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

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Abstract: *A new convergent synthetic procedure has been developed for preparation of lariat heptaribonucleotide* ***19****, modelling the lariat formed in Group II and nuclear pre-mRNA processing reaction (Splicing) The first three steps in this strategy involves the condensation of the appropriately protected 5′-O-levulinylated-cytidylyl(3′→5′)uridine-3′-phosphodiester* ***4*** *with the 3′, 5′-dihydroxy-6-N-(4-anisoyl)-2′-O-pixyl(9-phenylxanthen-9-yl)adenosine* ***14****, in presence of an activating agent, to give* ***15a*** *(49%) Chemospecific phosphorylation of 3′-OH of* ***15a*** *afforded the intermediate* ***15b*** *(92%) which was treated with mild acid to achieve a regiospecific removal of the 2′-O-pixyl group to give* ***15c*** *(91%) The fourth step is the introduction of the (2-cyanoethyl)-(2-(4-nitrophenyl)ethyl)phosphotriester moiety to the 2′-OH of the branch-point adenosine in* ***15c*** *in a single step, by using (2-cyanoethoxy)-(2-(4-nitrophenyl)ethoxy)-(diisopropylamino)phosphine, to give the crucial branch-point building block* ***15d*** *(58%), with two dissimilar vicinal phosphates at 2′- and 3′- of the branch-point* ***15d*** *was then condensed with the appropriately protected 5′-hydroxy-uridylyl(3′→5′)-(2′,3′-di-O-acetylcytidine)* ***11*** *to afford the fully protected intermediate* ***16a*** *(57%) Regiospecific deblocking of 2-cyanoethyl group from* ***16a*** *afforded the 2′-(2-(4-nitrophenyl)ethylphosphodiester* ***16b*** *(94%), which was condensed with the dimeric 5′-hydroxy-guanylyl(3′→ 5′)uridine-3′phosphotriester* ***13*** *to afford the fully protected* ***17a*** *(59%) The 5′-O-levulinyl and the 2-cyanoethyl groups were regiospecifically removed from* ***17a*** *successively to afford first* ***17b*** *(88%) and then the 5′-hydroxy-3′-phosphodiester block* ***17c*** *(68%)* ***17c*** *was allowed to undergo intramolecular phosphorylation, in presence of an activating agent, under a condition of high dilution to afford the fully protected lariat-RNA* ***18*** *(66%) which was then deprotected in four steps and purified to give the fully deprotected lariat-RNA* ***19*** *(29%) Detailed 500 MHz ^{1}H-NMR and 202 4 MHz ^{31}P-NMR studies, using Clean-TOCSY, DQF-COSY, NOESY & ^{31}P-^{1}H-NMR shift correlation techniques, have unequivocally established the purity and the structural integrity of lariat* ***19***

In the last few years, we have developed methodologies for the synthesis of branched RNAs 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] modelling the lariat intron formed at the penultimate step of Group II and nuclear pre-mRNA processing reaction (Splicing) in Eukaryotes (Scheme 1) We then subjected these synthetic branched RNAs to high-field NMR studies (500 and 600 MHz ^{1}H) to establish (1) why the conservation of nucleotide sequences is necessary at the Splice site of RNA intron [adenosine (A) as the branch-point nucleotide, guanosine (G) as the 2′→5′-linked nucleotide, and uridine (U) or cytidine (C) as the 3′→5′-linked nucleotide] for appropriate ligation of exons, and (2) what are their structural significance in the formation of lariat-RNA intron which is

shown to be an essential requirement for ubiquitous Splicing reaction. The comparative high-field NMR spectroscopic studies have established that solution conformation of these open-chained branched RNA models show distinct conformational changes upon addition of nucleotide units in any of the three 2'- , 3'- or 5'-terminals of 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] The solution structures of the branched nonamer and branched decamer has been shown to form a third category, distinct from the other shorter models[1t]

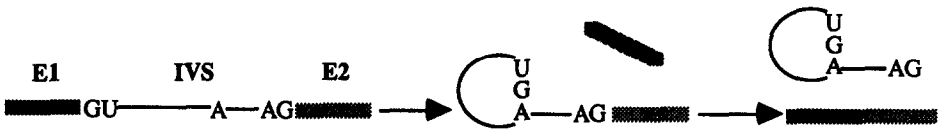


Figure 1. The two steps in Group II and Nuclear pre-mRNA Splicing (Step I) Substrate (E1-IVS-E2), A = Branch-point Nucleotide, the 2'-OH of the branch-point adenosine (A) attacks the 5'-phosphate of G residue connecting the E1 exon giving the Lariat intron which is still covalently linked to the exon E2 (Step II) Upon formation of the Lariat intron, 3'-OH of E1 attacks the 5'-phosphate of E2 giving the ligated exons (E1 + E2) as products. In Group II Splicing, no external source of energy such as ATP or GTP or a complexation with protein is required, only cofactor required is Mg^{2+} ion Nuclear mRNA splicing reaction requires both ATP and Mg^{2+} ion and it takes place within the spliceosome consisting of 50S to 60S complex of the pre-mRNA, four small ribonucleoprotein particles and as yet unknown number of associated protein factors

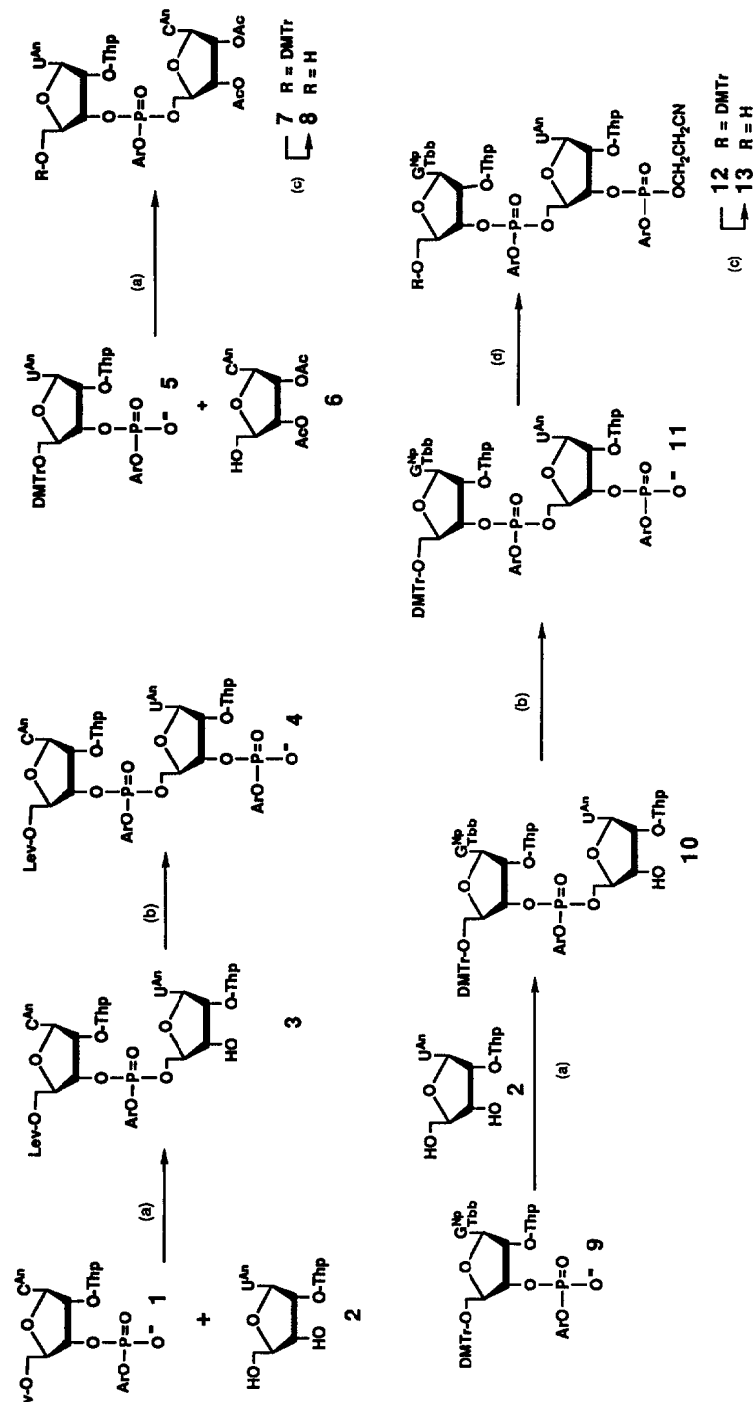
It has became clear from all these conformational studies[1a-u] that the open-chained branched RNA models adopt a rather loose and non-rigid conformation in solution It is likely that these open-chain branch-RNAs only partly mimic the natural lariat-RNAs because of lack of conformational constrain, ribonucleotide chain folding abilities and the restricted conformational space that are expected to be present in the real lariat-RNA structures Earlier, we have reported the synthesis of the smallest possible lariat-RNAs[1u,2], $C\overset{A}{\underset{G}{(U}}$ and $CU\overset{A}{\underset{G}{(U}}$ which have shown very rigid conformations, and clearly, they do not mimic the conformation of the natural counterpart. A more realistic synthetic lariat-RNA model that resemble the natural counterpart is the heptamer, $C^5U^4\text{-}A^1\overset{U^2C^6}{\underset{G^3U^7}{)}}$ **(19)** , which is 3′→5′ phosphodiester linked from the branch-point A to $U^4(3'\rightarrow5')C^5$ forming the "3′-tail", 5′→3′ phosphodiester linked to 3' of $U^2(5'\rightarrow3')C^6(5'\rightarrow3')U^7(5'\rightarrow3')G^3$ which is 5′→2′ phosphodiester linked to the branch-point A^1, giving a lariat-RNA Studies of the solution structures of a series of synthetic lariat RNAs of different loop sizes synthesised in this laboratory (tri-, tetra- and penta-nucleotidic loop containing lariat-RNA)[1u,v] have already started to reveal interesting effects of the size of the lariat-loop on the conformation of the constituent sugar and phosphate backbone compared to the open-chained counterparts These studies[1v] also show how the conformational strain change upon enlarging the loop-size and how they affect the Mg^{2+} binding properties Additionally, some constrained lariat-RNA loop show some interesting effects[1v] such as conformational isomerism and conformation dependent self-cleaving reactions as seen in self-cleaving RNA hammerhead loops [3a-f]

One of the most important considerations in the design and synthesis of lariat-RNAs is the careful choice of 5'- and phosphate protecting groups and the strategy for intramolecular phosphorylation leading to the lariat-loop For the synthesis of lariat-RNAs[1u,2], $U\overset{A}{\underset{G}{(U}}$ and $CU\overset{A}{\underset{G}{(U}}$, we chose to use 5'-levulinyl group protected

terminal guanosine and create the 2'-bis(cyanoethyl)phosphotriester function at the branch-point The final cyclization was carried out between the 5′-hydroxyl of the 5′-terminal guanosine moiety of the 5′→3′-branch and the branch point 2′-phosphodiester function, forming the trinucleotidic lariat-2′→5′-loop in  and  Note that this strategy allows ring expansion only through elongation in the 5′→3′-direction from the branch point nucleotide prior to the cyclization and is practical only for the synthesis of tri- and tetra-nucleotidic lariat-RNAs[1u,2] Since our ultimate goal is to expand the size of the loop of lariat-RNA such that it conformationally mimics the natural counterpart, we decided to develop a more efficient general synthetic strategy that would lead us to the synthesis of larger lariat-RNAs Clearly, this should be possible if the stategy allows chain elongation both in the 5′→3′ and 2′→5′-directions from the branch-point A We herein report the synthesis of the lariat-RNA heptamer **19** that mimics the sequence of the branch-site of the Group II intron bl 1 of yeast mitocondria[4]

