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Synthesis of Tetrameric Cyclic Branched-RNA (Lariat) Modelling the Introns of Group II and Nuclear Pre-mRNA Processing Reaction (Splicing)

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Abstract: *Convergent synthesis of cyclic branched tetraribonucleotide* **9**, *modelling the lariat of pre-mRNA processing reaction (Splicing), is reported. The first key step in the present strategy involves the condensation of the appropriately protected 5′-O-levulinyl-G3′p5′U3′ phosphodiester block* **4** *with the 3′,5′-dihydroxy-6-N-benzoyl-2′-O-pixyl(9-phenylxanthen-9-yl)adenosine* **5** *, in presence of an activating agent, to give* **6a** *(53%). Chemospecific phosphorylation of 3′-OH of* **6a** *afforded the intermediate* **6b** *(88%) which was treated with mild acid to achieve a regiospecific removal of the 2′-O-pixyl group to give compound* **6c** *(99%). The second key step involved the introduction of biscyanoethylphosphotriester moiety to the 2′-OH of the branch-point adenosine in* **6c**, *in one single step using (biscyanoethoxy)-(diisopropylamino)phosphine to give the crucial branch-point building block* **6d** *(68%), with two dissimilar vicinal phosphates at 2′- and 3′- of the branch-point.* **6d** *was then condensed with N-4-benzoyl-2′,3′-di-O-acetylcytidine to afford the fully protected intermediate* **7a** *(75%). The 5′-O-levulinyl protecting group was then specifically removed from* **7a** *by hydrazine hydrate to give the intermediate* **7b** *(83%) which was subsequently treated with a bulky tertiary amine to give the branch-point-2′-cyanoethylphosphodiester block* **7c** *(74%). A unique intramolecular 5′→2′ cyclization was then brought about in* **7c** *by treatment with an activating agent under a high dilution condition to afford the fully protected cyclic branched tetramer* **8** *(61%). This oligomer was then deprotected in the usual manner and purified to give the cyclic branched tetramer* **9** *in 30% isolated yield, the first synthetic lariat known to date. Detailed 500 MHz ^{1}H-NMR and 202.4 MHz ^{31}P-NMR studies on* **9** *have unequivocally established its purity. Detailed spectroscopic studies such as COSY, HOHAHA, ROESY & ^{31}P-^{1}H-NMR shift correlation spectroscopy have clearly established the structural integrity of the tetrameric lariat RNA.*

In the last few years we have been engaged in trying to understand the structural significance of formation of branched-RNA in the RNA Splicing reaction and the structural and conformational basis for the choice of adenosine as the branch-point nucleotide, guanosine as the 2'→5' linked nucleotide, and uridine or cytidine as the 3'→5' linked nucleotide. This has led us to develop methodologies for the synthesis of open-chained branched RNAs of various sizes such as branched trimer A^{G}_{C} [1a-d], branched tetramer UA^{G}_{C} [1e,f], branched pentamer A^{GU}_{CC} [1g] and a number of their analogues. A branched heptamer CUA^{GU}_{CC} [1g], a branched nonamer CUA^{GUG}_{UCA} [1h] and a branched decamer $CCUA^{GUG}_{UCA}$ [1h] have also been synthesized from this laboratory. Comparative high-field NMR spectroscopic studies have established that solution conformation of these open-chained branched RNAs show distinct conformational changes upon addition of nucleotide units in any of the three directions from the branch-point adenosine block. Thus, the structures of the branched trimer and pentamer are similar[1b,c,i,j,k] while the structures of branched tetramer[1k-n] closely mimic the structure of the

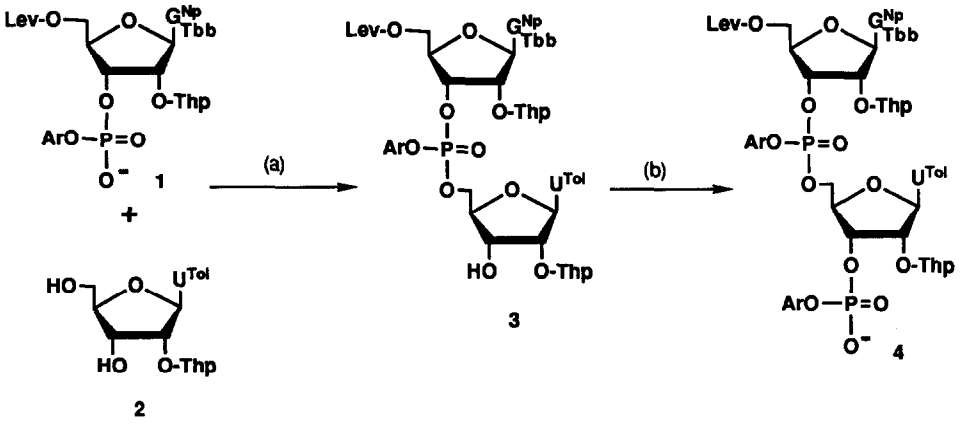
heptamer[1s]. During these conformational studies[1b,c,i-s] it became clear to us that the open-chained branch-RNA may only partly mimic and model the naturally-occurring lariat RNA formed in the Splicing reaction. This is primarily due to the fact that the conformational stress and strain inbuilt in the naturally-occurring lariat RNA owing to its cyclic nature is not reproduced in the synthetic open-chained branched RNA. Clearly, the true mimic and the model of the naturally-occurring lariat RNA is the synthetic cyclic counterpart in which the 3'-hydroxy of 2'→5' linked guanosine is cyclized to the 5'-hydroxyl group through a phosphodiester linkage. Next step would be to observe how and when the conformational stress and strain of the lariat RNA is released as the ring size of the lariat becomes larger! NMR spectroscopic studies on such cyclic lariat RNA and their analogues will reveal what effect the presence of the loop may have on its constituent sugar residues, the phosphate backbone and the base conformations, and how will this effect change upon enlarging the ring size! These are the rationals and questions that have directed our attention towards the synthesis of branched cyclic RNA. As a starting point, we have therefore chosen to regiospecifically synthesize the branched cyclic RNA which have all conserved nucleobases and constitute the smallest possible RNA lariat mimicing the naturally-occurring lariat intron: $\overbrace{p_{(5')}U_{(3')}p_{(5')}A^{(2')}_{(3')}p_{(5')}G_{(3')}}$ (**9**) .

All the synthetic strategies reported so far for the assembly of cyclic oligo(deoxy)ribonucleotides are based upon the phosphotriester approach, except for an early work by Khorana *et al* [2], who isolated cyclic oligomers and especially cyclic dithymidylic acid as by-products in the course of their work on synthesis of oligothymidylic acids using the phosphodiester approach. The intramolecular cyclization reactions in these reports are effected by condensing agents ($ArSO_2X$ type) under high dilution conditions, except for a recent work by Bonora *et al* [3] for the preparation of a number of cyclic oligodeoxyadenylic acids in which the cyclization was effected by 2-chlorophenyl-bis-O,O-(1-benzotriazolyl)phosphate. Hsu *et al* [4] described the synthesis of three cyclic diribonucleosidediphosphates r($\overbrace{ApAp}$), r($\overbrace{ApUp}$) and r($\overbrace{UpUp}$). van Boom *et al* synthesized r($\overbrace{GpGp}$)[5] and later his group prepared two cyclic tetra-RNAs, two cyclic hexa-DNAs and two cyclic octa-DNAs[6]. A solid phase synthesis was developed by Barbato *et al* [7,8] which produced up to hepta-cyclic-oligodeoxycytidylic acids. More recently Reese *et al* [9] described the synthesis of cyclic oligothymidylic acids together with a cyclic hexa-DNA of a mixed sequence.

