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NEW REGIOSPECIFIC SYNTHESIS OF THE "BRANCHED" TRI-, PENTA- & HEPTA-RIBONUCLEIC ACIDS WHICH ARE FORMED AS THE "LARIAT" IN THE PRE-mRNA PROCESSING REACTIONS [*Splicing*]

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Abstract: *Synthesis of branched triribonucleotides **14** & **19**, branched pentaribonucleotides **37** & **38** and branched heptaribonucleotide **41** are reported using the key intermediate **7** or **9**. The advantage of using **7** or **9** is that they circumvent the need for complementary protecting groups for the 2′-hydroxyl functions and for the internucleotidyl phosphodiesters in order to introduce the second phosphodiester bond specifically at the branch-point. All branched ribonucleotides **14**, **19**, **37**, **38** and **41** have been unambiguously characterized by ^{1}H- & ^{31}P-NMR spectroscopy.*

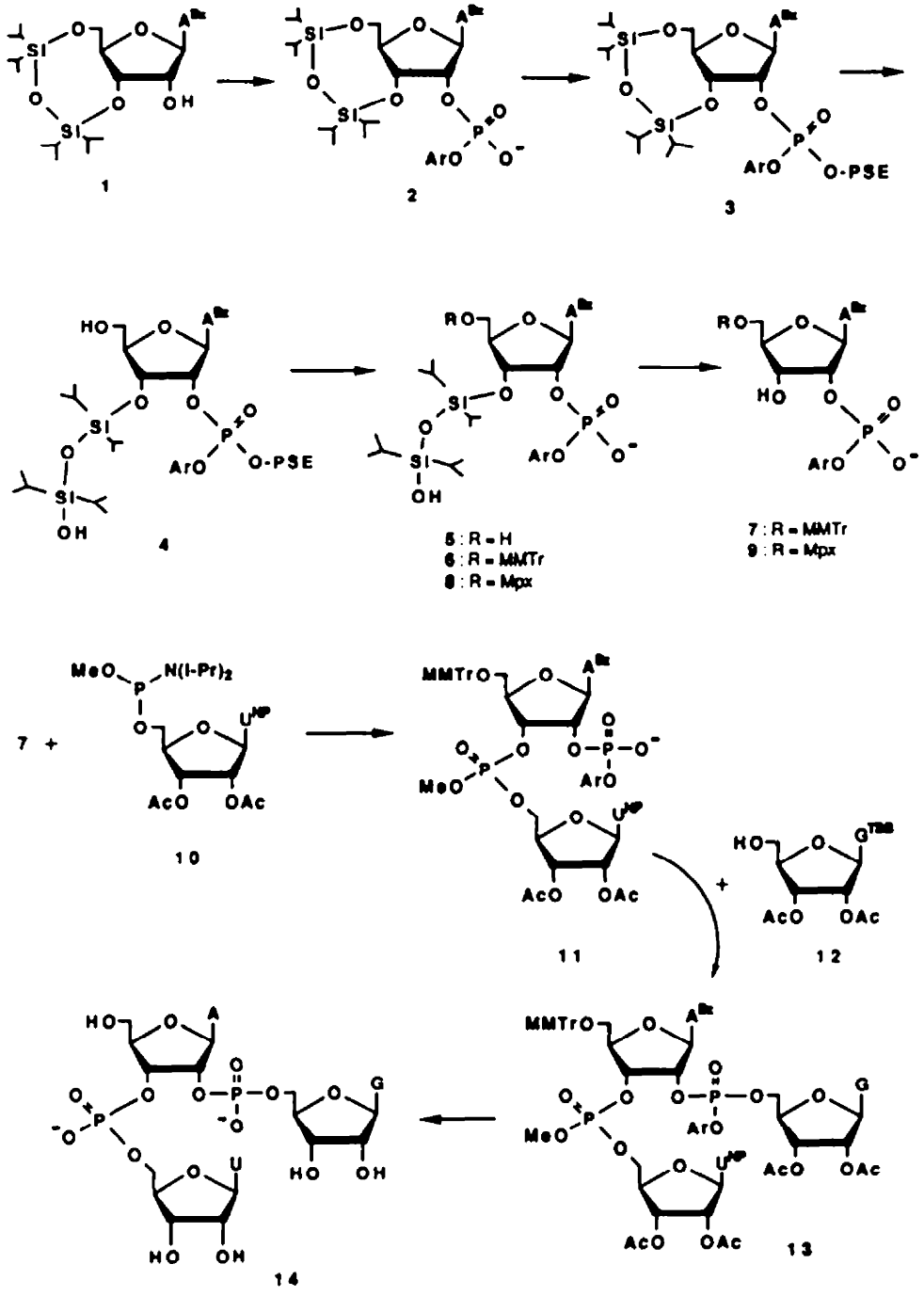
The cellular processing of pre-mRNA in eukaryotes (splicing), both from the nuclear messenger RNA and the Group II type messenger RNA precursors, involves the formation of a "lariat" structure[1-7]. These lariat RNA structures have adenosine as the unique branch-point residue which is connected to a guanosine residue through a 2′ → 5′ phophodiester bond, the 3′ → 5′ phosphodiester is linked up with a pyrimidine residue (uridine or cytidine) while the 5′ → 3′ phosphate residue is always either a uridine or a purine[3,5]. Our own interest is mainly directed toward understanding of the biological and conformational importance of the lariat formation in the the Group II and nuclear mediated splicing reactions. Considerable attention has been directed to the synthesis of the branched oligoribonucleotides[8-19], mainly owing to the inherent chemical problems encountered in the introduction of the 2′ → 5′ and 3′ → 5′ phosphodiester