Most of the procedures for the synthesis of the cyclic DNAs and RNAs, mainly because of their putative role in biological processes[5-8], have been based upon the phosphotriester approach[9] employing the intramolecular cyclization reaction of 5′-hydroxy function of an oligonucleotide to its 3′-phosphodiester function using an activating agent ($ArSO_2X$ type) under high dilution condition The cyclization reaction has also been carried out in a one-pot fashion by generation of the 5'-hydroxy-3'-phosphodiester intermediate *in situ* from an oligonucleotidic 3′, 5′-dihydroxy intermediate and aryl-bis-O,O-(1-benzotriazolyl)phosphate[10] The high dilution condition used for the intramolecular "tail biting" reaction (4-5 mM of the oligomer intermediate) is important in order to minimize any *intermolecular* polymerization The "*in situ* method" was used by Bonora *et al* [10] for preparation of a number of cyclic oligodeoxyadenylic acids In line with the "hydroxy phosphodiester method", Hsu *et al* [11] synthesized three cyclic diribonucleosidediphosphates r(ApAp), r(ApUp) and r(UpUp) Van Boom *et al* synthesized r(GpGp)[8] and later his group prepared two cyclic tetra-RNAs, two cyclic hexa-DNAs and two cyclic octa-DNAs[12] Reese *et al* [13] synthesized cyclic oligothymidylic acids together with a cyclic hexa-DNA of a mixed sequence Also by the 5'-hydroxy-3'-phosphodiester method, a solid phase synthesis was developed by Barbato *et al* [14,15] which produced up to cyclic hepta-oligodeoxycytidylic acids Khorana *et al*[16] isolated cyclic oligomers and especially cyclic dithymidylic acid as by-products in the course of their work on synthesis of oligothymidylic acids using his phosphodiester approach[17]

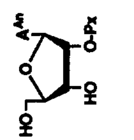
In our new strategy for the lariat-RNA synthesis, the fully protected pentameric intermediate **16a** is the key intermediate that can be chain extended in a flexible manner in the 2′→5′-direction from the branch-point A, by first converting it to 2′-phosphodiester (as in **16b**) and then can be coupled with various 5′-hydroxy nucleotide blocks [a dimer (**13**) or a trimer or an oligomer] The strategy was designed such that it allowed the use of the compatible and versatile combination of 5′-O-levulinyl- and 2-cyanoethylphosphotriester protecting group [1h,1u,2,18,19] in the assembly of **18** from **16a (16a →16b →17a→ 17b →17c →18)** Diastereomerically pure higher R_f 2'-O-tetrahydropyranyl (Thp) nucleoside blocks were used through out the synthesis of the lariat-RNA **19** The 4-anisoyl group was used for nucleobase protection, in order to increase the resistance of the protected intermediates against hydrazine[20] and tertiary amines required for the deprotection of 5'-levulinyl and cyanoethyl groups in the intermediates (vide infra) The 3′-phosphodiester block **4**, the dimeric 5′-hydroxy blocks **8** and **13** were synthesized according to standard phosphotriester protocols[9,21] The branch-point building block **15a** was prepared by the regioselective condensation of appropriate dimer-phosphodiester block



(a) MsCl (2.5 equiv), N-MeIm (5 equiv), pyridine, 45 min RT. (b) 2-chlorophenylphosphoro-bis-(1,2,4-triazolide) (1.5 equiv), pyridine / MeCN, 40 min. (c) CCl_3CO_2H (12 equiv), MeOH / CH_2Cl_2, 60 min, 0°C, RT. (d) $HOCH_2CH_2CN$ (1.5 equiv), MSNT (3 equiv), pyridine, 45 min RT.

Abbreviations. Ar = 2-Chlorophenyl Px = 9-phenylxanthen-9-yl, DMtr = 4,4'-dimethoxytrityl, Lev = levulinyl, Thp = tetrahydropyranyl Tbb = 4(t-butylbenzoyl) Np = 2-nitrophenyl, An = 4-ansoyl A = 9-adenanyl, C= 1-cytosunyl G = 9-guanunyl, U = 1-uracilyl G^{Np}_{Tbb} = N^2-((t-butylbenzoyl)-O^6-(2-nitrophenyl) 9-guanunyl, C^{An} = N^4-(4-ansoyl)-1-cytosunyl, U^{An} = N^3-(4 ansoyl)-1-uracilyl, A^{An} = N^6-(4-ansoyl)-9-adenunyl.

4 with 6-N-(4-anisoyl)-2′-O-pixyladenosine **14**[22] using 1-mesitylenesulfonyl chloride (MsCl) / N-methylimidazole (MeIm)[21] to give the key 3′-hydroxy block **15a** (49%, $\delta^{31}P$ = -7 06, -7 54, -7.23, -7 84, -7 93, -8 06,) Trimer **15a** was subjected to 3'-phosphorylation with 2-chlorophenylphosphoro-bis(1,2,4-triazolide) in the usual manner[23] to give the corresponding 3′-phosphodiester block **15b** (92%, $\delta^{31}P$ = -5 8 to -9 7). The 2'-O-depixylation of **15b** was carried out using trichloroacetic acid in 2% CH_3OH-CH_2Cl_2 at 0 °C[24] to give the 3′-phosphodiester-2′-hydroxy block **15c** (91%, $\delta^{31}P$ = -7 0 to -9 1). The choice of introducing a suitably protected 2'-phosphotriester function vicinal to the 3'-phosphodiester at the branch-point A in **15c** was based on the following considerations (1) After chain elongation in the 3′→5′ direction from the branch-point A, it should be possible to persue the chain elongation at the 2′→5′ direction with a 5'-hydroxy-3'-terminal phosphotriester block (such as **13**), which requires that at a specific stage of the synthetic transformations, one should be able to convert the 2'-phosphotriester function at the branch-point to the 2'-phosphodiester in a chemospecific manner (2) This means that the 2′→5′ internucleotidyl phosphate protecting group at the branch-point, generated upon chain elongation at the 2′→5′ direction, should be compatible with the removal condition of the 2-cyanoethyl group from the 3′-phosphotriester end of the 2′→5′ linked RNA tail Simple model reactions showed that neither the 2-chlorophenyl- nor 1,1,1,3,3,3-hexafluoro-2-propyl- group[25-28] can be easily introduced as a part of the 2′-phosphotriester function at the branch-point of **15c** through its reactions with the poorly reactive aryl-(2-cyanoethyl)-phosphoroamidites[25,26] or (1,1,1,3,3,3-hexafluoro-2-propyl)-(2-cyanoethyl)-phosphoro amidites[27] This is further aggravated by the relatively poor reactivity of 2'-OH group vicinal to the 3′-phosphodiester function at the branch-point A of **15c** The (2,2,2-trifluoroethyl)-(2-cyanoethyl)phosphate group was on the other hand easily introduced at 2'- of the branch-point in **15c** through a reaction with (2,2,2-trifluorethyl)-(2-cyanoethyl)-phosphoroamidite, but 2,2,2-trifluoroethyl group[29] turned out to be too stable under the conditions normally employed for the final removal of 2-chlorophenyl groups from arylphosphates[30,31] Although both are β-eliminating groups, the 4-nitrophenylethyl[32-37] has been shown to be quite compatible with the 2-cyanoethyl group[32], due to the differences in the acidities of the β-protons 2-cyanoethyl group is efficiently removable upon treatment of triethylamine (pK_a of the Et_3NH^+ ~11 0), while the 4-nitrophenylethyl group is completely stable under this condition, it is however removable by stronger organic nitrogen bases such as 1,8-diazabicyclo[5.4 0]undec-7-ene (DBU) (pK_a of $DBUH^+$ ~14) The 2-cyanoethyl-(2-(4-nitrophenyl)ethyl)phosphotriester function in **15d** was easily introduced by the reaction of **15c** with highly reactive (2-cyanoethoxy)-(2-(4-nitrophenyl)ethoxy)-(diisopropylamino)phosphine and tetrazole[38,39] in dimethylformamide-acetonitrile at room temperature followed by aqueous iodine oxidation to introduce vicinal phosphates the 3′-phosphodiester-2′-(2-cyanoethyl)-(2-(4-nitrophenyl) ethyl)phosphotriester block **15d** (58%, $\delta^{31}P$ = -4 7, [-7 0 to -9 5], ^{31}P NMR panel (a) in Fig 2, Ce/Npe-P ClPh-P = 1 3) The phosphine reagent was synthesized by reacting 2-cyanoethoxy-(bis-(diisopropylamino)) phosphine with 2-(4-nitrophenyl)ethanol (86%, δ ^{31}P = +147 8, +147.7) using a procedure reported for (bis-(2-cyanoethoxy))-(diisopropylamino)phosphine[39] Note that the vicinal phosphates at the branch-point in **15d** are differently protected in order to orchestrate different chemical reactivities toward alcohol (vide infra) the 3'-phosphate is a diester, protected partially with a 2-chlorophenyl group, and can easily react with an alcohol under an appropriate condition, while under the same condition the 2'-phosphate is a completely inert phosphotriester protected with two β-eliminating groups (2-cyanoethyl- and 4-nitrophenyl) Two distinct groups of phosphorous resonances in ^{31}P-NMR can be observed[1h,2] in the spectrum of each of the (2-cyanoethyl)-(2-(4-nitrophenyl)ethyl)phosphate or (2-(4-nitrophenyl)ethyl)phosphate containing intermediates (*i e* **15d, 16a, b,**



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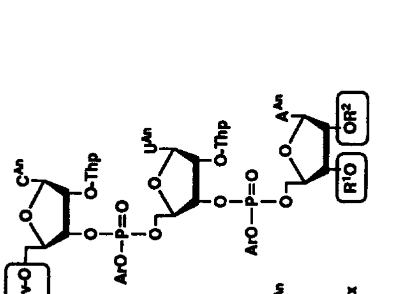
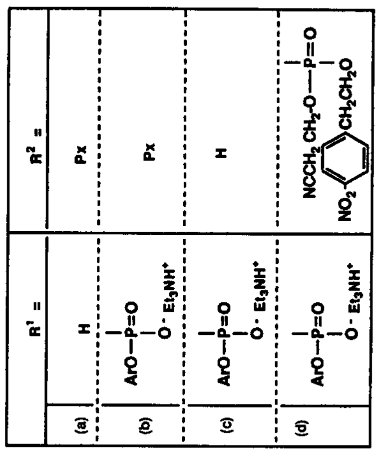
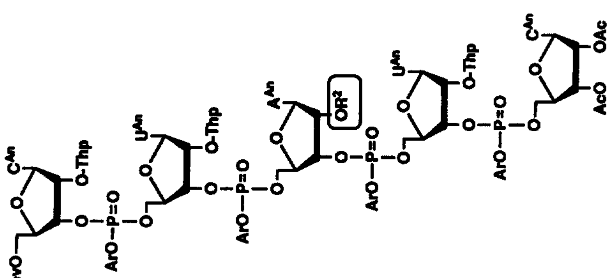
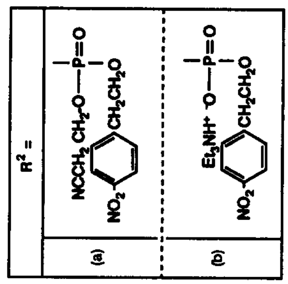