Following points[1h] may be noted regarding the present synthesis of lariat RNA, $\overbrace{p_{(5')}U_{(3')}p_{(5')}A^{(2')}_{(3')}pC}$ (**9**): *(i)* The levulinyl (Lev) protecting group on the 5'-hydroxyl function in all the intermediates up to **7a** was found to be compatible with the rest of the protecting groups necessary for this strategy, and it can be selectively removed over the cyanoethyl group of the 2'-biscyanoethylphosphotriester moiety at the branch-point in **7a**. *(ii)* Conveniently accessible 6-N-benzoyl-2′-O-pixyl[(9-phenylxanthen)-9-yl]adenosine **5**[10] has been found to be a crucially important building block in this strategy because it allowed the extension of the chain from the branch-point A in all three directions: 5′→3′, 3′→5′ and 2′→5′ . This has been possible through the highly regiospecific synthesis of the key intermediate **6a** due to a specific phosphorylation of the 3′-phosphodiester function in **4** with the 5′-hydroxyl group in **5**. *(iii)* The regiospecific removal of the 2′-O-pixyl group ($t_{99.9}$ = 15 min from 5′-O-Px derivative of adenosine in 80% aq. acetic acid)[11] at the branch-point over the 2′-O-Thp (tetrahydropyranyl) groups ($t_{1/2}$ = 69 min from 2′-O-Thp derivative of uridine in 80% aq. acetic acid)[12] in **6b** under a mild acidic condition to give **6c**. Note that only one of the two diastereomers of 2'-O-Thp protected blocks has been used in this work for being able to monitor the reaction on Tlc and by NMR spectroscopy in a straightforward manner. *(iv)* The introduction of the biscyanoethylphosphotriester

moiety at the 2′-hydroxyl group at the branch-point, vicinal to 3′-(2-chlorophenyl)phosphodiester moiety as in **6c** to give **6d** by the reaction with an excess of the key amidite reagent, (biscyanoethoxy)-(diisopropylamino)phosphine[13], and tetrazole under conditions normally employed in amidite chemistry[14]. *(v)* The oligomer **6d** is regiospecifically extended in the 3′-direction ["3′-tail"] by condensation with N-4-benzoyl-2′,3′-di-O-acetylcytidine to give **7a**. *(vi)* After successive removal of the 5'-O-levulinyl protecting group from **7a**, and the cyanoethyl group from the 2′-biscyanoethylphosphotriester moiety in **7b**, a unique 5'U→3'G intramolecular cyclization was initiated in **7c** under a high dilution condition using 1-mesitylene sulfonyl-3-nitro-1,2,4-triazole (MSNT)[15,16] as the condensing agent to give the protected lariat RNA **8**.

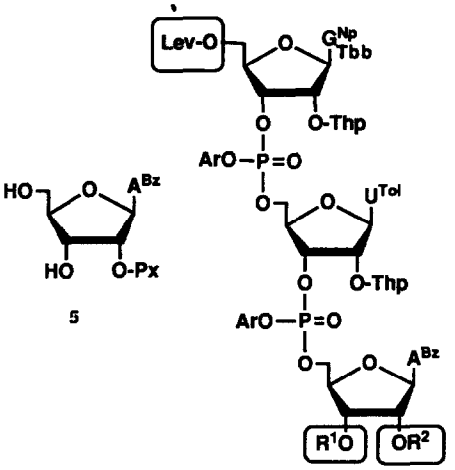
Synthesis of cyclic branched tetraribonucleotide 9: The 3′-phosphodiester block **4**, which was synthesized according to standard phosphotriester protocols[17,18], was condensed with the 3′,5′-dihydroxy block **5**[10] using 1-mesitylenesulfonyl chloride / N-methylimidazole[18] to give the key 3′-hydroxy block **6a** ($\delta^{31}P$ = -7.52, -7.57, -8.18) in 53% yield. Treatment of **6a** with *o*-chlorophenylphosphoro-bis-(1,2,4-



(a) (1) (1eq.), (2) (1 eq.), N-methylmidazole (5 eq.), MS-Cl (2.5eq.), pyridine, 40 min RT; (b) o-chlorophenyl-phosphoro-bis- (1,2,4-triazolide) (1.5 eq.), pyridine / MeCN 40 min RT;

triazolide)[19] gave the corresponding 3′-phosphodiester block **6b** in 88% yield [$\delta^{31}P$ = -7.47, -7.66 *(3′-phosphodiester)*, -7.81, -7.96, -8.06 *(3′→5′-phosphotriester)*]. The 2'-O-depixylation of **6b** was carried out using trichloroacetic acid in 2% CH_3OH-CH_2Cl_2 at 0 °C[20] to give the 3′-phosphodiester-2′-hydroxy block **6c** in a quantitative yield ($\delta^{31}P$ = -7.59, -7.69, -7.84, -7.98). Compound **6c** was treated with (biscyanoethoxy)-(diisopropylamino)phosphine[13] and tetrazole in dimethylformamide-acetonitrile at room temperature followed by aqueous iodine oxidation to give the 3′-phosphodiester-2′-biscyanoethylphosphotriester block **6d** in 68% yield ($\delta^{31}P$-NMR, Panel (a) in Fig. 1, Ce-P : ClPh-P = 1: 3). In the next step, a normal MSNT condensation reaction was carried out with compounds **6d** and N-4-benzoyl-2′3′-di-O-acetylcytidine to yield the fully protected oligomer **7a** [75 %, ($\delta^{31}P$-NMR, Panel (b) in Fig. 1, Ce-P : ClPh-P = 1: 3)]. The 5′-O-levulinyl protecting group was removed from compound **7a** by treatment with hydrazine hydrate in pyridine/acetic acid (3:2 v/v) in the usual way[21] to give **7b** in 83% yield ($\delta^{31}P$-NMR, Panel (c) in Fig. 1, Ce-P : ClPh-P = 1: 3). **7b** was treated with diisopropylethylamine in pyridine at 40 °C for ~1.5 h[22] to give the 5′-hydroxy-2′-

cyanoethylphosphodiester block **7c** in 74% yield (δ^{31}P-NMR, Panel (d) in Fig. 1, Ce-P : ClPh-P = 1: 3). Two distinct groups of phosphate resonances in ^{31}P-NMR can be observed in the spectrum of each of the cyanoethylphosphate containing oligomers[1h] (i.e **6d, 7a, 7b and 7c**). Simple integration of these two



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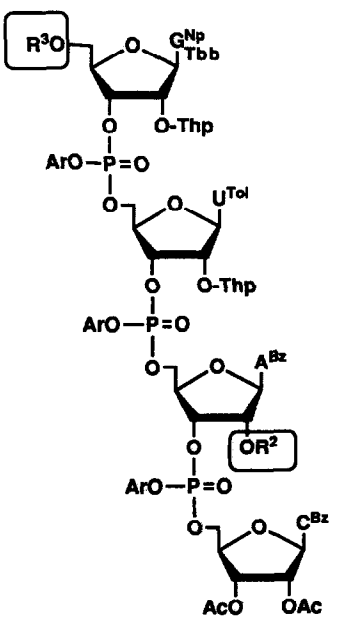
6a-d

	R^1 =	R^2 =
(a)	H	Px
(b)	ArO—P(=O)(O⁻ Et_3NH^+)—	Px
(c)	ArO—P(=O)(O⁻ Et_3NH^+)—	H
(d)	ArO—P(=O)(O⁻ Et_3NH^+)—	$NCCH_2CH_2O$—P(=O)($NCCH_2CH_2O$)—



7a-c

	R^2 =	R^3 =
(a)	$NCCH_2CH_2O$—P(=O)($NCCH_2CH_2O$)—	$CH_3COCH_2CH_2CO$-
(b)	$NCCH_2CH_2O$—P(=O)($NCCH_2CH_2O$)—	H
(c)	$NCCH_2CH_2O$—P(=O)(O⁻ Et_3NH^+)—	H

groups of phosphate resonances reveals the outcome of each reaction step[1h] [see Panels (a) - (e) in Fig. 1]. The intramolecular cyclization of **7c** was then brought about by addition of 9 equivalents of MSNT to a 4 mM