bond regiospecifically at the vicinal hydroxy groups of the branch-point adenosine. We have reported regiospecific synthesis of branched trimeric[10,16,20] and tetrameric[14] ribonucleotides, and have determined their solution conformations[16,20-25] by NMR spectroscopy. These studies have revealed that the conformation of the branch-point in a given branched oligoribonucleotide is entirely dictated by the neighbouring nucleobases. Thus the predominant conformational feature of the branched triribonucleotide core[16, 20-23, 25] is comprised of unnatural stacking between 2′ → 5′ linked nucleobases and the 3′-pyrimidine residue is free and apart from the branch-point adenosine while the conformation of a branched tetraribonucleotide resembles a distorted A-RNA helix fragment[23-25] which is easily recognized by an enzyme[11,26]. These conformational studies[16, 19-25] clearly raised an important question that what should be the minimum size of a branched oligoribonucleotide which would be able to mimic the conformation of a naturally-occurring lariat ? Clearly what is needed is systematic conformational studies with additional ribonucleotide residues in all three termini of the branched triribonucleotide core (*i.e.* 2′ → 5, 3′ → 5′ and 5′ → 3′) in order to understand (1) why branched RNAs are formed in splicing reaction? (2) does the free energy of activation for transesterification reactions in the self-splicing come from intron-folding ? (3) does the Mg^{2+} participate in stereochemical positioning of exons for the ligation step ? (4) why should guanosine residue play such an important role in precise transesterification reactions involved in splicing ? These questions have directed our efforts to develop synthetic procedures which would produce larger branched oligonbonucleotide in a pure state to study their conformations by ^{1}H-NMR spectroscopy.