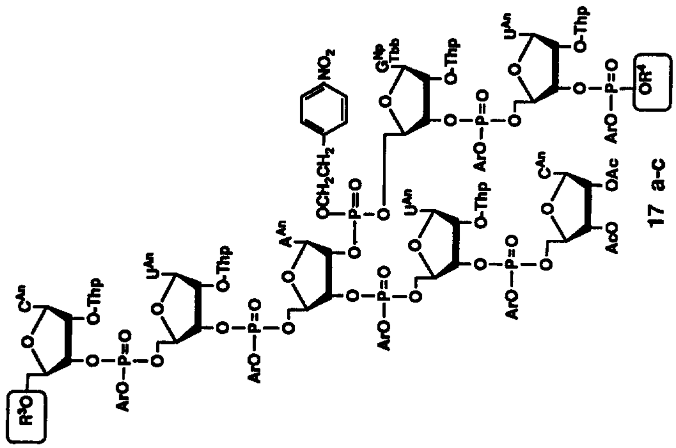
15a-d





16a, b





17 a-c

	R^3 =	R^4 =
(a)	$CH_3COCH_2CH_2CO$	CH_2CH_2CN
(b)	H	CH_2CH_2CN
(c)	H	$^+NHEt_3$

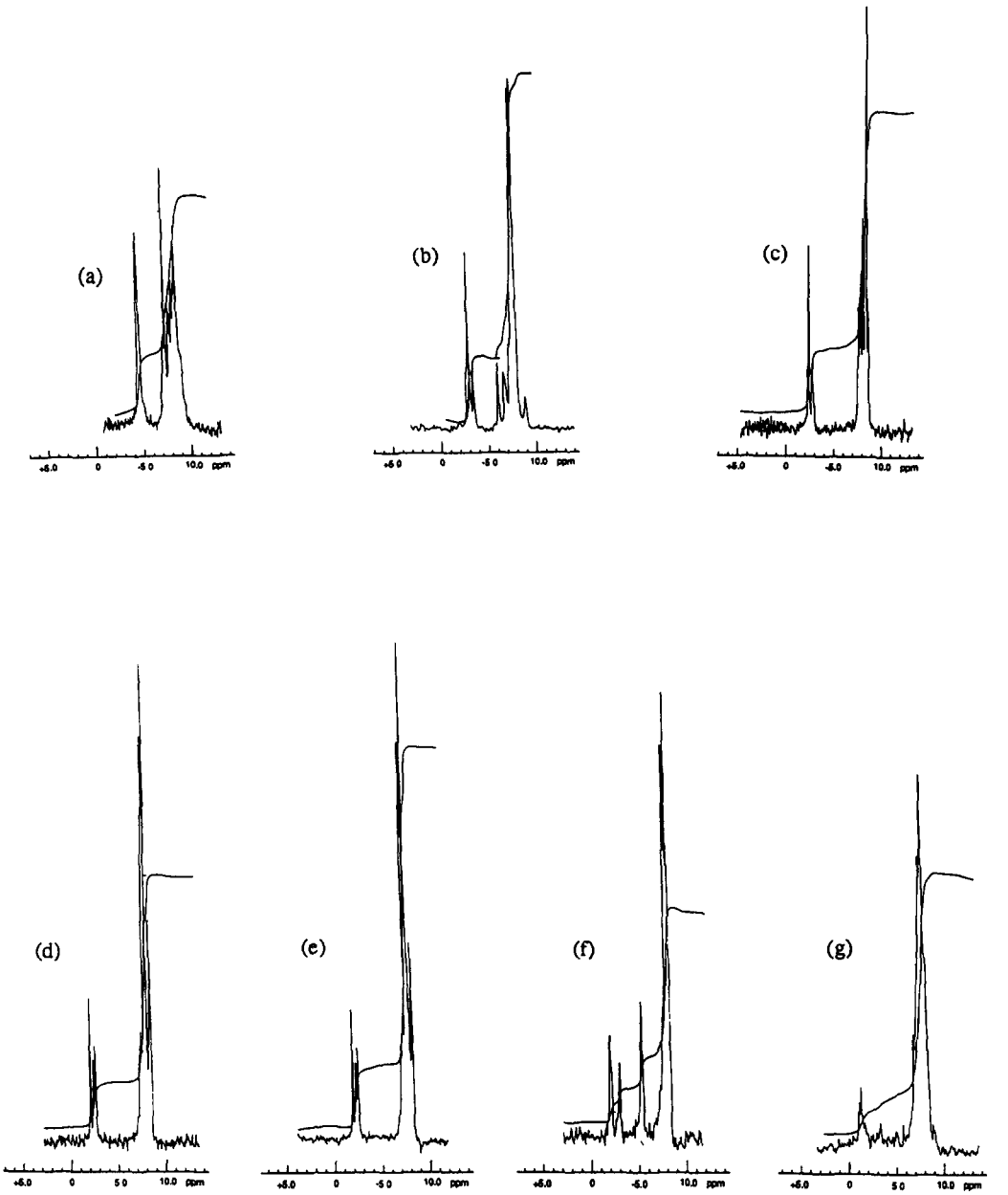
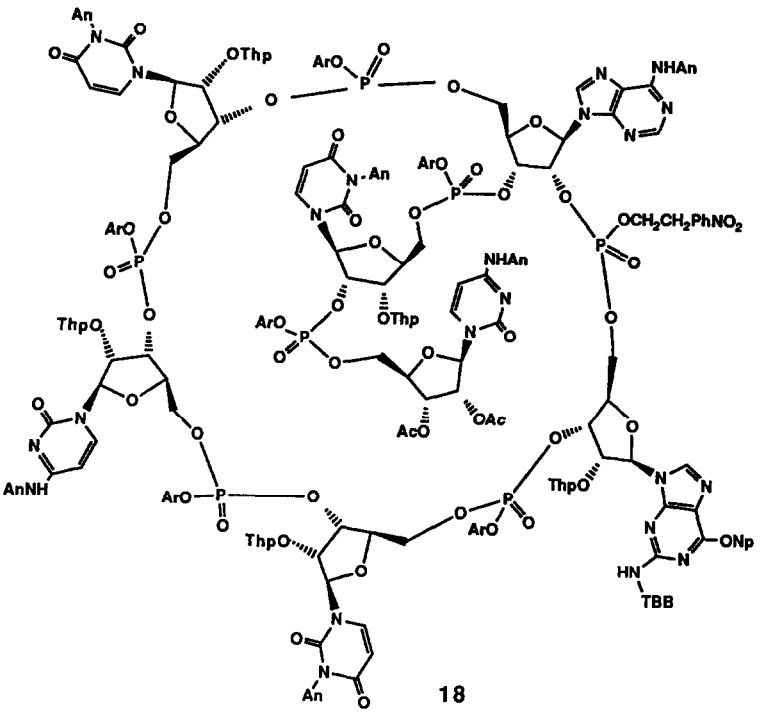


Fig. 2: ^{31}P-NMR spectra [36 MHz] of **15d** (Panel a) in $CDCl_3$+CD_3OD, **16a** (Panel b) in $CDCl_3$+CD_3OD, **16b** (Panel c) in $CDCl_3$+CD_3OD, **17a** (Panel d) in $CDCl_3$+CD_3OD, **17b** (Panel: e) in $CDCl_3$+CD_3OD, **17c** (Panel f) in $CDCl_3$+CD_3OD and **18** (Panel g) in $CDCl_3$+CD_3OD at 22 °C


An
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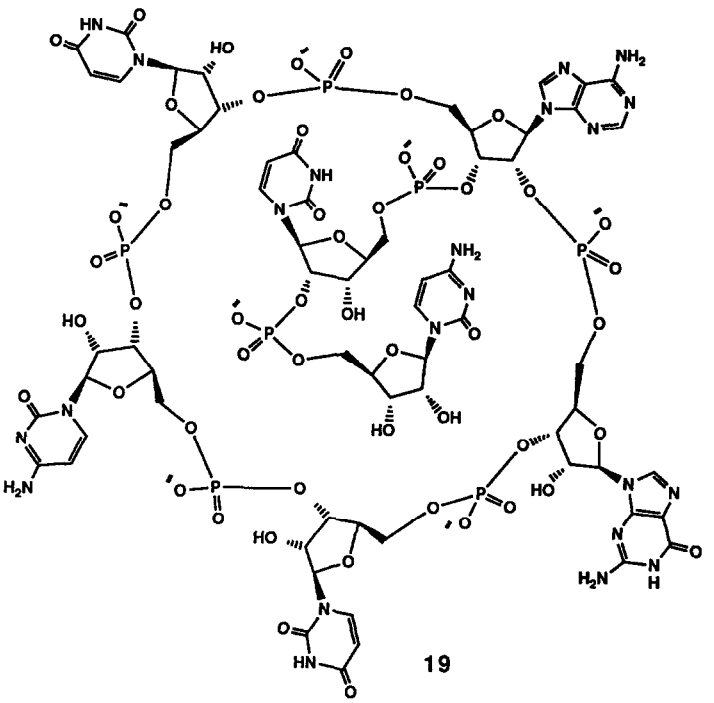
ArO
NHAn
N-An
OCH2CH2PhNO2
ThpO
AnNH
AcO
OAc
ONp
TBB

18


HO
NH2
OH
HN
H2N

19

17a-c, and **18**, vide infra) Simple integration of these two groups of phosphate resonances reveals the outcome of each reaction step[1h,2] [see Panels (a) - (g) in Fig. 2] In the next step, a 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MSNT)[30] condensation reaction was carried out between compounds **15d** and the dimeric 5′-hydroxy-uridylyl (3′→5′)-(2′, 3′-di-O-acetylcytidine) **8** to yield the fully protected pentamer **16a** [57 %, (δ^{31}P-NMR, Panel (b) in Fig. 2, Ce/Npe-P ClPh-P = 1 4)] **16a** was then treated with excess triethylamine in pyridine for 4.5 h at ~20 °C to give the decyanoethylated 2′-(2-(4-nitrophenyl)ethyl)phosphodiester block **16b** [94 %, (δ^{31}P-NMR, Panel (c) in Fig. 2, Npe-P · ClPh-P = 1· 4)]. At this point, pentamer **16b** was extended in the 2′→5′ -direction by a condensation with MSNT for 6 h at ~20 °C using the dimeric 5′-hydroxy-*guaninylyl*(3′→5′)*uridine*-3′-(2-cyanoethyl)-(2-chlorophenyl)phosphotriester **15** to afford **17a** [59 %, (δ^{31}P-NMR, Panel (d) in Fig. 2, Ce/Npe-P : ClPh-P = 1 6)] The 5′-O-levulinyl protecting group was removed from **17a** by treatment with hydrazine hydrate in pyridine/acetic acid (3 2 v/v) for 5 min at ~20 °C to give **17b** [88 %, (δ^{31}P-NMR, Panel (e) in Fig 2, Ce/Npe-P ClPh-P = 1 6)] **17b** was then treated with excess of triethylamine in pyridine for 3 h at ~20 °C to give the decyanoethylated (2′→5′)-(2-(4-nitrophenyl)ethyl)phosphotriester-3′-(2-chlorophenyl)phosphodiester **17c** [68 %, (δ^{31}P-NMR, Panel (b) in Fig 2, Npe-P ClPh-P-diester ClPh-P-triester = 1. 1 5)]. **17c** was then subjected to the intramolecular phosphorylation reaction in pyridine solution (4 mM) in presence of of MSNT (14 equiv, 18 h) at ~20 °C to give the fully protected heptameric lariat-RNA **18** [68 %, (δ^{31}P-NMR, Panel (g) in Fig. 2, Npe-P · ClPh-P = 1· 6)] Fully protected lariat-RNA **18** was then deprotected in four steps *(i)* DBU in pyridine for 5 h at ~20 °C to remove the 2-(4-nitrophenyl)ethyl group from the 2′→5′-phosphotriester, *(ii)* TMG-aldoximate[30,31] in dioxane water for 45 h at ~20 °C to remove the 2-chlorophenyl groups from the phosphates and the O^6-(2-nitrophenyl) group of the guanine, *(iii)* concentrated ammonia for 7 days at ~20 °C to remove the acyl groups and *(iv)* 80% aqueous acetic acid for 24 h at ~20 °C to remove the 2′-O-Thp groups The crude product (see Hplc profile in Fig 3a) was purified and isolated by DEAE-Sephadex A-25 column chromatography followed by semi-preparative RP-HPLC (see the analytical Hplc profile in Fig 3b) and Dowex ion-exchange column chromatography (see experimental section for details) to finally give pure Na^+ salts of cyclic branched heptamer **19** in 29% (432 A_{260} o d units) isolated yield A solution of **19** (~1 0 A_{260} o d unit) was digested with Snake venom phosphodiesterase in tris-hydrochloride buffer (pH 8 0) at 37 °C for 48 h followed by digestion with alkaline phosphatase gave the mixture of all constituent adenosine, guanosine, uridine and cytidine in a correct ratio as quantitated by Hplc (see experimental)

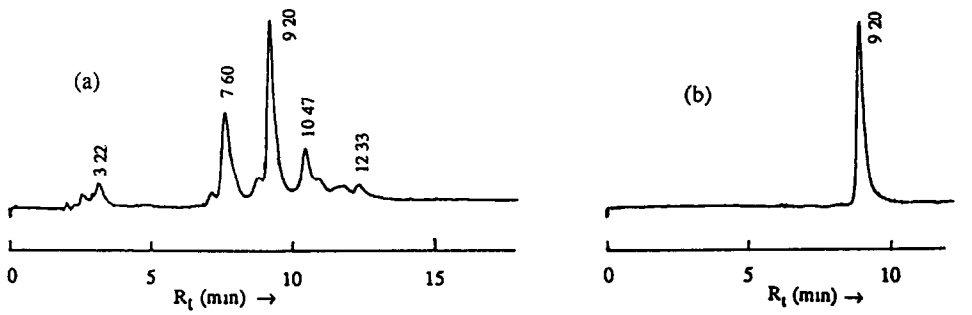


Fig. 3: (a) HPLC-chromatogram (Nucleosil C18, 5μ) of the crude deprotection mixture, eluted with 0 1Mtriethylammonium acetate (TEAA)/ acetonitrile (MeCN) (0 40% B in 30 min, 1ml/min; A = 5% MeCN in 0 1M TEAA, pH 7 0, B = 50% MeCN in 0 1M TEAA, pH 7 0) The desired product **19** eluted at R_t = 9.20 min, (b) HPLC- chromatogram (on the same column using the same gradient) of **19** as its triethylammonium salt after purification on DEAE-Sephadex and semi-preparative RP-HPLC

Characterization of Lariat-RNA, $C^5U^4\langle A^1 \langle U^2C^6 \rangle G^3U^7\rangle$ (19), by ^{1}H-NMR & ^{31}P-NMR Spectroscopy.