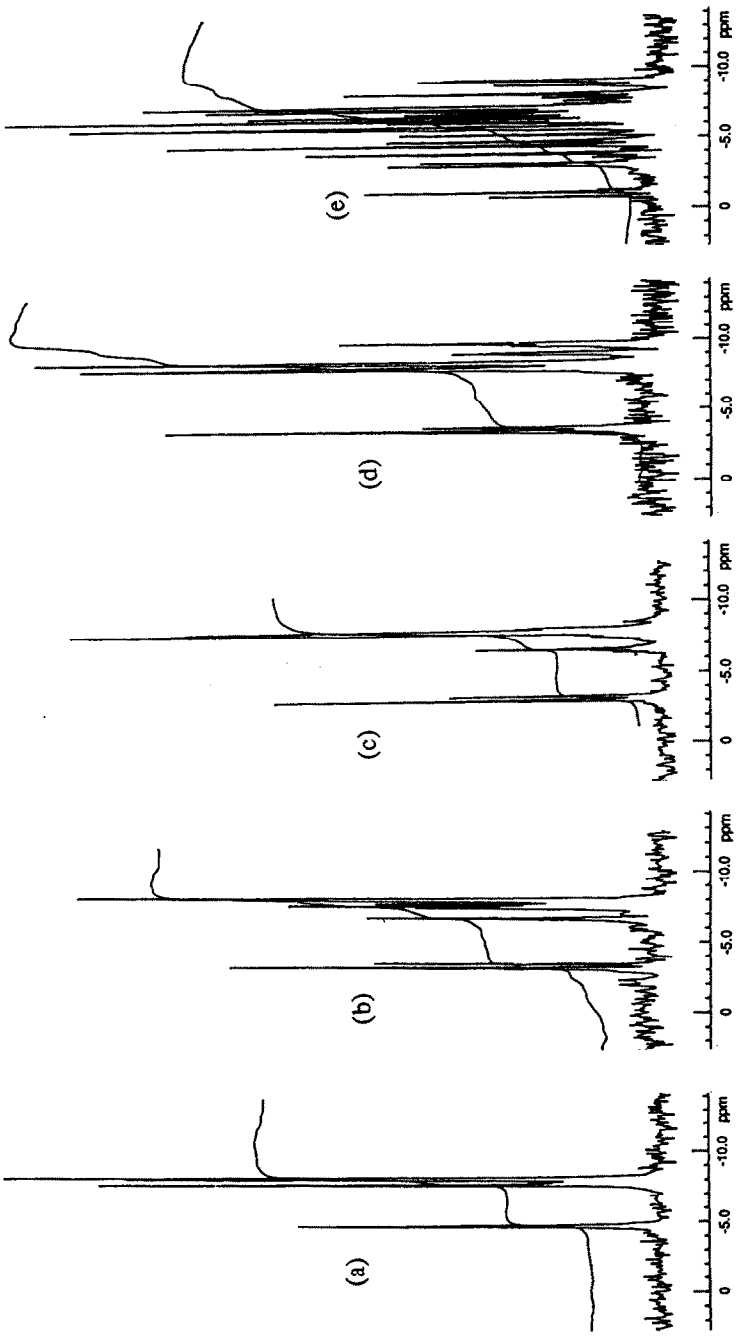
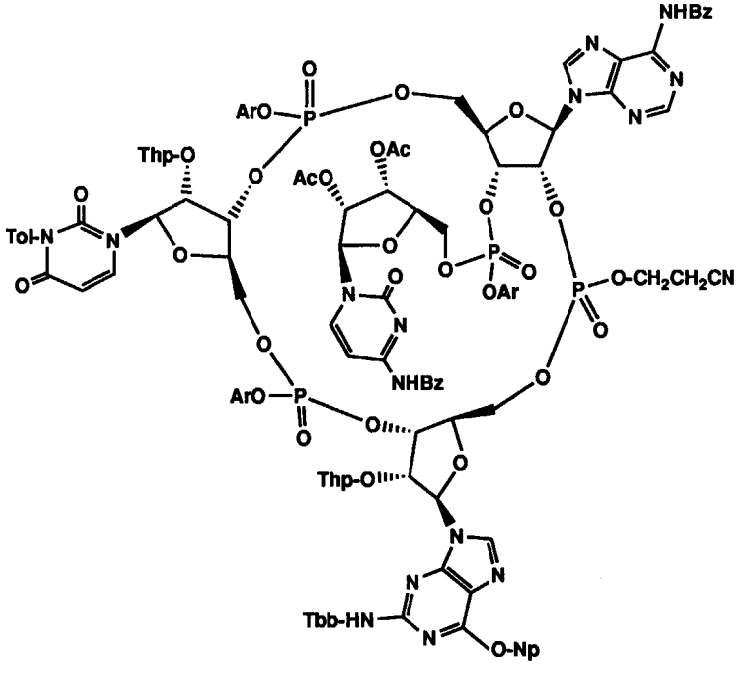


Fig. 1: ^{31}P-NMR spectra [36 MHz] of **6d** (Panel: a) in $CDCl_3$+CD_3OD, **7a** (Panel: b) in $CDCl_3$, **7b** (Panel: c) in $CDCl_3$, **7c** (Panel: d) in $CDCl_3$+CD_3OD and **8** (Panel: e) in $CDCl_3$ at 22 °C



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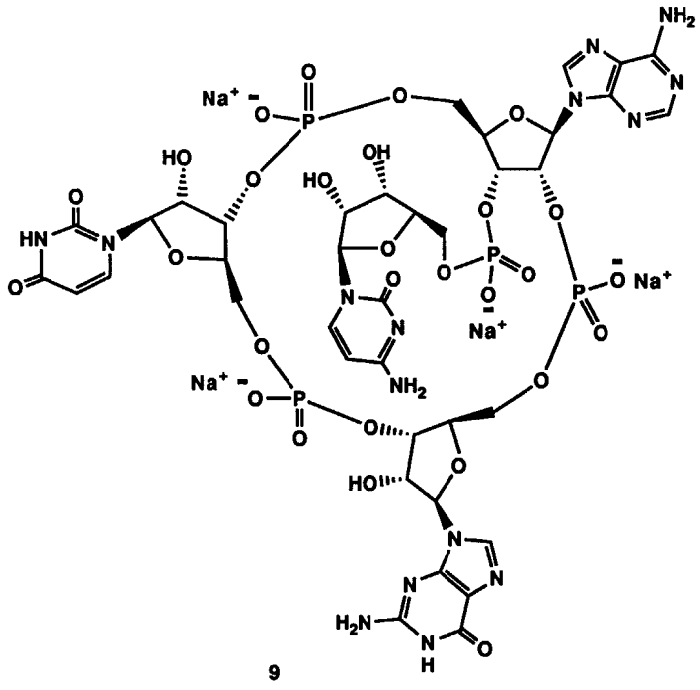

9

Abbrevations: Ar = 2-Chlorophenyl; Px = 9-phenylxanthen-9-yl; Lev = levulinyl; Thp = tetrahydropyranyl; $G^{Np}{}_{Tbb}$ = N^2-(4-(t-butyl)benzoyl)-O^4-(2-nitrophenyl)-9-guaninyl; C^{Bz} = N^4-benzoyl-1-cytosinyl; U^{Tol} = N^3-(4-toluoyl)-1-uracilyl; A^{bz} = N^6-benzoyl-9-adeninyl; A = 9-adeninyl; C= 1-cytosinyl; G = 9-guaninyl; U = 1-uracilyl, Tbb = 4-(t-butyl)benzoyl; Np = 2-nitrophenyl; Tol = 4-toluoyl

pyridine solution of **7c**, after over night stirring at room temperature, to give the fully protected cyclic branced tetramer **8** in 61% yield [δ^{31}P-NMR, Panel (e) in Fig. 1]. Compound **8** was then deprotected in the usual manner and the product was isolated from the product mixture by DEAE-Sephadex column chromatography followed by semi-preparative RP-HPLC (Fig. 2) and Dowex ion-exchange column chromatography to finally give pure Na^+ salt cyclic branched tetramer **9** (see experimental section for details) in 30% yield (432 A_{260} o.d units) which has been subsequently spectroscopically characterized (*vide infra*).

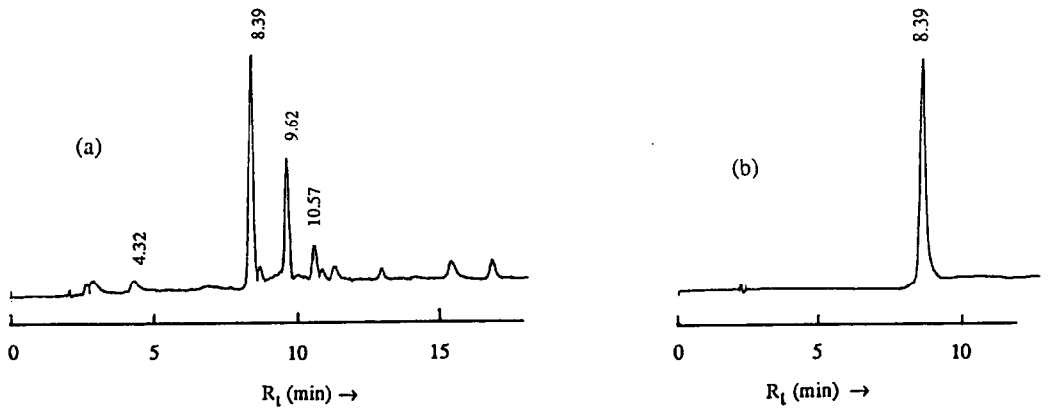

Fig. 2 : (a) HPLC-chromatogram (Nucleosil C18, 5μ) of the crude deprotection mixture, eluted with 0.1M triethylammonium acetate/acetonitrile (0-40%B in 30 min, 1ml/min; A = 5% MeCN in 0.1M TEAA, pH 7.0, B = 50% MeCN in 0.1M TEAA, pH 7.0). The desired product **9** eluted at R_t = 8.39 min; (b) HPLC-chromatogram (on the same column using the same gradient) of the cyclic branched tetramer **9** as its triethylammonium salt after purification on DEAE-Sephadex and semi-preparative RP-HPLC.