We herein report a new procedure for the synthesis of the branched heptaribonucleotide **41**, which extends in all of three directions of 2′ → 5′, 3′ → 5′ and 5′ → 3′ from the core branched trimer **14**. This heptamer **41** corresponds to the sequence at the branch-site of the Group II intron bI1 from the yeast mitochondria[27].

It may be noted that the synthetic strategies for branched oligoribonucleotides which have been reported until now[8-17] are only suitable for the synthesis of the branched trimer or an oligomer extending only at the 5′ → 3′ direction from the branch-point

A*. Recently, Hata *et al* has reported[18] the synthesis of a branched hexamer using N^6-benzoyl-5′-O-monomethoxytrityl-adenosine-3′-dicyanoethylphosphoro-2′-S-phenylphosphorothioate as the key intermediate. The synthesis of this intermediate was however performed[8,18] in seven steps with low overall yield, one of the important steps in the latter strategy involved the separation of N^6-benzoyladenosine-2′-phosphorodianilidate from its 3′-isomer (37 %) which was formed during the deprotection of 3′,5′-O-tetraisopropyl-1,3-disilyl group by fluoride ion[8].

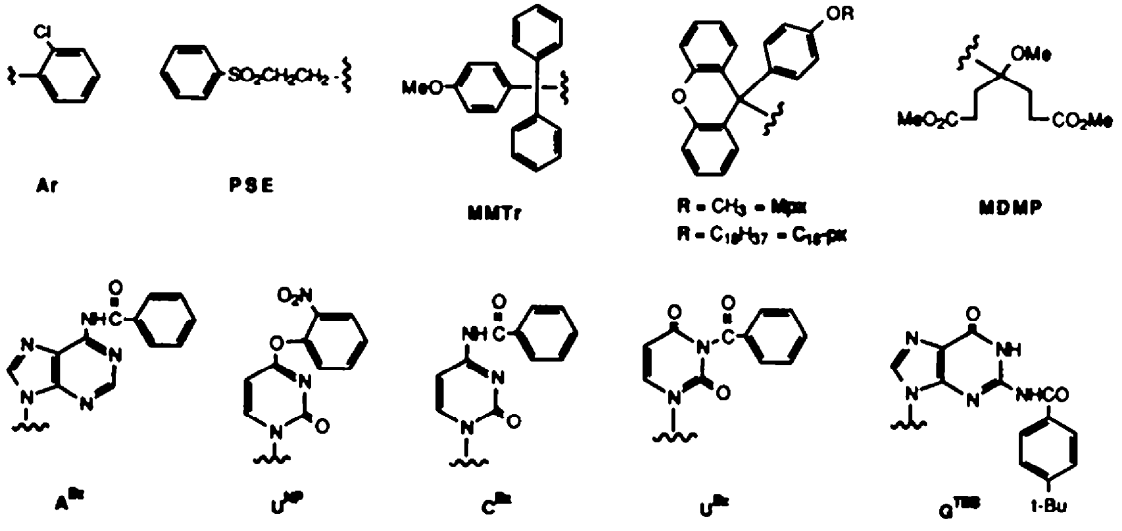
Clearly, the sequential introduction of phosphates at the vicinal 2′- and 3′-hydroxyls at the branch-point A* is a mandatory chemical requirement in order to be able to synthesize branched RNA by *regiospecific incorporation* of 2′ → 5′ and 3′ → 5′ phosphodiester-linked nucleobases. This demand necessarily addresses to a central chemical problem in RNA synthesis that involves the phosphate group vicinal to a hydroxyl function. Indeed, it is the chemical nature of this phosphate group which dictates the nucleophilic reactivity of the vicinal hydroxyl function toward the phosphate[28-30]. Such a reaction is clearly a



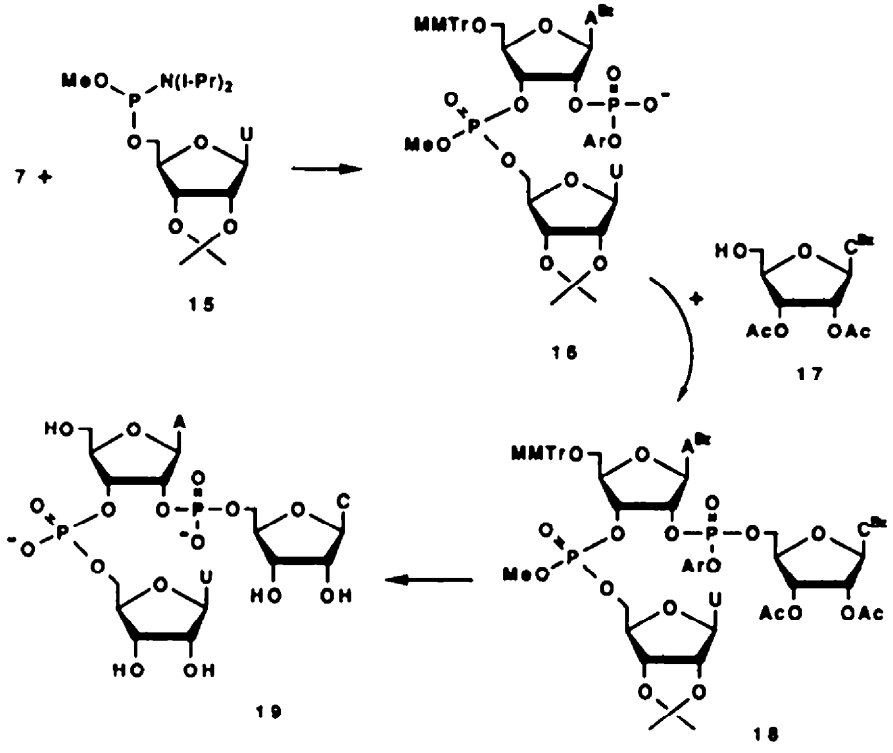
dominant feature with a phosphotriester which is vicinal to a hydroxyl group in mononucleotides[28] or in oligonucleotides[29, 30]. Todd *et al* first reported such neighbouring group participations in isomerically pure mononucleotides under acidic, basic and neutral conditions[28]. The importance of such neighbouring 2′- OH attack on the vicinal 3′-phosphotriester has been clearly illustrated in oligonucleotide synthesis[29,30]. The recent study on the synthesis of branched oligomer using the H-phosphonate methodology was unsuccessful, owing to the reaction involving neighbouring group participation[31]. It has, however, clearly emerged that the phosphodiester, with a vicinal hydroxyl group, is reasonably stable, even during the treatment of fluoride ion which has been demonstrated in our previous work in the synthesis of branched nucleotides[10,14,20] along with other works done elsewhere[11,12,17].