All sugar protons, non-exchangeable nucleobase protons and phosphorus resonances were assigned (Table 1) using several 1D and 2D experiments, such as clean-TOCSY[40], DQF-COSY[41], NOESY[42] and ^{1}H-^{31}P inverse correlation[43] (^{1}H at 500 MHz) This full assignment of NMR resonances in Table 1 itself suggest the presence of seven sugar residues, seven nucleobase moieties and seven phosphodiester linkages. From 1D experiments (Figs 4A-C), it was possible to assign the H6 and H5 protons belonging to either uridine ($^3J_{H6H5}$ = 8 1 Hz) or cytidine ($^3J_{H6H5}$ = 7.6 Hz) residues. The TOCSY spectra (Fig. 5) clearly showed seven oscillatory relays arising from H1' protons of seven constituent pentose sugar residues It was then possible to connect the aromatic protons of each nucleobase with its respective sugar residue from the NOESY spectra (Fig 6). Thus, from both H8A^1 and H8G^3 it was possible to observe crosspeaks to their respective H1' proton The pyrimidine residues could be also assigned by the crosspeaks between their respective H6 and H2' or H3' protons Fig 4D shows the 1D ^{31}P-NMR (202 45 MHz) spectrum in which all phosphate resonances connecting the H-3' of the first sugar residue and H5'/5" of the second pentose sugar residues (H-3'-O-P-O-5'/5"-H) are shown This unequivocal assignment of phosphate resonances has been possible using the ^{1}H-^{31}P correlated spectra (Fig 7). The cyclic nature of the lariat-RNA loop in **18** is clearly evident from the fact that the key intramolecular phosphorylation reaction of the 5'-terminal hydroxyl group of C^6 residue to the 2´→5´-linked-3'-terminal phosphodiester of U^7 in **17c**, should produce a new type of U^7(3'p5')C^6 phosphodiester moiety which should be clearly observable in ^{31}P-NMR of the deprotected lariat-RNA **19** This has indeed been observed at δ 0 90, which in ^{1}H-^{31}P inverse correlation spectroscopy has been found to correlate with the H3' of U^7 (at δ 4 46) and the H5' /5" of C^6 (at δ 4 05 and 3 96) as would be expected from the the loop structure of **19**, thus providing the straightforward spectroscopic evidence that the lariat-loop had indeed been formed (see the legend of Fig 7 for a list of all other ^{1}H-^{31}P correlations observed)

Table 1 : ^{1}H-NMR (500 MHz) assignments (reference δ (H_2O) = 4 7 ppm) of lariat heptamer (**19**) based on 1D and 2D NMR experiments such as Clean TOCSY, NOESY, and DQF-COSY

Residue	H1'	H2'	H3'	H4'	H5'	H5"	H5	H6	H8	H2
A^1	5 89	5 10	4 87	4 26	4 08	3 74	-	-	7 94	7 73
U^2	5 41	3 98	4 33	3 98	3 73	3 73	5 57	7 42	-	-
G^3	5 50	4 70	4 52	4 29	4 01	4 01	-	-	7 62	
U^4	5 58	4 15	4 34	4 23	4 15	3 97	5 57	7 57	-	-
C^5	5 66	3 99	4 07	4 06	3 99	3.88	5 81	7 65	-	-
C^6	5 75	4 16	4 43	4 20	4 05	3 97	5 78	7 64	-	-
U^7	5 84	4 22	4 46	4 31	4 22	4 02	5 71	7 71	-	-

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 or 202 MHz in the same solvent using 85 % phosphoric acid or cAMP as external standard Thin layer chromatography was carried out using pre-coated Merck silica gel F_{254} TLC or HPTLC plates in the following -methanol mixtures· (A) 90 10 (v/v), (B) 80·20 (v/v) Dry pyridine was obtained by distillations

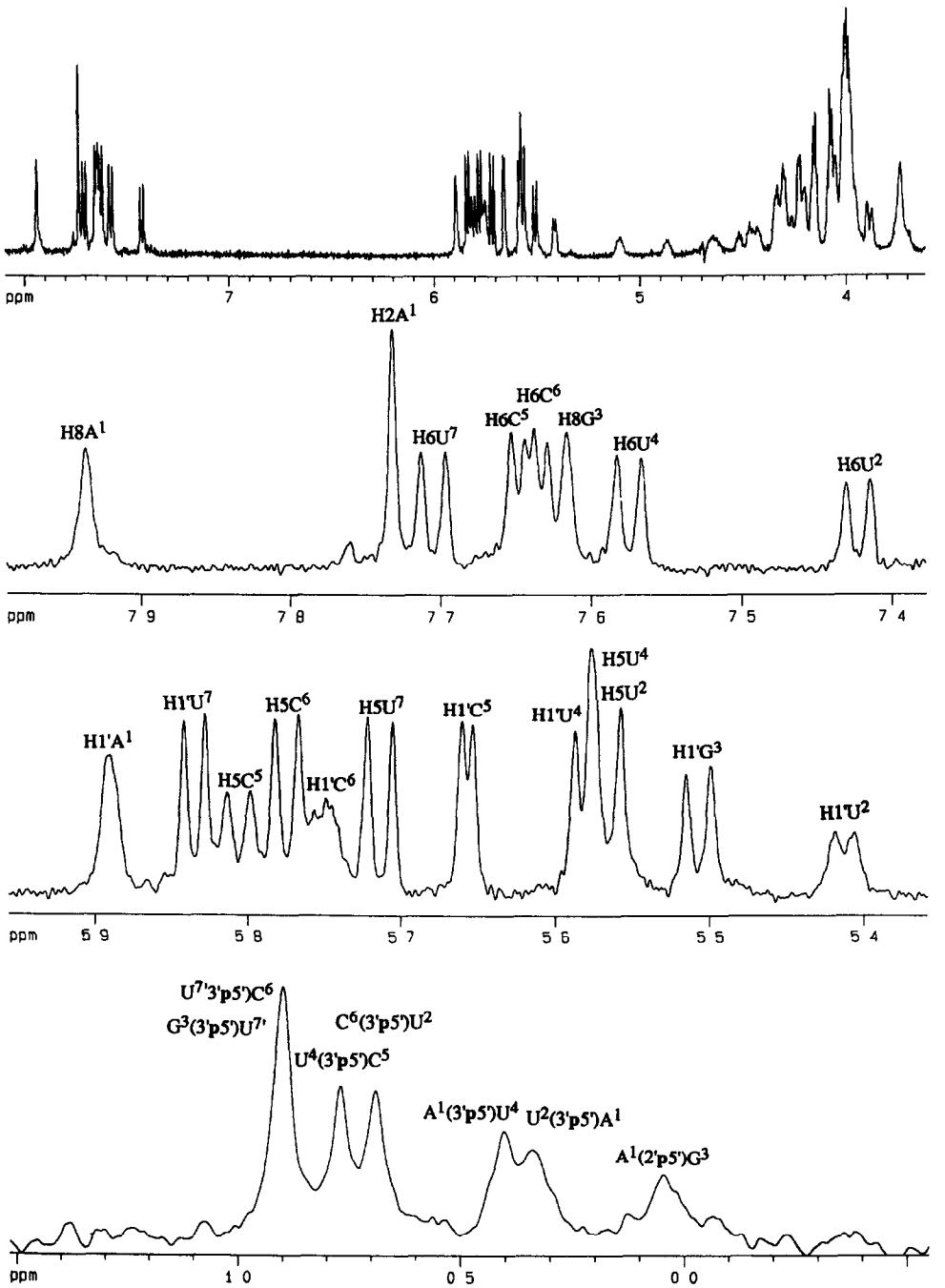


Figure 4. (A) 500 MHz ^{1}H-NMR spectrum of lariat heptamer (**19**) (1 7 mM) recorded at 284 K, (B) Expansion of aromatic region (δ 8 0 - 7 425 ppm) showing full assignments, (C) Expansion of anomeric region (δ 5 95 - 5 425 ppm) showing full assignments, (D) 1D ^{31}P-NMR (202 45 MHz) NMR spectrum of lariat heptamer (**19**) δ 0 90 [G^{3}(3'**p**5')U^{7}], δ 0 90 [U^{7}(3'**p**5')C^{6}], δ 0 77 [U^{4}(3'**p**5')C^{5}], δ 0 69 [C^{6}(3'**p**5')U^{2}], δ 0 40 [A^{1}(3'**p**5')^{4}], δ 0 34 [U^{2}(3'**p**5')A^{1}], δ 0 05 [A^{1}(2'**p**5'G)^{3}]