Characterization of Lariat RNA $\overbrace{p(5')U(3')p(5')A^{(2')pG(3')}_{(3')pC}}$ (9) by 500 MHz ^{1}H-NMR and 202.4 MHz ^{31}P-NMR Spectroscopy. The 1D ^{1}H-NMR at 500 MHz of cyclic $\overbrace{pU(5')pA^{p(2')G}_{p(3')C}}$ is shown in Fig. 3A at 298K in ~6 mM 99.98% D atom D_2O solution. Figs. 3B and 3C show the expansions of the aromatic and anomeric regions, respectively, of 1D ^{1}H-NMR spectra. H-6 and H-5 of uridine appear as a doublet of ~ 8.1 Hz while H-6 and H-5 of cytidine appear as a doublet of ~ 7.6 Hz. This showed that the H6 and H5 of uracil protons absorbed at δ8.00 (d, $J_{5,6}$ = 7.6 Hz), and δ5.82 (d, $J_{5,6}$ = 8.1 Hz), respectively. The cytosine H6 and H5 protons absorbed at δ7.63 (d, $J_{5,6}$ = 8.1 Hz, H6) and δ5.84 (d, $J_{5,6}$ = 7.6 Hz, H5), respectively. H8G abosorbs at δ7.77. H2A and H8A abosorb at δ8.19 and 8.46, respectively. All aromatic protons have been correlated to its own anomeric proton by rOe connectivities in a ROESY experiment shown in Fig. 4. A COSY experiment (not shown) has established connectivities to the vicinally coupled protons while a HOHAHA experiment (Fig. 5) has unequivocally shown connectivities through the J-relays amongst all mutually coupled protons within each sugar unit. Assignment of all aromatic and anomeric protons are thus shown in Table 1. A perusal of H5'/5" chemical shifts of all four sugar residues show that they all absorb within a range of 0.20 ppm between δ4.20-4.00 suggesting that they are each covalently linked with a phosphodiester group. Note that we

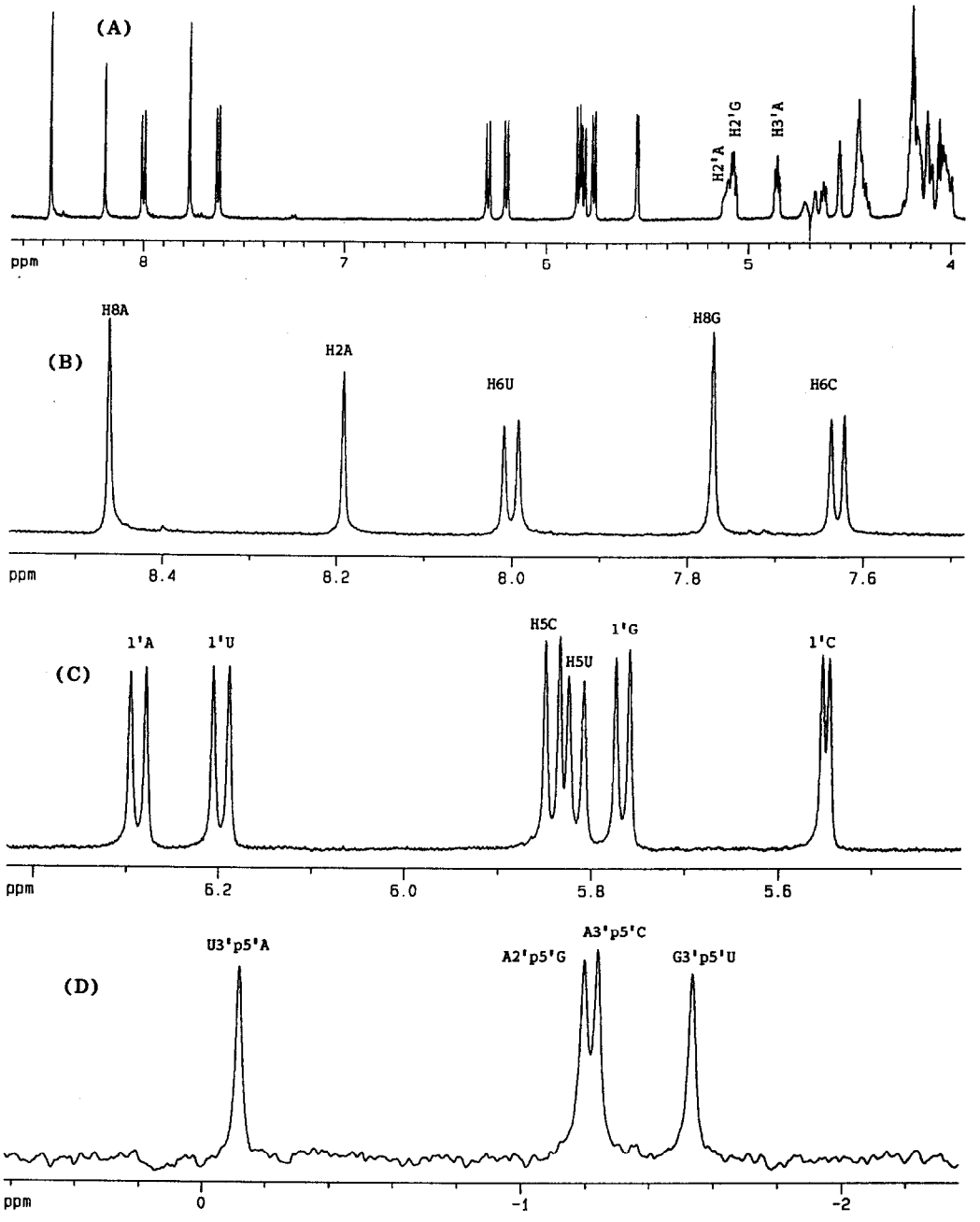

Fig. 3 : *Panel A* shows the one dimensional ^{1}H-NMR spectra at 500 MHz of branched cyclic-RNA **9** in 99.98%D D_2O (6 mM) at 298K, DOH as internal reference at $\delta = 4.7$ ppm. *Panels B & C* show the expansion of aromatic and anomeric regions, respectively, with full assignments based on COSY; HOHAHA, and ROESY experiments. *Panel D* shows the 202.45 MHz ^{31}P-NMR spectra of **9** as its Na+ salt in 99.98%D D_2O (6 mM) at 298K (cAMP as an external reference).

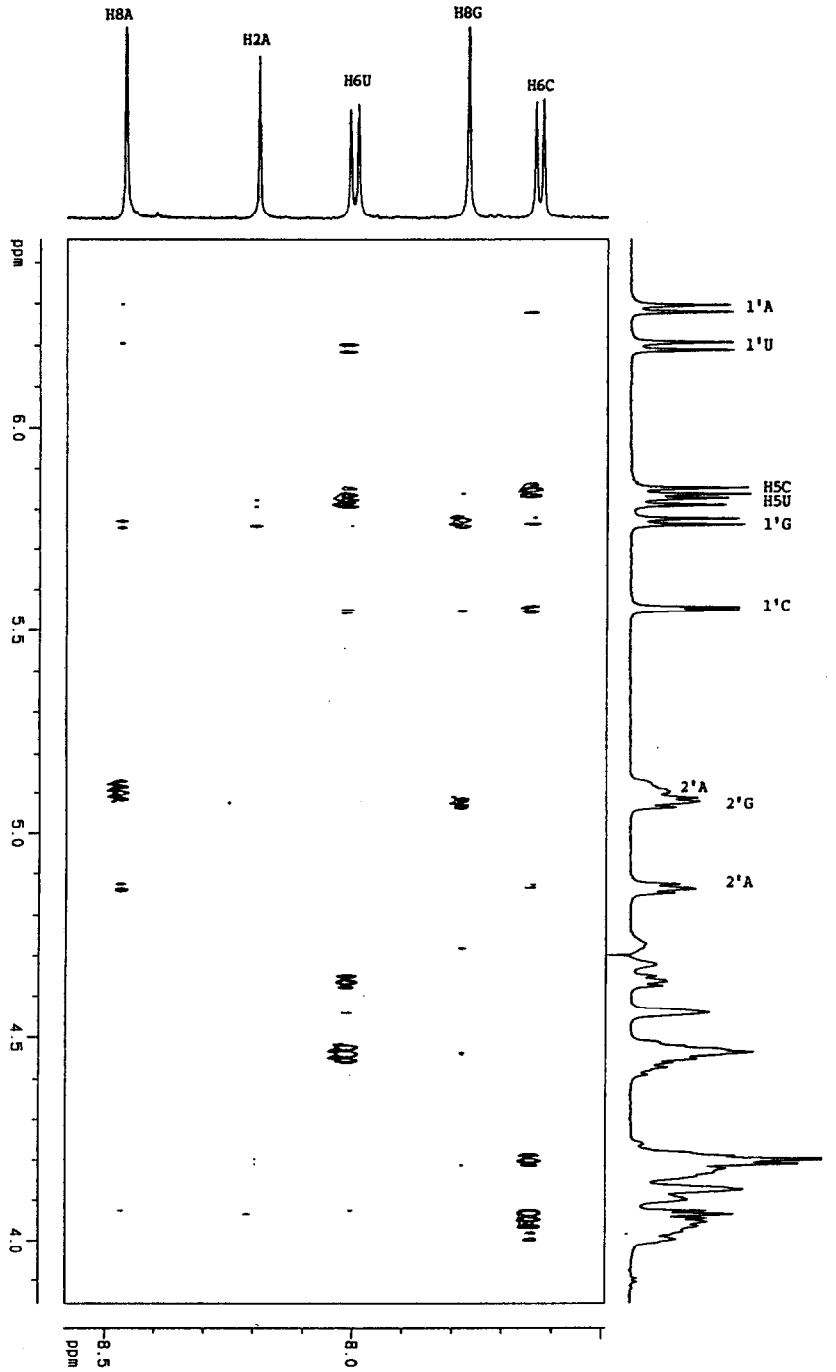

Fig. 4 : ROESY spectrum of branched cyclic-RNA 9 at 298K (6 mM) in D_2O in the phase sensitive mode [mixing time 500 ms, 4K data points, 256 experiments of 64 scans, 2K x 512 real data points in F2 and F1 dimensions , respectively, processed by window function $sine^2$ in both dimensions].