In this paper, we report the synthesis of N^6-benzoyl-5′-O-(4-methoxytrityl)adenosine-2′-(o- chlorophenyl)phosphate 7 and the 5′-O-[9-(p-anisoyl)xanthen-9-yl-] analogue 9 as the key intermediate which allow the phosphate chain extension in all three directions [2′ → 5′, 3′ → 5′and 5′ → 3′]. The use of the key intermediate such as 7 or 9 also circumvents the need for complementary protecting groups for 2′-hydroxyl functions and for internucleotide phosphodiesters in order to introduce the branching phosphodiester bond at the branch-point adenosine residue *specifically* during the course of the synthesis. A model study (^{31}P-NMR) of the stability of key intermediate 7 or 9 has shown that they are stable under the condition used for the phosphoramidite condensation [tetrazole (7 equiv) in dry acetonitrile at ~ 20 °C][35]. Even under a slightly basic condition [N,N-diisopropylethylamine (3 equiv.) in dry acetonitrile] after 2 h at 20 °C, compound 7 or 9 showed only ~ 5% phosphate isomerization which was detectable by ^{31}P-NMR. This indicated that the compound 7 or 9 might be suitable for our purpose to be used as the key intermediate in the synthesis of the branched oligonucleotides. In order to demonstrate the utilities of 7 and 9 in the synthesis of branched oligoribonucleotides, we herein report the synthesis of two branched trimers **14** & **19**, two pentamers **37** & **38**, and a heptamer **41**. We first converted 3′, 5′-bis-protected-N^6-benzoyladenosine 1 into its 2′-phosphodiester 2 and then to the 2′-phosphotriester 3 [o-chlorophenyl and 2-phenylsulfonylethyl (PSE) as the phosphate protecting groups] in good yields. Then the 5′-hydroxyl group from 3 was specifically released by the treatment with 0.2 M aqueous HCl in dioxane to produce compound 4 [^{31}P-NMR: -8.34 and -8.59] in 82 % yield[14,32,33]. The presence of the 2′-phosphotriester vicinal to the 3′-O-silyl group, as in 4, greatly increased the stability of the 3′-O-silyl ether bond toward the acidic treatment[34]. The PSE group was then removed by treatment with triethylamine to generate the phosphodiester 5 in 90 % yield [^{31}P-NMR: -7.32]. The compound 5 upon reaction with 4-methoxytrityl (MMTr) chloride or 9-chloro-9-(p-anisoyl)xanthen (Mpx-Cl) at the 5′ end gave 6 [^{31}P-NMR: -6.62] and 8 [^{31}P-NMR: -6.69] in 98 % and 89 % yields respectively. They were subsequently treated with 0.2 M n-tetrabutylammonium fluoride in dry THF, giving the key intermmediates 7 [^{31}P-NMR: -6.37] and 9 [^{31}P-NMR: -6.27] in 95 % and 89 % yields respectively.

As the test of our strategy, we first condensed the 7 with the 2′, 3′-di-O-acetyl- 5′-methyl diisopropylphosphoramidite **10**, using the standard phosphiteamidite methodology[35] which gave the dimer with 2′, 3′-diphosphates **11** in 70 % yield [^{31}P-NMR: -0.66, -1.07, -6.81 and -7.30]. The dimer **11** was then condensed with the 5′-hydroxyl block **12**, forming a *fully*