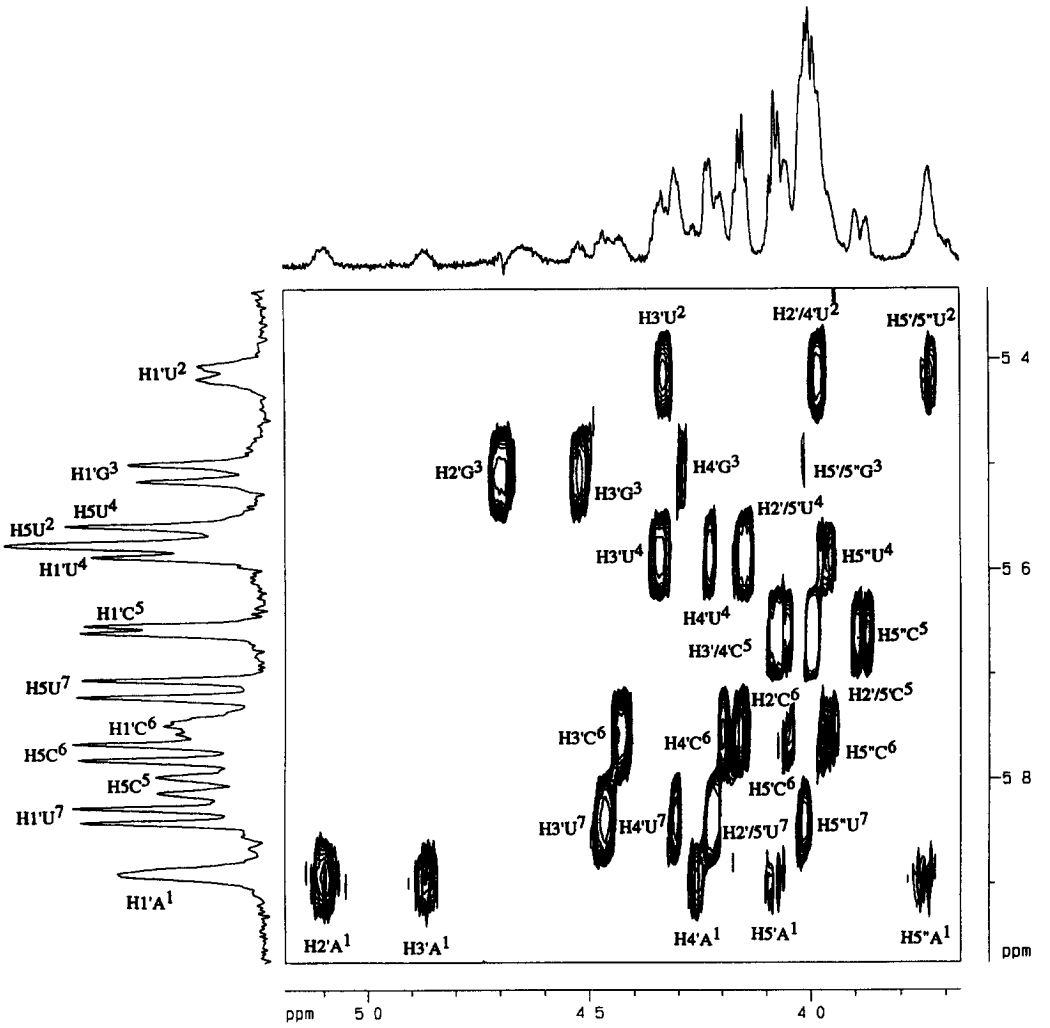


Figure 5 Clean-TOCSY spectra of the lariat heptamer (**19**) recorded at 284 K with a sweep width of 4000 Hz 4K data points was used in F2 and 256 experiments of 64 scans in F1 Quadrature detection in F1 was achieved with TPPI A $sine^2$ window was applied in both dimensions before zero-filling and Fourier transformation to give a 2K * 1K matrix All seven sugar residues are easily assigned in the spectra [reference. δ (H_2O) = 4.7 ppm] See Table 1

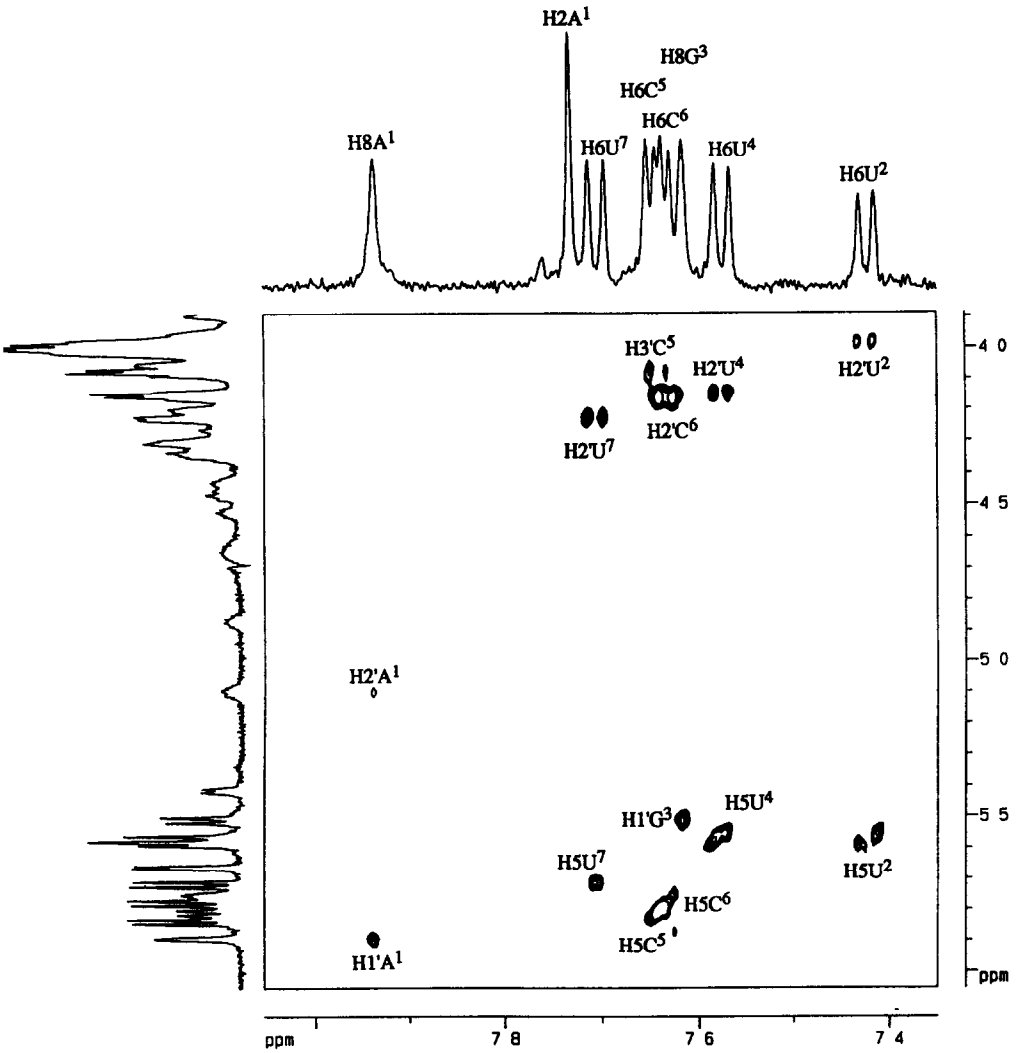


Figure 6 NOESY spectra of the lariat heptamer (**19**) recorded at 284 K with a mixing time of 200 ms A sweep width of 4000 Hz was used for 4K data points in F2 and 256 experiments of 96 scans in F1 Quadrature detection in F1 was achieved with TPPI A sine^2 window was applied in both dimensions before zero-filling and Fourier transformation to give a 2K * 1K matrix From the spectra it was possible to assign all sugar residues with the help of the crosspeaks between H6 and H2'/3' for pyrimidines and between H8 and H1' for purines [reference δ (H_2O) = 4 7 ppm] See Table 1

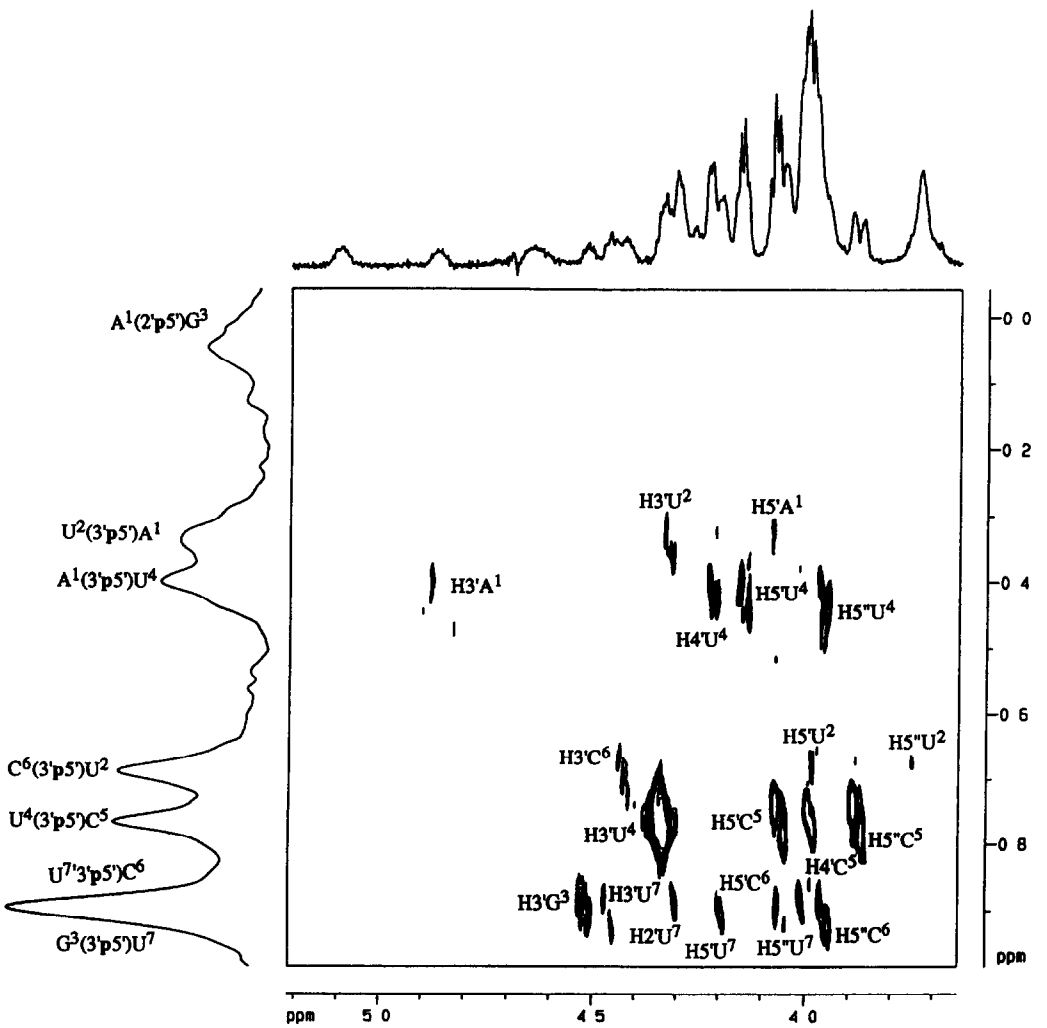


Figure 7. ^{1}H-^{31}P inverse correlated spectra of the lariat heptamer (**19**) The spectra was recorded in the absolute value mode with a sweep width of 4000 Hz in F2 and 1400 Hz in F1 4K data points was used in F2 and 256 experiments of 72 scans in F1 A sine^2 window was applied in both dimensions before zero-filling and Fourier transformation to give a 2K * 1K matrix. The following six sets of ^{1}H-^{31}P correlations have been found (1) H3'G^3(4 52 ppm)**p**(0 90 ppm)H5'U^7(4 22 ppm) & H5"U^7(4 02 ppm), (2) H3'U^7(4 46 ppm)**p**(0 90 ppm)H5'C^6(4 05 ppm) & H5"C^6(3 96 ppm), (3) H3'U^4(4 34 ppm)**p**(0 77 ppm)H4'C^5(3 99 ppm) & H5'C^5(3 99 ppm) & H5"C^5(3 88 ppm), (4) H3'C^6(4 43 ppm)**p**(0 69 ppm)H4'U^2(3 98 ppm) & H5'/H5"U^2(3 73 ppm), (5) H3'A^1(4 87 ppm)**p**(0 40 ppm)H4'U^4(4.23 ppm) & H5'U^4(4 15 ppm) & H5"U^4(3 97 ppm), (6) H3'U^2(4 33 ppm)**p**(0 34 ppm)H5'A^1(4 08 ppm) Referenced against water (4 7 ppm) in F2 and against cAMP (external reference 0 0 ppm) in F1 In ^{1}H-^{31}P inverse correlation spectrum, the H2'A^1**p**H5'/5"G^3 was not detectable The presence of H2'A^1**p**H5'/5"G^3 linkage can be however substantiated from the following line of reasoning (1) The presence of 2'-phosphodiester in the sugar moiety of A^1 was evident from the down-field shift of H2' A^1 at δ 5 10 which is most down-field amongst all H2' of the other six pentose residues [note that corresponding chemical shift is also found in the lariat tetramer and pentamer[2]] (2) From DQF-COSY spectrum (not shown) performed with and without ^{31}P decoupling, it was possible to measure the $^{3}J_{HP}$ = 4 Hz between 5'-phosphate of G^3 and its H5'/H5" (3) The H5'/H5" of G^3 absorbs at δ4 01 which is again typical for a 5'-$\underline{CH_2}$-OPO$_3$-

from CaH_2 and TsCl. Acetonitrile was distilled from P_2O_5 under argon. Dimethylformamide, triethylamine and DBU were distilled from CaH_2 under argon 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 LDC equipment with ConstaMetric Pump model III and Gradient Master was used for analytical HPLC 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. All reactions were carried out at ~20°C, unless otherwise specified. After silica gel column chromatography, some phosphodiester blocks had to be subjected to a 0 2M NH_4CO_3 - CH_2Cl_2 extraction to enhance the resolution of their ^1H & ^{31}P-NMR spectra