have earlier unequivocally characterized the corresponding open-chained branched tetramer U(5')pA$^{p(2')G\ 1p}_{p(3')C}$ and have shown that the 5' and 5" protons of 5'-terminal uridine residue, which does not have a phosphodiester group absorbed at δ3.59 and 3.51, respectively. A quick evaluation of $J_{1',2'}$ in all anomeric protons show that three sugar residues of adenosine, uridine and guanosine involved in the cyclic structure have $J_{1',2'}$ couplings of 8.5, 8.5 and 7.2 Hz, respectively, suggesting that these sugar residues are locked in 2'-*endo*-3'-*exo* (South) conformations. We have subsequently performed a two dimensional ^1H, ^{31}P-correlation experiment in order to establish unequivocally the presence of a covalently linked phosphodiester group connecting the 5'-terminal uridine (A5'→3'U) and 3'-terminal guanosine (A5'→2'G) residues in the lariat RNA **9**. Fig 3D shows the 1D ^{31}P-NMR spectra, and Fig. 6 show the proton detected inverse ^1H, ^{31}P-correlation experiment in the absolute mode at 298K in D_2O solution (~6 mM). Note that the connectivities of all four phosphates to the respective 2' or 3' proton of the first sugar residue to the 5'/5" of the second sugar have indeed been found in the ^1H, ^{31}P-correlation experiment (Fig. 6). For example, the phosphate resonance at δ -1.532 is found to have J-couplings with 5'/5"U, 4'U, 3'G and 2'G suggesting that it can be unequivocally assigned to G3'**p**5'U in the target compound **9**. Clearly such ^1H, ^{31}P couplings are only possible if the U and G residues are covalently linked through a phosphodiester residue between 3'G to 5'/5"U confirming the cyclic nature of the target molecule **9**. The long-range couplings of 3'→5'-phosphate to 4'U and 2'G in G3'**p**5'U moiety are clearly due to coupling through the "W" pathway owing to conformationally rigid South conformation of both participating sugar residues. A similar kind of observed J-couplings of phosphate resonances at δ -1.235, -1.193 and -0.116 with their appropriate 2' or 3'-linked sugar and 5'/5"-linked sugar residues (Fig 6) also clearly confirm that they are A3'**p**5'C, A2'**p**5'G and U3'**p**5'A, respectively.

Table 1: Chemical shifts of all aromatic and sugar protons of lariat RNA $\overline{p(5')U(3')p(5')A^{(2')p(5')G(3')}_{(3')p(5')C}}$ **(9)** at 298K in 99.98% D atom D_2O solution (~6 mM) at 500 MHz (internal reference DOH at δ4.7)

Nucleotide residues/ Protons	Adenosine (A)	Guanosine (G)	Cytidine (C)	Uridine (U)
H1'	6.29	5.77	5.55	6.20
H2'	5.10	5.07	4.05	4.45
H3'	4.85	4.71	4.14	4.63
H4'	4.67	4.46	4.03	4.55
H5'	4.18	4.00	4.20	4.16
H5"	4.11	4.00	4.20	4.10
H2	8.19	-	-	-
H8	8.46	7.77	-	-
H6	-	-	7.63	8.00
H5	-	-	5.84	5.82

Further work is now in progress to synthesize cyclic branched oligomers with an increased number of nucleotide units both in the 2'→5'-loop of the branch-point A and in its 3'-"tail" .

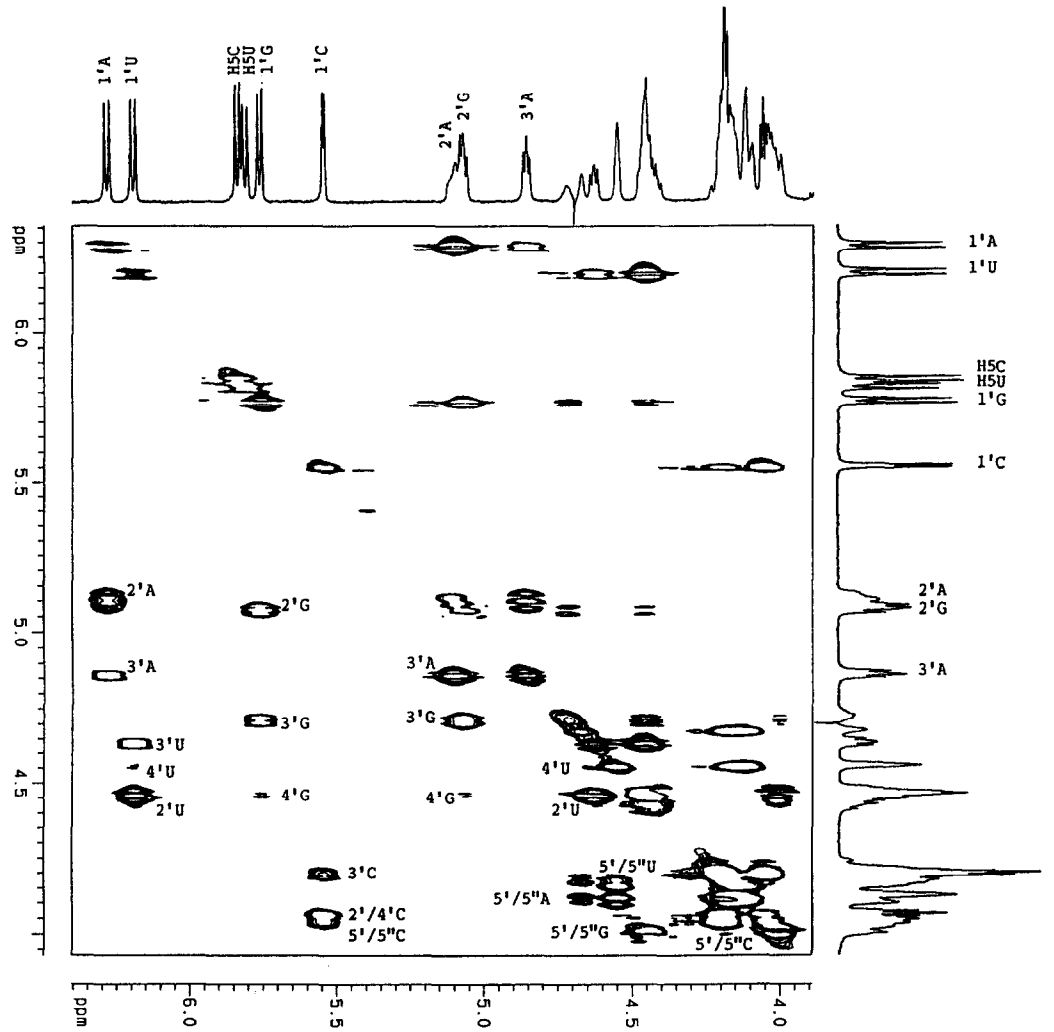

Fig. 5 : Expansion of the 500 MHz NMR homonuclear Hartmann-Hahn spectrum (HOHAHA) of branched cyclic-RNA **9** at 298K in D_2O (6 mM) in the phase sensitive mode [4K data points, 256 experiments of 64 scans, 2K x 512 real data points in F2 and F1 dimensions, respectively, processed by window function $sine^2$ in both dimensions].

