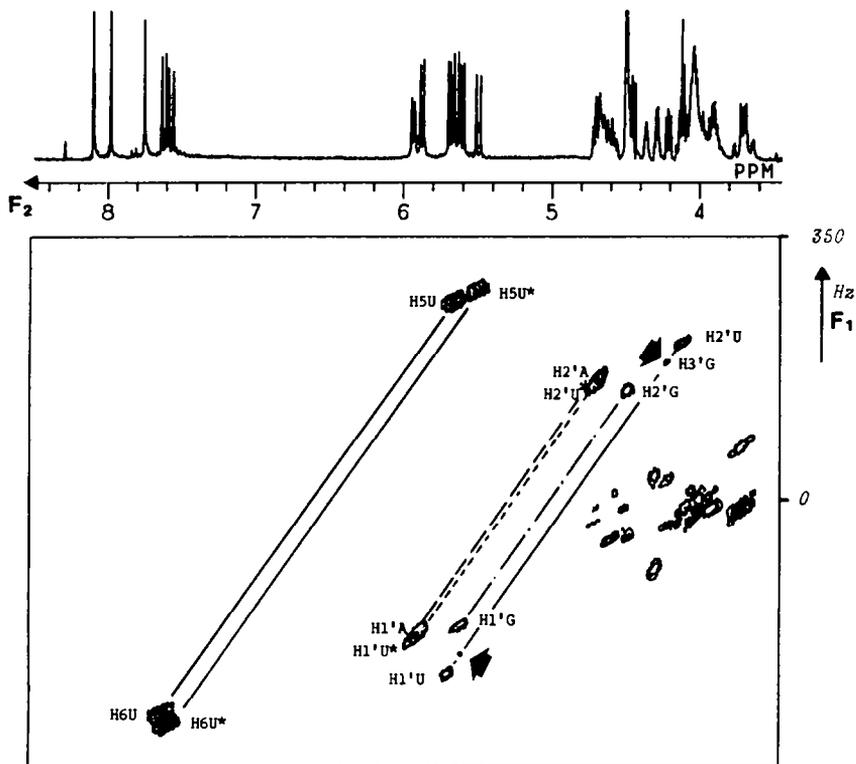


Figure 8 : Two dimensional DOSE-SECSY experiment for $A3'p5'U^*2'p5'G$ ($3'p5'U$) (35) at 270 MHz in 2H_2O at 308 K
 [Experimental conditions : reading pulse 135° , $\Delta t_1 = 0.5$ ms, $F_1 = 1000$ Hz, $F_2 = 2200$ Hz, 96 FIDs were accumulated for each 256 experiments. The resolution was 3.9 Hz / point in F_1 and 3.9 Hz / point in F_2 after zero filling to $256 \times 2K$. A sine-bell window was applied. The spectrum was symmetrized. Note the $^4J_{H,H}$ coupling between $H1'G$ and $H3'G$].

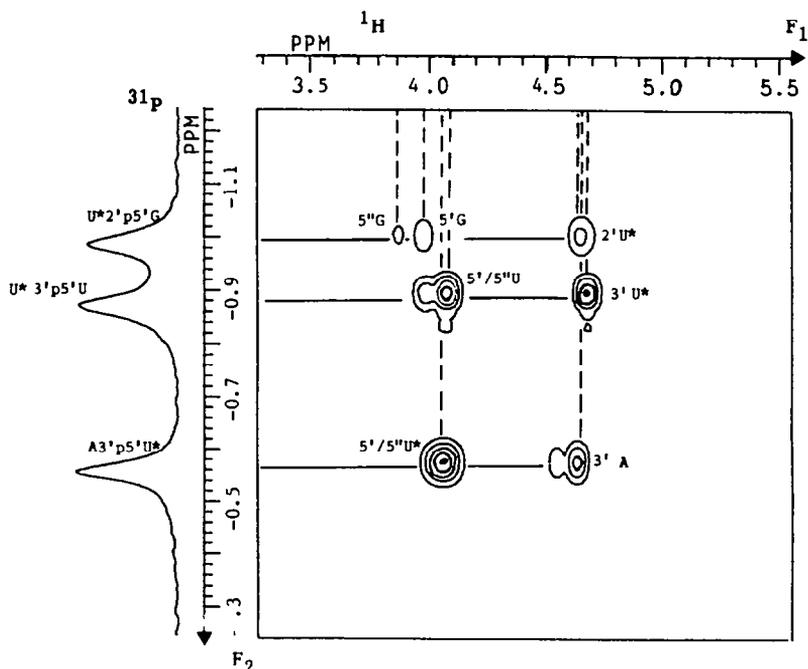


Figure 9 : Two dimensional $^{31}P/^1H$ correlation experiment for $A3'p5'U^*2'p5'G$ ($3'p5'U$) (35) at 109.25 MHz [$T = 303$ K in 2H_2O]. The two delays were at 58 ms and 29 ms.

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