Compound 3: 5′-O-Levulinyl-2′-O-tetrahydropyranyl-4-N-(4-anisoyl)-cytidine-3′-triethylammonium(2-chlorophenyl)phosphate[19,44] *(HighRf)* **1** (1 25 g, 1.55 mmol) was condensed with 2′-O-tetrahydropyranyl-N-3-(4-anisoyl)-uridine[9,45] *(HighRf)* **2** (0 68 g, 1.47 mmol) in dry pyridine (4.5 ml / mmol) by use of 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MSNT)[9] (1.38 g, 4 65 mmol) The reaction mixture was then stirred for 60 min 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 crude *High Rf - High Rf* - isomer of **3** (1 3 g) ^1H-NMR ($CDCl_3$)· 8 25-6 88 (m, 15H) arom , CH-6, CH-5 & UH-6, 6 04-5 68 (m, 3H) CH-1′, UH-1′& UH-5; 5 07 (m, 2H) CH-2′ & CH-3′, 4 90-4 05 (m, 10H) sugar protons + 2 x tetrahydropyranyl-, 3 87 (s, 6H) 2 x -COPhO*CH*$_3$, 3.57 (m, 4H) 2 x tetrahydropyranyl, 2 70 (m, 4H) -CO*CH*$_2$*CH*$_2$COCH$_3$, 2 20, 2 19 (2 x s, 3H) -COCH$_2$CH$_2$CO*CH*$_3$, 1 54 (m, 12H) 2 x tetrahydropyranyl, ^{31}P-NMR($CDCl_3$)· -7.01, -7 64 ppm.

Compound 4: Crude **3** (1.3 g) dissolved in dry pyridine (9 ml) was stirred with 0.2 M acetonitrile solution of *o*-chlorophenylphosphoro-bis-(1,2,4-triazolide)[23] (9.3 ml, 1.85 mmol) for 40 min. Aqueous ammonium bicarbonate work up followed by silica gel column chromatography (2-8% ethanol in CH_2Cl_2) afforded the **4** (NH_4^+-salt, 1.05 g, 0.75 mmol, 48%, calc.from **2**), R_f: 0 67 (B); ^1H-NMR ($CDCl_3$+CD_3OD)·8 22-6 88 (m, 19H) arom., CH-6, CH-5, & UH-6; 6 07-5 55 (m, 3H) CH-1′, UH-1′ & UH-5, 5 16-4 16 (m, 12H) sugar protons & 2 x tetrahydropyranyl, 3.87, 3 85 (2 x s, 6H) 2 x -COPhO*CH*$_3$, 3 71-3 32 (m, 4H) 2 x tetrahydropyranyl, 2 69 (m, 4H) -CO*CH*$_2$*CH*$_2$COCH$_3$, 2 20, 2 17 (2 x s, 3H) -COCH$_2$CH$_2$CO*CH*$_3$, 1 50 (m, 12H) 2 x tetrahydropyranyl, ^{31}P-NMR ($CDCl_3$+CD_3OD) -7 17, -8 35, -8 74, -9 20 ppm

Compound 7: 5′-O-(4,4′-dimethoxytrityl)-2′-O-tetrahydropyranyl-3-N-(4-anisoyl)-uridine-3′-triethyl ammonium(2-chlorophenyl)phosphate[44] *(HighRf)* **5** (1 14 g, 1 50 mmol) was condensed with N-3-(4-anisoyl)-2′,3′-di-O-acetyl-cytidine **6** (576 mg, 1 25 mmol) in dry pyridine (5 ml / mmol) by addition of N-methylimidazole (595 µl, 7 5 mmol) and 1-mesitylenesulfonylchloride (MsCl)[21] (819 mg, 3.75 mmol) The reaction mixture was then stirred for 60 min. Aqueous ammonium bicarbonate work up followed by silica gel column chromatography (0-3% ethanol in CH_2Cl_2) afforded **7** (1 52 g, 1 09 mmol, 87%) R_f 0 55 (A), ^1H-NMR ($CDCl_3$) 8.05-6.78 (m, 28H) arom., CH-6, CH-5 & UH-6, 6.16-5 97 (m, 2H) CH-1′& UH-1′, 5.48-5 22 (m, 4H) CH-2′, 3′, UH-3′&UH-5, 4 84 (m, 2H) UH-2′& tetrahydropyranyl, 4 65-4 19 (m, 4H) CH-4′, 5′, 5′′&UH-4′, 3 88, 3 86 (2 x s, 6H) 2 x -COPhO*CH*$_3$, 3 79, 3 69 (2 x s, 6H) 2 x OCH$_3$, 3 63 (m, 4H) UH-5′, 5′′& tetrahydropyranyl, 2 08, 2 06 (2 x s, 6H) 2 x -COCH$_3$, 1 56 (m, 6H) tetrahydropyranyl, ^{31}P-NMR ($CDCl_3$) -7 37, -7 84 ppm

Compound 8: The DMTr-dimer block **7** (1 52 g, 1 09 mmol) was dissolved in half of the calculated total volume of 2% EtOH-CH_2Cl_2 solution and chilled to 0°C in an ice bath Trichloroacetic acid (TCA) (1 78 g, 10 9 mmol) was dissolved in the second half of the 2% EtOH-CH_2Cl_2 solution and chilled to 0°C prior to pouring it into the diester solution. The final concentration of TCA was 0 055M[24]. After stirring for 60 min the reaction was quenched by a small amount of pyridine and then it was subjected to aqueous ammonium bicarbonate work up Silica gel chromatography (2-4% ethanol in CH_2Cl_2) afforded **8** (1 12 g, 1 02 mmol, 94%), Rf· 0 49 (A), ^1H-NMR ($CDCl_3$+CD_3OD) 8 23-6 86 (m, 15H) arom , CH-6, CH-5 & UH-6, 6 08-5 95 (m, 2H) CH-1′& UH-1′, 5 84 (d, J = 8 54Hz, 1H) UH-5, 5 66-5 30 (m, 3H) CH-2′, 3′& UH-3′, 4 89-4 23 (m, 6H) UH-2′, 4′, CH-4′, 5′, 5′′ & tetrahydropyranyl, 3 97 (m, 2H) UH-5′, 5′′, 3 86 (s, 6H) 2 x -COPhO*CH*$_3$, 3 63 (m, 2H) tetrahydropyranyl, 2 09 (s, 6H) 2 x -COCH$_3$, 1 51 (m, 6H) tetrahydropyranyl, ^{31}P-NMR ($CDCl_3$)· -6 71, -7 76 ppm

Compound 10: 5′-O-(4,4′-dimethoxytrityl)-2′-O-tetrahydropyranyl-2-N-(*t*-butylbenzoyl)-6-O-(2-nitrophenyl) guanosine-3′-triethylammonium (2-chlorophenyl)phosphate[44] **9** *(High Rf)* (4 57 g, 4 09 mmol) was condensed with 2′-O-tetrahydropyranyl-N-3-(4-anisoyl)-uridine[45] *(High Rf)* **2** (1 74 g, 3 76 mmol) in presence of MsCl (2 23 g, 10 2 mmol) and N-methylimidazole (1 63 ml, 20 5 mmol) in dry pyridine (20 ml) for 60 min After

aqueous ammonium bicarbonate work up, silica gel column chromatography (0-2% EtOH, 0 25% pyridine / CH_2Cl_2) was performed, which afforded crude **10** (4.31 g). ^{1}H-NMR ($CDCl_3$): 8.18-6.69 (m, 30H) arom UH-6 & GH-8; 6.29 (d, $J_{1'2'}$ = 7.33Hz, 1H) GH-1'; 5.93 (m, 2H) UH-1'& UH-5, 5 69-5 43 (m, 2H) GH-2'& 3', 4.69-4.18 (m, 8H) UH-2', 3', 4', 5', 5'', GH-4'& 2 x tetrahydropyranyl, 3 84 (s, 3H) -COPhO*CH*$_3$; 3.70, 3.68 (2 x s, 6H) 2 x -OCH_3; 3.49 (m, 6H) GH-5',5''& 2 x tetrahydropyranyl; 1 46 (m, 12H) 2 x tetrahydropyranyl, 1.29 (s, 9H) *t-butyl*PhCO-, ^{31}P-NMR ($CDCl_3$). -7.66 ppm.

Compound 11: Crude compound **10** (4.31 g) was stirred with 0 2 M acetonitrile solution of *o*-chlorophenylphosphoro-bis-(1,2,4-triazolide) (22.2 ml, 4.43 mmol) in dry pyridine (28 ml) for 40 min Aqueous ammonium bicarbonate work up and silica gel column chromatography (3-7% EtOH / 0.25% pyridine / CH_2Cl_2) afforded **11** (3 41 g, 1 95 mmol, 52% calc. from **2**) R_f 0.74 (B); ^{1}H-NMR ($CDCl_3$): 8 27-6 60 (m, 34H) arom , UH-6 & GH-8, 6 28 (m, 1H) GH-1', 6 07 (d, $J_{1'2'}$= 5 37Hz, 1H) UH-1', 5 93-5 73 (m, 2H) GH-2' & UH-5, 5.44 (m, 1H) GH-3', 5 05-4.31 (m, 8H) UH-2', 3', 4', 5', 5'', GH-4'& 2 x tetrahydropyranyl, 3.85 (s, 3H) -COPhO*CH*$_3$; 3.68 (s, 6H) 2 x -OCH_3, 3 48 (m, 6H) GH-5',5''& 2 x tetrahydropyranyl, 1.49 (m, 12H) 2 x tetrahydropyranyl, 1 28 (s, 9H) *t-butyl*PhCO-; ^{31}P-NMR ($CDCl_3$) -6.25, -7 57, -8 03 ppm

Compound 12: Compound **11** (3 41 g, 1.95 mmol) was condensed with 2-cyanoethanol (200 µl, 2 93 mmol) in presence of MSNT (1.73 g, 5 85 mmol) in dry pyridine (10 ml) Aqueous ammonium bicarbonate work up and silica gel column chromatography (0-2% EtOH / 0 25% pyridine / CH_2Cl_2) afforded **12** (3.32 g, 1 95 mmol, quant) R_f 0.69 (A); ^{1}H-NMR ($CDCl_3$) 8 37-6 61 (m, 34H) arom , UH-6 & GH-8, 6 31-6 06 (m, 2H) GH-1'& UH-1'; 5.93 (d, J = 8.3Hz, 1H) UH-5, 5.72 (m, 1H) GH-2', 5 47 (m, 1H) GH-3'; 4.80-4 29 (m, 10H) UH-2', 3', 4', 5', 5'', GH-4', 2 x tetrahydropyranyl & -O*CH*$_2$CH$_2$CN, 3 84 (s, 3H) -COPhO*CH*$_3$, 3.69, 3.68 (2 x s, 6H) 2 x -OCH_3, 3.50 (m, 6H) GH-5', 5''& 2 x tetrahydropyranyl; 2 78 (m, 2H) -OCH$_2$*CH*$_2$CN, 1 49 (m, 12H) 2 x tetrahydropyranyl, 1 28 (s, 9H) *t-butyl*PhCO-, ^{31}P-NMR ($CDCl_3$) -7 03, -7 91, -8.11, -8 15, -8 20, -8.25 ppm.