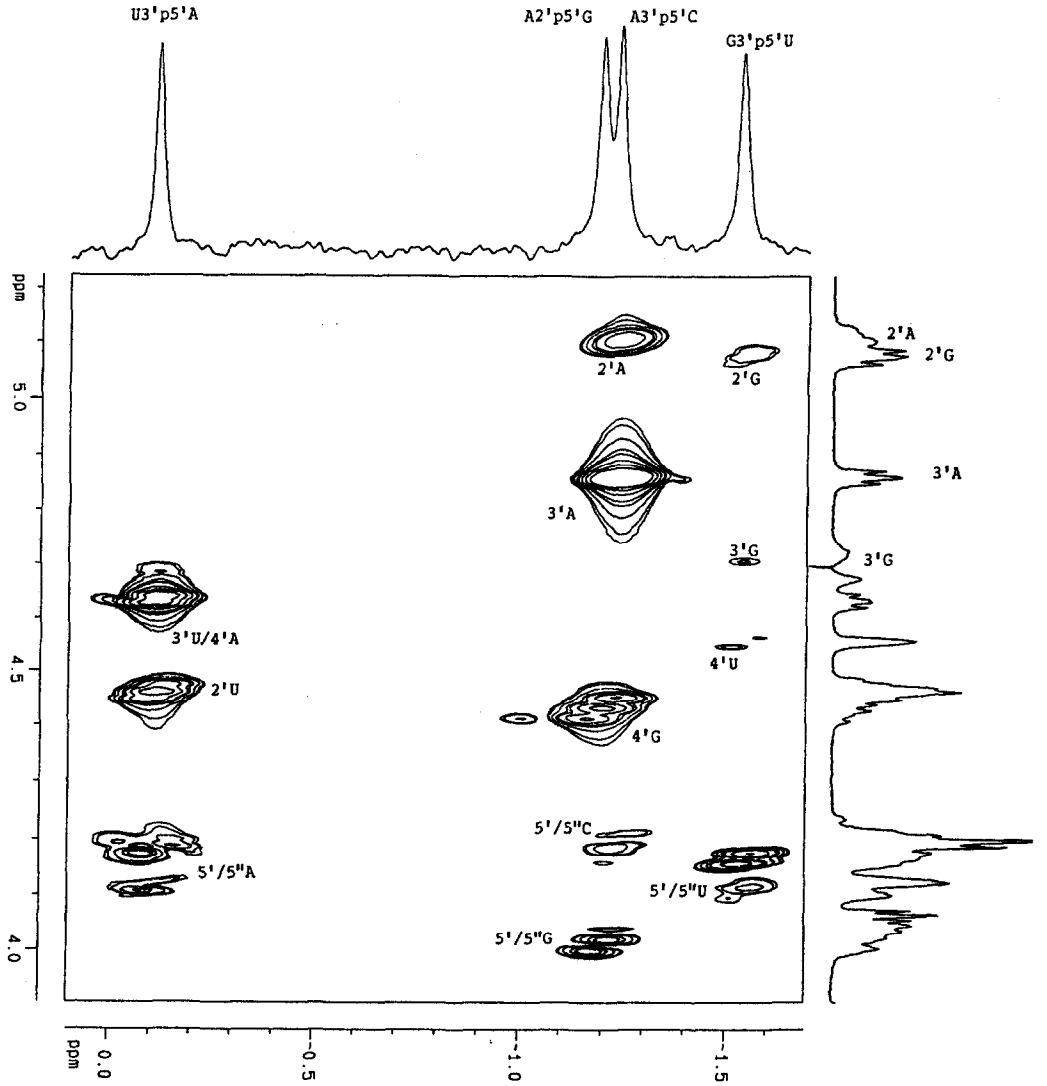

Fig. 6 : Two dimemsional ^{31}P-^{1}H chemical shifts correlation spectrum (at 500 MHz ^{1}H and 202.45 MHz ^{31}P) of branched cyclic-RNA **9** in D_2O at 298K (6 mM) in the absolute mode [4K data points, 256 experiments of 64 scans, 2K x 512 real data points in F2 and F1 dimensions, respectively, processed by window function sine^2 in both dimensions].

EXPERIMENTAL

^{1}H-NMR spectra were recorded in δ scale with Jeol FX 90 Q and Bruker AMX-500 spectrometers at 90 and 500 MHz respectively, using TMS or H_2O (set at 4.7 ppm) as internal standards. ^{31}P-NMR spectra were recorded at 36 and 202 MHz in the same solvent using 85 % phosphoric acid or cAMP as external standard. TLC was carried out using pre-coated silica gel F_{254} plates in the following dichloromethane-methanol mixtures: (A) 98: 2 (v/v), (B) 95: 5 (v/v), (C) 90: 10 (v/v), (D) 80: 20 (v/v). Dry pyridine was obtained by distillations over CaH and TsCl. Acetonitrile was distilled from P_2O_5 under argon. Dimethylformamide was distilled over CaH. The column chromatographic separations of all the protected intermediates were carried out using Merck G 60 silica gel. DEAE-Sephadex A-25 from Pharmacia was used for the ion exchange chromatography. A Gilson equipment with Pump Model 303, Manometric Module Model 802C and Dynamic Mixer 811B connected to a Dynamax computer program for gradient control was used for semi-preparative RP-HPLC separations. 2′-O-Thp derivatives of all nucleosides used in this work were separated and used in a diastereomerically pure form which are designated as "Low R_f" or "High R_f" subsequently in the following experimental section.

Compound 3: 5′-O-Levulinyl-2′-O-tetrahydropyranyl-2-N-(4-(t-butyl)benzoyl)-6-O-(2-nitrophenyl)guanosine-3′-triethylammonium(2-chlorophenyl)phosphate[23,24] (Low R_f) **1** (1.36 g, 1.31 mmol) was condensed with 2′-O-tetrahydropyranyl-N-3-(4-toluoyl)-uridine[17,25] (Low R_f) **2** (583 mg, 1.31 mmol) in dry pyridine (6ml / mmol) by addition of N-methylimidazole (521 µl, 6.55 mmol)) followed by addition of 1-mesitylenesulfonyl chloride (715 mg, 3.28 mmol). The reaction mixture was then stirred for 40 min at room temperature. Aqueous ammonium bicarbonate work up (partition between concentrated ammonium bicarbonate solution and dichloromethane) followed by silica gel column chromatography (0-3% ethanol in CH_2Cl_2) afforded **3** (1.73 g). R_f: 0.49 (C); ^{1}H-NMR ($CDCl_3$): 8.67-6.87 (m, 18H) arom., GH-8 & UH-6; 6.23 (d, $J_{1'2'}$= 3.91Hz, 1H) GH-1′; 6.06 (d, $J_{1'2'}$= 4.63Hz, 1H) UH-1′; 5.78 (d, J = 7.81Hz, 1H) UH-5; 5.17 (m, 1H) GH-2′; 4.90-4.11 (m, 11H) sugar protons + tetrahydropyranyl-; 3.84-3.30 (m, 4H) tetrahydropyranyl-; 2.58 (m, 4H) $CH_3COCH_2CH_2CO$-; 2.40 (s, 3H) -COPhCH_3; 2.09 (s, 3H) $CH_3COCH_2CH_2CO$-; 1.50 (m, 12H) tetrahydropyranyl-; 1.31 (s, 9H) *t-butyl*PhCO-; ^{31}P-NMR($CDCl_3$): -7.54 ppm.

Compound 4: Compound **3** (1.73 g) in dry pyridine (13 ml) was treated with 0.2 M acetonitrile solution of *o*-chlorophenylphosphoro-bis-(1,2,4-triazolide) (8.25 ml, 1.65 mmol) for 40 min. Aqueous ammonium bicarbonate work up followed by silica gel column chromatography (2-7% ethanol in CH_2Cl_2) afforded **4** (1.49 g, 69%, calc.from **1**); R_f: 0.25 (C); ^{1}H-NMR ($CDCl_3$+CD_3OD): 8.17-6.84 (m, 22H) arom., GH-8 & UH-6; 6.23 (d, $J_{1'2'}$= 5.13Hz, 1H) GH-1′, 6.03 (d, $J_{1'2'}$= 5.26Hz, 1H) UH-1′; 5.79 (d, J = 8.54Hz, 1H) UH-5; 5.63 (m, 1H) UH-2′; 5.40 (m, 1H) GH-2′; 4.97 (m, 1H) UH-3′; 4.70-4.16 (m, 9H) sugar protons + tetrahydropyranyl-; 3.71-3.32 (m, 4H) tetrahydropyranyl-; 3.02 (q, 6H) CH_2 of triethylammonium; 2.58 (m, 4H) $CH_3COCH_2CH_2CO$-; 2.40 (s, 3H) -COPhCH_3; 2.06 (s, 3H) $CH_3COCH_2CH_2CO$-; 1.50 (m, 12H) tetrahydropyranyl-; 1.30 (s, 9H) *t-butyl*PhCO-; 1.25 (t, 9H) CH_3 of triethylammonium; ^{31}P-NMR ($CDCl_3$+CD_3OD): -7.91, -8.13 ppm.

Compound 6a The 3′-phosphodiester block **4** (1.57 g, 0.95 mmol) was condensed with 6-N-benzoyl-2′-O-pixyladenosine[10] **5** (555 mg, 0.89 mmol) in dry pyridine (8 ml) by addition of distilled N-methylimidazole (379 µl, 4.76 mmol) followed by addition of 1-mesitylenesulfonyl chloride (519 mg, 2.38 mmol). The reaction mixture was then stirred for 50 min at room temperature. Aqueous ammonium bicarbonate work up followed by silica gel column chromatography (using the silica gel pre-washed with 1% Et_3N - CH_2Cl_2 mixture followed by washing with pure CH_2Cl_2) with 0-3% ethanol/0.5% pyridine in CH_2Cl_2 afforded **6a** as a white powder after co-evaporation with toluene and cyclohexane (1.01g, 53%). R_f: 0.59 (C); ^{1}H-NMR ($CDCl_3$+lutidine): 9.18 (br, 1H) NH; 8.68, 8.60, 8.56 (3 x s, 1H) AH-8; 8.17-6.42 (m, 27H) arom., GH-8, AH-2 & UH-6; 6.21 (d, $J_{1'2'}$= 4.63Hz, 1H) GH-1′; 6.13-5.79 (m, 4H) AH-1′, UH-1′, UH-2′, & UH-5; 5.29 (m, 1H) GH-2′; 5.01 (m, 1H) UH-3′; 4.82 (m, 1H) AH-2′; 4.70-4.17 (m, 14H) sugar protons & tetrahydropyranyl-; 3.60-3.20 (m, 5H) tetrahydropyranyl- & AH-3′; 2.58 (m, 4H) $CH_3COCH_2CH_2CO$-; 2.40 (s, 3H) -COPhCH_3; 2.06 (s, 3H) $CH_3COCH_2CH_2CO$-; 1.50 (m, 12H) tetrahydropyranyl-; 1.27 (s, 9H) *t-butyl*PhCO-; ^{31}P-NMR ($CDCl_3$+lutidine): -7.52, -7.57, -8.18 ppm.

Compound 6b: Compound **6a** (1.01 g, 0.47 mmol) in dry pyridine (5 ml) was treated with 0.2 M acetonitrile solution of *o*-chlorophenylphosphoro-bis-(1,2,4-triazolide) (4.67 ml, 0.93 mmol) for 60 min. Aqueous ammonium bicarbonate work up followed by silica gel column chromatography (using the silica gel pre-washed with 1% Et_3N - CH_2Cl_2 mixture followed by washing with pure CH_2Cl_2) with 0-7% EtOH/1% pyridine in CH_2Cl_2 afforded **6b** as a white powder after co-evaporation with toluene and cyclohexane (1.0 g, 88%). R_f: 0.74 (D); ^{1}H-NMR ($CDCl_3$+CD_3OD+2,6-lutidine): 8.61, 8.54 (2 x s, 1H) AH-8; 8.20-6.47 (m, 45H) arom., GH-8, AH-2 & UH-6; 6.25-5.60 (m, 4H) AH-1′, UH-1′, UH-2′, & UH-5; 5.34 (m, 1H) GH-2′; 5.14-4.90

(m, 2H) AH-2′& UH-3′; 4.78-4.05 (m, 14H) sugar protons & tetrahydropyranyl-; 3.50 (m, 4H) tetrahydropyranyl-; 3.02 (q, 6H) CH_2 of triethylammonium; 2.58 (m, 4H) $CH_3COCH_2CH_2CO$-; 2.40 (s, 3H) -COPhCH_3; 2.06 (s, 3H) $CH_3COCH_2CH_2CO$-; 1.54 (m, 12H) tetrahydropyranyl-; 1.27 (s, 9H) *t-butyl*PhCO-; 1.25 (t, 9H) CH_3 of triethylammonium; ^{31}P-NMR ($CDCl_3$+CD_3OD+lutidine): -7.47, -7.66, -7.81, -7.96, 8.06 ppm.

Compound 6c : The 3′-phosphodiester block **6b** (1.0 g, 0.41 mmol) was dissolved in 2% EtOH-CH_2Cl_2 and chilled to 0 °C in an ice bath. Trichloroacetic acid (TCA) (668 mg, 4.1 mmol) was dissolved in the second half of the 2% EtOH-CH_2Cl_2 solution and chilled to 0 °C prior to addition into the above solution of **6b**. The final concentration of TCA was 0.055 M. After stirring for 20 min, the solution was poured into 0.2M ammonium bicarbonate solution which was saturated with ammonium chloride and acidified with dry ice and this aqueous phase (pH ~6.5) was extracted with dichloromethane (3 x 50 ml). The organic phase was dried in magnesium sulfate and filtered and evaporated. The residue was dissolved in a small amount of dichloromethane and pipetted into a diethylether-hexane solution (150 ml) (2:1 v/v). The precipitate was centrifuged, the supernatant was decanted and the white pixyl-free solid was dried *in vacuo* (891 mg, quantitative). R_f: 0.67 (D); ^{1}H-NMR ($CDCl_3$+CD_3OD): 8.72 (s, 1H) AH-8; 8.20-7.01 (m, 32H) arom., GH-8, AH-2 & UH-6; 6.25-6.06 (m, 3H) AH-1′, UH-1′, GH-1′; 5.89-5.67 (m, 2H) UH-5, UH-2′; 5.34 (m, 1H) GH-2′; 5.14-4.90 (m, 2H) AH-2′& UH-3′; 4.89-4.05 (m, 14H) sugar protons & tetrahydropyranyl-; 3.50 (m, 4H) tetrahydropyranyl-; 3.02 (q, 6H) CH_2 of triethylammonium; 2.58 (m, 4H) $CH_3COCH_2CH_2CO$-; 2.40 (s, 3H) -COPhCH_3; 2.07 (s, 3H) $CH_3COCH_2CH_2CO$-; 1.54 (m, 12H) tetrahydropyranyl-; 1.29 (s, 9H) *t-butyl*PhCO-; 1.25 (t, 9H) CH_3 of triethylammonium; ^{31}P-NMR ($CDCl_3$+CD_3OD): -7.59, -7.69, -7.84, -7.98 ppm.

Compound 6d. Bis(2-cyanoethoxy)-(diisopropylamino)phosphine[13] (1.0 g, 4.07 mmol) was weighed into a dry 100 ml round bottomed flask and dry 15% dimethylformamide / acetonitrile solution (14 ml) was added under argon (argon balloon). Then dry and sublimed tetrazole (855 mg, 12.2 mmol) was added under stirring, and it rapidly went into solution followed by a quick formation of a precipitate. After 3 min stirring, solid 2′-hydroxy-3′-phosphodiester block **6c** (891 mg, 0.41 mmol) was added to the colorless suspension and the clear reaction solution was then stirred for 40 min at room temperature under argon. A solution of 0.1M I_2 / tetrahydrofuran / pyridine / H_2O (7:2:1 v/v/v) (43 ml) was added and the reaction solution was stirred for 15 min, poured into 0.1M sodium thiosulfate / concentrated ammonium bicarbonate solution and extracted three times with dichloromethane. The pyridine-free gum obtained after toluene co-evaporation of the organic residue was then purified by short silica gel column chromatography (2-9% EtOH in CH_2Cl_2) to finally give the 3′-phosphodiester-2′- biscyanoethoxyphosphotriester block **6d** as a white powder after co-evaporation with toluene and cyclohexane (659 mg, 68%). R_f: 0.67 (D); ^{1}H-NMR ($CDCl_3$+CD_3OD): 8.69 (s, 1H) AH-8; 8.33-6.86 (m, 32H) arom., AH-2, GH-8 & UH-6; 6.35 (m, 1H) AH-1′; 6.26-5.00 (m, 7H) GH-1′, UH-1′, UH-5 & sugar protons; 4.68-4.15 (m, 18H) sugar protons, tetrahydropyranyl-& 2 x -OCH_2CH_2CN; 3.47 (m, 4H) tetrahydropyranyl-; 3.05 (q, 6H) CH_2 of triethylammonium; 2.87-2.51 (m, 8H) 2 x -OCH$_2CH_2$CN & $CH_3COCH_2CH_2CO$-; 2.40 (s, 3H) -COPhCH_3; 2.07 (s, 3H) $CH_3COCH_2CH_2CO$-; 1.46 (m, 12H) tetrahydropyranyl-; 1.29 (s, 9H) *t-butyl*PhCO-; 1.25 (t, 9H) CH_3 of triethylammonium; ^{31}P-NMR ($CDCl_3$+CD_3OD): -4.66, -7.66, -7.84, 8.06, -8.15 ppm.