Compound 13· Deprotection of the 5'- 4,4'-dimethoxytrityl group of **12** (3 32 g, 1 95 mmol) was done in the same way as for **7** Silica gel chromatography (0-3% ethanol in CH_2Cl_2) afforded **13** (2 65 g, 1 89 mmol, 97%), Rf 0 51 (A), ^{1}H-NMR ($CDCl_3$+CD_3OD): 8.36-6 89 (m, 21H) arom , UH-6 & GH-8, 6.15 (m, 2H) GH-1'& UH-1', 5.89, 5 79 (2 x d, J = 8.06Hz, 8 55Hz, 1H) UH-5, 5.49-5 06 (m, 3H) GH-2', 3' & UH-3', 4 81-4 31 (m, 10H) UH-2', 3', 4', 5', 5'', GH-4', 2 x tetrahydropyranyl & -O*CH*$_2$CH$_2$CN; 4 00 (m, 2H) GH-5',5'', 3 86 (s, 3H) -COPhO*CH*$_3$, 3 39 (m, 4H) 2 x tetrahydropyranyl, 2.81 (t, J = 6 59Hz, 2H) -OCH$_2$*CH*$_2$CN, 1.51 (m, 12H) 2 x tetrahydropyranyl, 1 31 (s, 9H) *t-butyl*PhCO-, ^{31}P-NMR ($CDCl_3$) -6 93, -7 01, -8 08, -8 13, -8 20, -8 59, -8 76 ppm

Compound 15a The 3'-phosphodiester block **4** (*High Rf - High Rf* (1 05 g, 0 75 mmol) was condensed with 6-N-(4-anisoyl)-2'-O-pixyladenosine[22] **14** (467 mg, 0 71 mmol) in dry pyridine (5 ml) by addition of N-methylimidazole (299 µl, 3 75 mmol) and MsCl (410 mg, 1 88 mmol) The reaction mixture was then stirred 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 0-3% ethanol/0 5% pyridine in CH_2Cl_2 afforded **15a** as a white powder after co-evaporation with toluene and cyclohexane (738 mg, 0.365 mmol, 49%), R_f 0.70 (A), ^{1}H-NMR ($CDCl_3$+2, 6-lutidine) 8.56 (m 1H) AH-8, 8 22-6 40 (m, 37H) arom , AH-2, CH-6, CH-5 & UH-6, 6 05-5 68 (m, 4H) CH-1', UH-1', AH-1' & UH-5, 5 12-4 16 (m, 16H) sugar protons & 2 x tetrahydropyranyl, 3 88, 3 86 (2 x s, 9H) 3 x -COPhO*CH*$_3$, 3 60-3 30 (m, 5H) 2 x tetrahydropyranyl & AH-3', 2 68 (m, 4H) -CO*CH*$_2$*CH*$_2$COCH$_3$, 2 19, 2 16 (2 x s, 3H) -COCH$_2$CH$_2$CO*CH*$_3$; 1 58 (m, 12H) 2 x tetrahydropyranyl, ^{31}P-NMR ($CDCl_3$+lutidine). -7 06, -7 54, -7 23, -7 84, -7 93, -8 06 ppm

Compound 15b: Compound **15a** (738 mg, 0 365 mmol) in dry pyridine (3 ml) was treated with 0 2 M acetonitrile solution of *o*-chlorophenylphosphoro-bis-(1,2,4-triazolide) (5 48 ml, 1.09 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 **15b** as a white powder after co-evaporation with toluene and cyclohexane (NH_4^+-salt, 746 mg, 0 334 mmol, 92%), R_f 0.74 (B), ^{1}H-NMR ($CDCl_3$+CD_3OD+2,6-lutidine)· 8 59 (m, 1H) AH-8, 8 39-6 47 (m, 41H) arom , AH-2, CH-6, CH-5 & UH-6, 6.35-5 70 (m, 5H) arom , AH-1', UH-1', CH-1', UH-2', & UH-5, 5 37-4 07 (m, 16H) sugar protons & 2 x tetrahydropyranyl, 3 91, 3 89, 3 86 (3 x s, 9H) 3 x -COPhO*CH*$_3$, 3 40 (m, 4H) 2 x tetrahydropyranyl, 2 58 (m, 4H) -CO*CH*$_2$*CH*$_2$COCH$_3$, 2 20, 2 14, 2 09 (3 x s, 3H) -COCH$_2$CH$_2$CO*CH*$_3$, 1 60 (m, 12H) 2 x tetrahydropyranyl, ^{31}P-NMR ($CDCl_3$+CD_3OD+lutidine) -5 78 to -9 71 ppm

Compound 15c : The 3'-phosphodiester block **15b** (746 mg, 0 334 mmol) was dissolved in half of the calculated total volume of 2% EtOH-CH_2Cl_2 solution and chilled to 0 °C in an ice bath Trichloroacetic acid

(TCA) (655 mg, 4.01 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 **15b**. The final concentration of TCA was 0.055 M. After stirring for 60 min at 0 °C, 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* (NH_4^+-salt, 605 mg, 0.305 mmol, 91% of **15c**). R_f: 0.65 (C); ^{1}H-NMR ($CDCl_3$+CD_3OD). 8.68 (s, 1H) AH-8, 8.36-6.86 (m, 28H) arom., AH-2, CH-6, CH-5 & UH-6; 6.00 (m, 3H) AH-1′, CH-1′ & UH-1′; 5.77, 5.74 (2 x d, J = 8.12Hz, 8.06Hz, 1H) UH-5, 5.19-4.13 (m, 16H) sugar protons & 2 x tetrahydropyranyl, 3.87 (s, 9H) 3 x -COPhO*CH*$_3$, 3.50 (m, 4H) 2 x tetrahydropyranyl, 2.68 (m, 4H) -CO*CH*$_2$*CH*$_2$COCH$_3$, 2.19, 2.16 (s, 3H) -COCH$_2$CH$_2$CO*CH*$_3$, 1.44 (m, 12H) 2 x tetrahydropyranyl, ^{31}P-NMR ($CDCl_3$+CD_3OD) -7.08 to -9.13 ppm

Compound 15d. (2-cyanoethoxy)-(2-(4-nitrophenyl)ethoxy)-(diisopropylamino)phosphine (1.12 g, 3.05 mmol) was weighed into a dry 50 ml round bottomed flask and dry 15% dimethylformamide / acetonitrile solution (10 ml) was added under argon (argon balloon). Then dry and sublimed tetrazole (640 mg, 9.13 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 **15c** *(High Rf - High Rf)* (605 mg, 0.305 mmol) was added to the 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) (32 ml) was added and the reaction solution was stirred for 15 min, poured into 0.1M sodium thiosulfate / concentrated ammonium bicarbonate solution (50 ml) and extracted with dichloromethane (3 x 50 ml). The pyridine-free gum obtained after toluene co-evaporation of the organic residue was then purified by short silica gel column chromatography (2-8% EtOH in CH_2Cl_2) to finally give the 3′-phosphodiester-2′-(2-cyanoethoxy)-(2-(4-nitrophenyl)ethoxy)phosphotriester block **15d** as a white powder after co-evaporation with toluene and cyclohexane (NH_4^+-salt, 402 mg, 0.177 mmol, 58%); R_f: 0.69 (B), ^{1}H-NMR ($CDCl_3$+CD_3OD) 8.65 (s, 1H) AH-8, 8.34-6.84 (m, 32H) arom., AH-2, CH-6, CH-5 & UH-6, 6.23 (m, 1H) AH-1′; 6.10-5.69 (m, 4H) CH-1′, UH-1′, UH-5 & AH-2′, 5.13-4.14 (m, 18H) sugar protons, 2 x tetrahydropyranyl -O*CH*$_2$CH$_2$CN & -O*CH*$_2$CH$_2$PhNO$_2$, 3.88, 3.87 (2 x s, 9H) 3 x -COPhOCH$_3$, 3.50 (m, 4H) 2 x tetrahydropyranyl-, 3.05 (m, 2H) -OCH$_2$*CH*$_2$PhNO$_2$, 2.87-2.47 (m, 8H) -OCH$_2$*CH*$_2$CN, -OCH$_2$*CH*$_2$PhNO$_2$ & -CO*CH*$_2$*CH*$_2$COCH$_3$, 2.19, 2.17 (2 x s, 3H) -COCH$_2$CH$_2$CO*CH*$_3$, 1.51 (m, 12H) 2 x tetrahydropyranyl, ^{31}P-NMR ($CDCl_3$+CD_3OD) -4.74, [-7.05 to -9.52] ppm

Compound 16a. A mixture of **15d** (402 mg, 0.177 mmol) and compound **8** *(High Rf)* (291 mg, 0.266 mmol) was co-evaporated with dry pyridine and redissolved in dry pyridine (0.8 ml). MSNT (367 mg, 1.24 mmol) was added and the reaction mixture was stirred for 6 h. Aqueous ammonium bicarbonate work up followed by silica gel column chromatography (2-4% EtOH in CH_2Cl_2) gave the fully protected*all-High Rf* - isomer of pentamer pentamer **16a** as a white powder after co-evaporation with toluene and cyclohexane (336 mg, 0.101 mmol, 57%), R_f 0.55 (A), ^{1}H-NMR ($CDCl_3$) 9.23 (br, 1H) NH, 8.70 (m, 1H) AH-8, 8.34-6.85 (m, 47H) arom., AH-2, 2 x CH-6, 2 x CH-5 & 2 x UH-6, 6.47 (m, 1H) AH-1′, 6.15-5.49 (m, 11H) 2 x CH-1′, 2 x UH-1′, 2 x UH-5, AH-2′, -3′, 1 x CH-2′, -3′ & UH-3′, 5.10-4.03 (m, 27H) sugar protons, 3 x tetrahydropyranyl, -O*CH*$_2$CH$_2$CN & -O*CH*$_2$CH$_2$PhNO$_2$, 3.84 (s, 15H) 5 x -COPhO*CH*$_3$, 3.40 (m, 6H) 3 x tetrahydropyranyl, 2.98-2.45 (m, 8H) -OCH$_2$*CH*$_2$CN, -OCH$_2$*CH*$_2$PhNO$_2$ & -CO*CH*$_2$*CH*$_2$COCH$_3$, 2.20, 2.18, 2.16 (3 x s, 3H) -COCH$_2$CH$_2$CO*CH*$_3$, 2.07 (s, 6H) 2 x -COCH$_3$, 1.50 (m, 18H) 3 x tetrahydropyranyl, ^{31}P-NMR ($CDCl_3$+CD_3OD) [-2.61 to -3.29] & [-5.86 to -8.79] ppm

Compound 16b: Compound **16a** (336 mg, 0.101 mmol) was treated with dry triethylamine (281 µl, 2.02 mmol, 20% conc. in dry pyridine (1:1 ml)) and stirred for 4.5 h. The reaction mixture was diluted with dry pyridine and evaporated and then co-evaporated with toluene. Silica gel column chromatography (4-8% EtOH / CH_2Cl_2) afforded the pentamer **16b** (312 mg, 0.095 mmol, 94%), R_f: 0.42, 0.40 (A), ^{1}H-NMR ($CDCl_3$+CD_3OD) 8.65 (m, 1H) AH-8, 8.44-6.81 (m, 47H) arom., AH-2, 2 x CH-6, 2 x CH-5 & 2 x UH-6, 6.34 (m, 1H) AH-1′, 6.15-5.30 (m, 11H) 2 x CH-1′, 2 x UH-1′, 2 x UH-5, AH-2′, -3′, 1 x CH-2′, -3′ & 1 x UH-3′, 5.20-4.17 (m, 24H) sugar protons, 3 x tetrahydropyranyl, -O*CH*$_2$CH$_2$CN & -O*CH*$_2$CH$_2$PhNO$_2$, 3.89, 3.86 (2 x s, 15H) 5 x -COPhO*CH*$_3$, 3.40 (m, 6H) 3 x tetrahydropyranyl, 2.85-2.45 (m, 6H) -OCH$_2$*CH*$_2$PhNO$_2$ & -CO*CH*$_2$*CH*$_2$COCH$_3$; 2.19, 2.16 (2 x s, 3H) -COCH$_2$CH$_2$CO*CH*$_3$, 2.07 (s, 6H) 2 x -COCH$_3$, 1.50 (m, 18H) tetrahydropyranyl, ^{31}P-NMR ($CDCl_3$+CD_3OD) -2.37, -2.76, [-7.57 to -8.62] ppm