Compound 7a. A mixture of **6d** (659 mg, 0.274 mmol) and 2′,3′-di-O-acetyl-4-N-benzoylcytidine (180 mg, 0.417 mmol) was co-evaporated with dry pyridine and redissolved in dry pyridine (2 ml). 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MSNT) (495 mg, 1.67 mmol) was added and the reaction mixture was stirred for 6 h at room temperature. Aqueous ammonium bicarbonate work up followed by silica gel column chromatography (2-5% EtOH in CH_2Cl_2) gave the fully protected oligomer **7a** as a white powder after co-evaporation with toluene and cyclohexane (560 mg, 75%); R_f: 0.51 (D); ^{1}H-NMR ($CDCl_3$): 9.11 (br, 1H) NH; 8.75, 8.74, 8.69 (3 x s, 1H) AH-8; 8.46-7.15 (m, 39H) arom., AH-2, GH-8, CH-6, CH-5 & UH-6; 6.39 (m, 1H) AH-1′; 6.24-5.19 (m, 10H) UH-5, anomeric & sugar protons; 4.79-4.01 (m, 21H) sugar protons, tetrahydropyranyl & 2 x -OCH_2CH_2CN 3.42 (m, 4H) tetrahydropyranyl-; 2.87-2.51 (m, 8H) 2 x -OCH$_2CH_2$CN & $CH_3COCH_2CH_2CO$-; 2.40 (s, 3H) -COPhCH_3; 2.11 (s, 3H) $CH_3COCH_2CH_2CO$-; 2.06 (s, 6H) 2 x -$COCH_3$; 1.43 (m, 12H) tetrahydropyranyl-; 1.29 (s, 9H) *t-butyl*PhCO-; ^{31}P-NMR ($CDCl_3$): -3.07, -3.37, -6.61, 7.32, -7.42, -7.59, -7.81, -7.91 ppm.

Compound 7b. 7a (268 mg, 0.1 mmol) was dissolved in dry pyridine (1 ml). Then 0.5 M hydrazine hydrate (1 mmol) in pyridine/acetic acid (3:2 v/v) (1 ml) was added, and after 5 min stirring at room temperature the reaction was quenched by addition of pentane-2,4-dione (103 µl, 1 mmol). The reaction mixture was then poured into concentrated ammonium bicarbonate solution and extracted with dichloromethane (3 x 50 ml). The pyridine-free gum obtained after evaporation and co-evaporation with toluene was purified by silica gel chromatography (3-5%EtOH in CH_2Cl_2) to give **7b** as a white powder after co-evaporation with toluene and cyclohexane (216 mg, 83%); R_f: 0.50 (C); ^{1}H-NMR ($CDCl_3$): 9.24 (br, 1H) NH; 8.75, 8.73 (2 x s, 1H) AH-8; 8.41-7.04 (m,

39H) arom., AH-2, GH-8, CH-6 CH-5 & UH-6; 6.41 (m, 1H) AH-1´; 6.15-5.00 (m, 10H) UH-5, anomeric & sugar protons; 4.81-3.95 (m, 21H) sugar protons, tetrahydropyranyl & 2 x -O*CH*$_2$CH$_2$CN; 3.50 (m, 4H) tetrahydropyranyl-; 2.67 (m, 4H) 2 x -OCH$_2$*CH*$_2$CN; 2.40 (s, 3H) -COPh*CH*$_3$; 2.07 (s, 6H) 2 x -COCH$_3$; 1.43 (m, 12H) tetrahydropyranyl-; 1.30 (s, 9H) *t-butyl*PhCO-; ^{31}P-NMR ($CDCl_3$): -3.07, -3.27, -3.34, -6.44, -6.62, -7.71,-7.74, -7.84, -7.91, -8.08 ppm.

Compound 7c. The 5´-hydroxy oligomer block **7b** (216 mg, 0.084 mmol) was co-evaporated with dry pyridine and redissolved in dry pyridine (850 µl). The solution was warmed to 40 °C in an oil bath and then dry diisopropylethylamine (363 µl, 2.08 mmol) was added and stirred for 90 min. Volatile matters were then evaporated, and the residue was co-evaportated with toluene. The organic residue was purified by short silica gel column chromatography using EtOH-CH_2Cl_2 gradients as mobile phases (4-12% EtOH in CH_2Cl_2) to give the 5´-hydroxy-2´-phosphodiester block **7c** as a white powder after co-evaporation with toluene and cyclohexane (165 mg ,74%). R_f: 0.35, 0.46 (C); ^{1}H-NMR ($CDCl_3$): 8.68 (s, 1H) AH-8; 8.42-6.97 (m, 39H) arom., AH-2, GH-8, CH-6 CH-5 & UH-6; 6.31 (m, 1H) AH-1´; 6.25-5.00 (m, 10H) UH-5, anomeric & sugar protons; 4.77-4.22 (m, 19H) sugar protons, tetrahydropyranyl & -O*CH*$_2$CH$_2$CN; 3.50 (m, 4H) tetrahydropyranyl-; 2.49 (br t, 2H) -OCH$_2$*CH*$_2$CN; 2.40 (s, 3H) -COPh*CH*$_3$; 2.10, 2.07 (2 x s, 6H) 2 x -COCH$_3$; 1.43 (m, 12H) tetrahydropyranyl-; 1.31 (s, 9H) *t-butyl*PhCO-; ^{31}P-NMR ($CDCl_3$): -3.15, -3.42, -7.59, -7.64,-7.69, -7.88, -8.08, -8.74, -8.86, -9.32, -9.49, -9.57 ppm.

Fully protected cyclic branched RNA tetramer 8. The 5´-hydroxy-2´-phosphodiester block **7c** (151 mg, 56.7 µmol) was co-evaporated with dry pyridine and redissolved in dry pyridine (14.2 ml, 4 mM). Then MSNT (151 mg, 510 µmol) was added and the reaction solution was stirred for 14 h at room temperature. Aqueous ammonium bicarbonate work up followed by silica gel column chromatography (2-5% EtOH in CH_2Cl_2) afforded the title compound **8**. Yield: 87 mg (61%); R_f: 0.61 (C); 9.33, 9.11 (2 x br, 1H) NH; 8.72, 8.67 (2 x s, 1H) AH-8; 8.59-7.03 (m, 39H) arom., AH-2, GH-8, CH-6 CH-5 & UH-6; 6.54-5.30 (m, 10H) UH-5, anomeric & sugar protons; 4.94-4.05 (m, 19H) sugar protons, tetrahydropyranyl & -O*CH*$_2$CH$_2$CN; 3.50 (m, 4H) tetrahydropyranyl-; 2.53 (m, 2H) -OCH$_2$*CH*$_2$CN; 2.40 (s, 3H) -COPh*CH*$_3$; 2.06 (s, 6H) 2 x -COCH$_3$; 1.43 (m, 12H) tetrahydropyranyl-; 1.27 (s, 9H) *t-butyl*PhCO-; ^{31}P-NMR ($CDCl_3$): -0.90 to -9.27 ppm (29 peaks).

Deprotection of 8 to 9: Fully protected lariat **8** (87 mg, 34.5 µmol) was dissolved in dioxane : water (8:2 v/v) solution (10 ml). *syn*-4-nitrobenzaldoxime (229 mg, 1.38 mmol) and 1,1,3,3-tetramethylguanidine (155 µl, 1.24 mmol) were added. After stirring for 26 h at room temperature the solvents were removed by evaporation *in vacuo* and concentrated ammonia (40 ml, d = 0.9) was added. The reaction mixture was stirred for 6 days at room temperature and was then evaporated and co-evaporated with distilled water. The residue was treated with 80% aq. acetic acid (30 ml) for 24 h at room temperature. After evaporation and co-evaporation with distilled water the residue was dissolved in distilled water (20 ml) and extracted with diethylether. **Purification of 9:** The aqueous phase was evaporated and the residue redissolved in 0.001 M ammonium bicarbonate (BDH Chemicals Ltd, Poole, England) buffer and applied to a DEAE-Sephadex column (2 x 25 cm, HCO_3^- form). The column was eluted with a linear gradient 0.001 - 0.5 M - 0.75 M ammonium bicarbonate solution (500ml/1000ml/500ml respectively, pH 7.5). The peak containing the product eluted with at *ca.* 0.6 M buffer concentration and contained a shoulder on the right side. The main peak (672 A_{260} o.d units of which ca. 70% represents the product) was collected separately from the shoulder peak (175 A_{260} o.d units of which ca. 10% represents the product) and the two parts were evaporated and co-evaporated with distilled water a few times to remove the salt and then lyophilized. 5-7 mg batches of the main peak material were dissolved each in 5% MeCN in 0.1M triethylammonium acetate (TEAA) at pH 7.0 (900-1000 µl) and were injected onto a semi-preparative Spherisorb S5ODS2 column (8 x 250 mm) equilibrated in 5% acetonitrile in 0.1M TEAA. Gradient elution with acetonitrile in 0.1M TEAA (0-35% solvent B (B = 50% acetonitrile in 0.1M TEAA) in 45 min, 1 ml/min) resolved the desired peak,which eluted first (Fig. 2a) with base-line separation (Detector was set at 254 nm). The purified material (Fig. 2b) was collected, evaporated and then lyophilized several times until the TEAA salt was removed (monitored by ^{1}H-NMR). The title compound was obtained as a sodium salt by elution through a Dowex column (1 x 20 cm, Na^+ form) with distilled water.The aqueous solution was pooled and evaporated, the residue was redissolved in a small amount of distilled water and lyophilized (432 A_{260} o.d. units, 30%). ^{31}P-NMR ($CDCl_3$): δ -1.532 (G3'**p**5'U), -1.235 (A3'**p**5'C),, -1.193 (A2'**p**5'G) and -0.116 (U3'**p**5'A).

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