Compound 17a: 16b (312 mg, 0.095 mmol) and **13** (243 mg, 0.142 mmol)] were co-evaporated with dry pyridine and redissolved in dry pyridine (0.48 ml). (MSNT) (197 mg, 0.67 mmol) was added and the reaction mixture was stirred for 6 h. Aqueous ammonium bicarbonate work up followed by silica gel column chromatography of the organic residue (0-5 % EtOH-CH_2Cl_2) gave the fully protected oligomer **17a** as a white

powder after co-evaporation with toluene and cyclohexane. (277 mg, 59 %), R_f 0.53 (A); ^{1}H-NMR($CDCl_3$) 9 45 (br, 1H) NH, 8.70 (m, 1H) AH-8; 8 33-6.84 (m, 69H) arom., AH-2, GH-8, 2 x CH-6, 2 x CH-5 & 3 x UH-6; 6 37 (m, 1H) AH-1′; 6.15-4.90 (m, 19H) 3 x UH-5, anomeric & sugar protons, 4.80-4.01 (m, 30H) sugar protons, 3 x tetrahydropyranyl, -O*CH*$_2$CH$_2$CN & -O*CH*$_2$CH$_2$PhNO$_2$; 3.83 (s, 18H) 6 x -COPhO*CH*$_3$, 3.30 (m, 10H) 5 x tetrahydropyranyl; 2 90-2.45 (m, 8H) -OCH$_2$*CH*$_2$CN, -OCH$_2$*CH*$_2$PhNO$_2$ & -CO*CH*$_2$*CH*$_2$COCH$_3$, 2.18, 2.14 (2 x s, 3H) -COCH$_2$C H$_2$CO*CH*$_3$; 2.05, 2.02 (2 x s, 6H) 2 x -COCH$_3$; 1 46 (m, 30H) tetrahydropyranyl; 1 26 (s, 9H) *t-butyl*PhCO-; ^{31}P-NMR ($CDCl_3$+CD_3OD)· -2.00, -2.29, -2.51 & [-7 35 to -8 35] ppm.

Compound 17b. 17a (277 mg, 0 056 mmol) was dissolved in dry pyridine (0.5 ml). Then 0.5 M hydrazine hydrate (10 equiv) in pyridine/acetic acid (3.2 v/v)[46] (1.12 ml) was added to the reaction mixture and after 5 min stirring, the reaction was quenched by addition of pentane-2,4-dione (58 µl, 10 equiv.) The reaction mixture was then subjected to aqueous ammonium bicarbonate work up. The pyridine-free gum obtained after evaporation and co-evaporation with toluene was then purified by silica gel chromatography (3-5% EtOH in CH_2Cl_2) to give **17b** as a white powder after co-evaporation with toluene and cyclohexane: (239 mg, 88%), R_f 0 49 (A), ^{1}H-NMR ($CDCl_3$) 9 46 (br, 1H) NH; 8.67 (s, 1H) AH-8, 8 33-6.84 (m, 69H) arom , AH-2, GH-8, 2 x CH-6, 2 x CH-5 & 3 x UH-6, 6.37 (m, 1H) AH-1′, 6.17-4.95 (m, 19H) 3 x UH-5, anomeric & sugar protons; 4.85-4.10 (m, 28H) sugar protons, 5 x tetrahydropyranyl, -O*CH*$_2$CH$_2$CN & -O*CH*$_2$CH$_2$PhNO$_2$; 3 83 (s, 18H) 6 x -COPhO*CH*$_3$, 3.78 (m, 2H) CH-5′, 5″, 3.39 (m, 10H) 5 x tetrahydropyranyl, 2.92-2.61 (m, 8H) -OCH$_2$*CH*$_2$CN, -OCH$_2$*CH*$_2$PhNO$_2$ & -CO*CH*$_2$*CH*$_2$COCH$_3$; 2.05, 2 02 (2 x s, 6H) 2 x -COCH$_3$; 1 46 (m, 30H) 5 x tetrahydropyranyl; 1 26 (s, 9H) *t-butyl*PhCO-; ^{31}P-NMR ($CDCl_3$+CD_3OD) -1.98, -2 29, -2 54 & [-7 15 to -8 38] ppm

Compound 17c: 17b (239 mg, 0 049 mmol) was treated with dry triethylamine (136 µl, 20 equiv , 20% concentration in dry pyridine, 0 55 ml) and stirred for 3 h The reaction mixture was diluted with dry pyridine and evaporated and then co-evaporated with toluene Preparative thin-layer chromatography (14%EtOH / CH_2Cl_2 as eluent) afforded **17c** as a white powder after co-evaporation with toluene and cyclohexane: (NH_4^+-salt, 160 mg, 68%). R_f 0 28 (A); ^{1}H-NMR ($CDCl_3$ +CD_3OD) 8 67 (s, 1H) AH-8, 8 47-6 79 (m, 69H) arom , AH-2, GH-8, 2 x CH-6, 2 x CH-5 & 3 x UH-6; 6 38 (m, 1H) AH-1′, 6 17-4.95 (m, 19H) 3 x UH-5, anomeric & sugar protons, 4.90-4 10 (m, 26H) sugar protons, 5 x tetrahydropyranyl & -O*CH*$_2$CH$_2$PhNO$_2$; 3.84 (s, 18H) 6 x -COPhO*CH*$_3$; 3 78 (m, 2H) CH-5′, 5″; 3.38 (m, 10H) 5 x tetrahydropyranyl; 3.01 (m, 2H) -OCH$_2$*CH*$_2$PhNO$_2$; 2 07, 2 04 (2 x s, 6H) 2 x -COCH$_3$; 1.43 (m, 30H) 5 x tetrahydropyranyl, 1 26 (s, 9H) *t-butyl*PhCO-; ^{31}P-NMR ($CDCl_3$+CD_3OD) -1.90, -2 09, -2 88, 5 18 & [-7 18 to -8 13] ppm

Fully protected heptameric cyclic branched RNA 18: The 5′-hydroxy 3′-phosphodiester block **17c** (160 mg, 0 033 mmol) was co-evaporated with dry pyridine and redissolved in dry pyridine (250 ml / mmol, 4mM) Then MSNT (137 mg, 0 46 mmol, 14 equiv) was added to the reaction solution and was then stirred for 18 h Aqueous ammonium bicarbonate work up followed by silica gel column chromatography (2-5% EtOH in CH_2Cl_2) afforded the title compound **18** as white a powder after co-evaporation with toluene and cyclohexane (105 mg, 0 022 mmol, 66%); R_f 0 44 (A), 8.79 (s, 1H) AH-8, 8.59-6 84 (m, 69H) arom., AH-2, GH-8, 2 x CH-6, 2 x CH-5 & 3 x UH-6, 6 52 (m, 1H) AH-1′, 6 25-5 00 (m, 19H) 3 x UH-5, anomeric & sugar protons, 4 90-4 10 (m, 28H) sugar protons, 5 x tetrahydropyranyl & -O*CH*$_2$CH$_2$PhNO$_2$, 3.83 (s, 18H) 6 x -COPhO*CH*$_3$, 3 31 (m, 10H) 5 x tetrahydropyranyl; 2 62 (m, 2H) -OCH$_2$*CH*$_2$PhNO$_2$, 2 04 (s, 6H) 2 x -COCH$_3$; 1 44 (m, 30H) tetrahydropyranyl, 1 26 (s, 9H) *t-butyl*PhCO-, ^{31}P-NMR ($CDCl_3$+CD_3OD).-1 07, -1 24, -3 32 & [-6 91 to -9.11] ppm.

Deprotection of 18 to 19: Fully protected lariat **18** (105 mg, 22 µmol) was dissolved in dry pyridine (0 2 ml) followed by addition of 0.5M DBU / pyridine solution (440 µl, 220 mmol) and the reaction mixture was stirred for 5 h, after which all of **18** had reacted and resulted in a new lower Rf spot on TLC (solvent A), due to regioselective removal of the 4-nitrophenylethyl group The reaction was then quenched by addition of 1M acetic acid / pyridine solution (220 µl, 220 mmol) After a few minutes, the mixture was poured into water and extracted by dichloromethane (4 x 25 ml). Adjustment of the pH with ammonium bicarbonate helped clearify the colloidal water phase The organic phase was dried through magnesium sulphate and evaporated and coevaporated with dioxane. The residue was dissolved in dioxane / water (8·2 v/v) solution (6 7 ml) and to this solution, *syn*-4-nitrobenzaldoxime (256 mg, 1 54 mmol) and 1,1,3,3-tetramethylguanidine (173 µl, 1 38 mmol) were added After stirring for 45 h at room temperature the solvents were removed by evaporation *in vacuo* and concentrated ammonia (45 ml, d = 0 9) was added The reaction mixture was stirred for 7 days at room temperature and was then evaporated and co-evaporated with distilled water The residue was treated with 80% aq acetic acid (40 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 (3 x 20 ml) and the water

phase was evaporated to dryness **Purification of 19:** DEAE-Sephadex A-25 column chromatography was carried out by redissolving the residue in 0.001 M ammonium bicarbonate (BDH Chemicals Ltd, Poole, England) buffer and applied to a DEAE-Sephadex A-25 column (2 x 25 cm, HCO_3^- form) and eluted with a linear gradient 0 001 M - 0 5 M - 0.8 M ammonium bicarbonate solution (500ml/1000ml/500ml, pH 7 5) The main peak eluted between 0.7 and 0 8 M and contained a shoulder on the front side, which was collected separately (front fraction, 249 A_{260} units) The rest of the main peak was divided in two equal parts (middle- and rear fractions, 366 and 249 A_{260} units respectively). These two latter fractions (contained 75-80% of product, judged from analytical HPLC runs) were purified by high pressure semi-preparative Spherisorb S5ODS2 column chromatography 5-7 mg batches of lyophilized material obtained from the middle- and rear fractions were dissolved each in 5% MeCN in 0 1M triethylammonium acetate (TEAA) at pH 7.0 (900-1000 µl) in Eppendorf tubes, centrifuged and were then 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 (0-5% solvent B (B = 50% acetonitrile in 0 1M TEAA) in 20 min, 1 ml/min) resolved the desired peak with base-line separation (detector was set at 254 nm) The purified material was collected, evaporated and then lyophilized several times (~9 x 1 ml) until the TEAA salt was removed (monitored by ^{1}H-NMR) Yield of pure triethylammonium salt of **19**. 430 A_{260} units, 18 5 mg, 6.36 µmol, 29%

The title compound **19** was obtained as a sodium salt by elution through a Dowex column (1 x 20 cm, Na^+ form) with distilled water at 2 °C The eluted water was collected in a 50 ml round bottom flask which was cooled in ethanol / dry ice bath. The freezed aqueous solution was lyophilized and the white silky residue was redissolved in a small amount of distilled water, swiftly transferred to a 10 ml screw cap bottle and lyophilized. **Enzymatic digestion of deprotected lariat 19:** *Crotalus adamanteus* snake venom phosphodiesterase (SVPD) (0 008 units) in 0.1M Tris-hydrochloride buffer at pH 8 0 (50 µl) was added to a solution of lariat **19** (1 o d in 50 µl) in an Eppendorf tube The resulting solution was kept for 60 min at 37 °C by which time all of lariat **19** had been digested to mononucleotide blocks ($t_{1/2}$ = 7 min HPLC -gradient· 0-40% B, 30 min, 1 ml / min A = 0 1M TEAA / 5% MeCN, B = 0 1M TEAA / 50% MeCN) Alkaline phoshatase (0 01 units) in 0 1M Tris-hydrochloride buffer at pH 8 0 (40 µl) was added to the SVPD solution and the mixture was kept for 18 h at 37 °C HPLC quantitation of the resulting nucleoside mixture was carried out using the gradient 0-50% B, 30 min, 1 ml / min A = 0 01M TEAA, B = 0.01M TEAA / 20% MeCN, which showed the following ratio for Cytidine (R_t = 3 87'). Uridine (R_t = 4.72') Guanosine (R_t = 9 42') Adenosine (R_t = 14 23') are 0 89 2 09 0 82 · 1 0 (calculated for **19** 0 94 1 97 . 1 11 1 0)

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