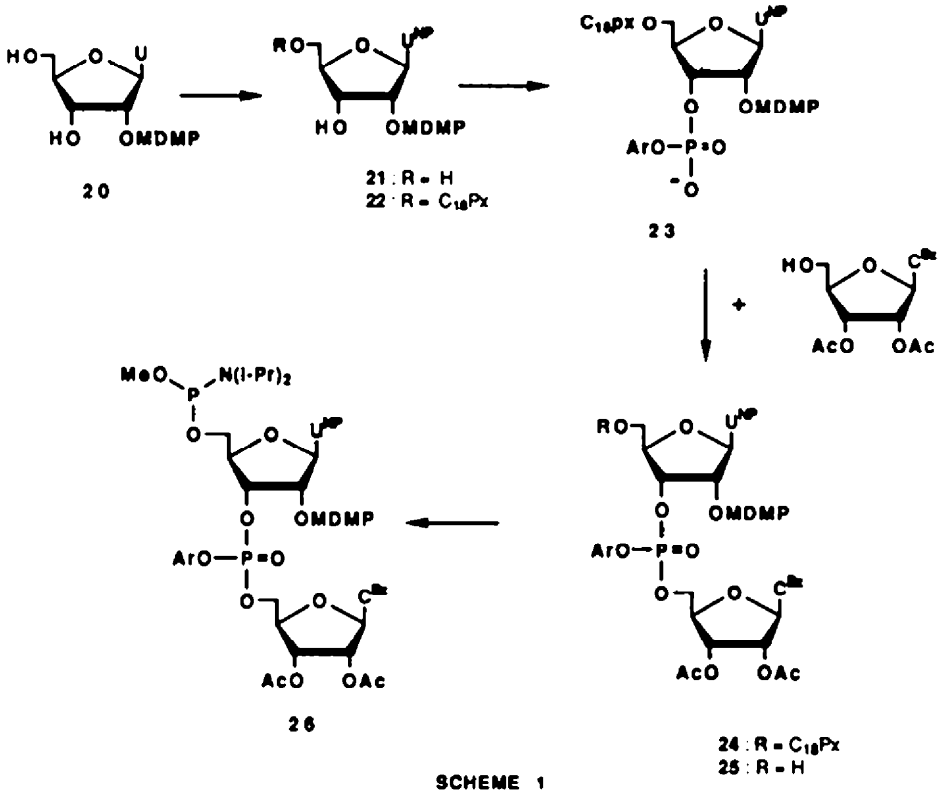
protected branched trimer **13** in 92 % yield [^{31}P-NMR: -0.15, -0.51, -0.56, -1.03, -8.18, -8.40, -8.62 and -8.86]. After the usual deprotection[36] and purification on a DEAE-Sephadex A-25 column chromatography, the naturally-occurring[1-7] branched trimer core **14** was obtained in 48 % yield. Compound **14** was then fully characterized by the ^{1}H- and ^{31}P-NMR spectroscopic study and found to be identical with the branched-trimer that we synthesized using an independent procedure[10]. Using the above methodology, we have also synthesized another branched trinucleotide **19** [**7** + **15** → **16** (78 %); **16** + **17** → **18**



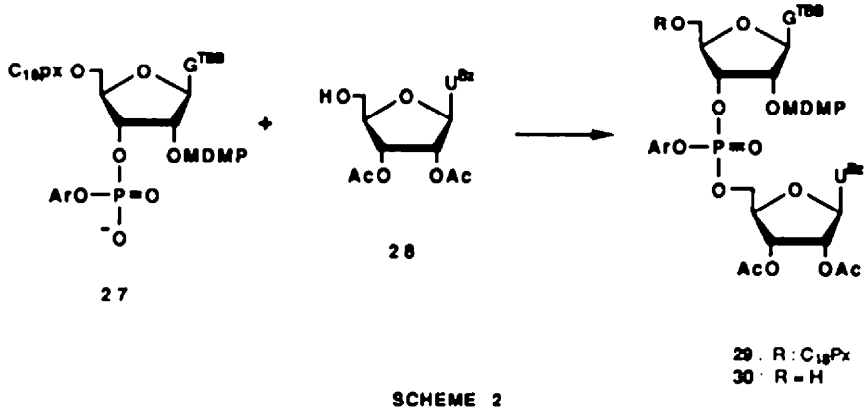
(63 %); **18** → **19** (30 %); see experimental section for details] which also has been characterized spectroscopically *(vide infra)*.

For the synthesis of the heptamer **41**, we have employed the building block **9** in which the 5′-hydroxyl function is being protected with the 9-anisoylxanthen-9-yl- (Mpx)[37]. The decision to employ the 5′-O-Mpx group was based on earlier experiences[37-39] that it could be safely and selectively removed [0.05 M trichloroacetic acid in chloroform-ethanol mixture, 98:2, v/v at 0 °C; $t_{99.5\%}$ ~2 h] from a fully protected oligonucleotide with 3-methoxy-1,5-dicarbomethoxypentane-3-yl [MDMP][38] as the 2′-OH protecting group. Compound **9** was coupled with the fully-protected dimeric 5′-methyldiisopropyl phosphoramidite **26** [*Scheme 1*: **20** → **21** → **22** → **23**, **17** + **23** → **24** → **25** → **26**] giving the trimer with a 2′-phosphodiester at the branch-point, as shown in **35**, in 71 % yield [^{31}P-NMR: -0.66, -0.88, -1.02, -1.63, -6.52, -6.62, -6.78, -7.00, -7.05, -7.32, -7.57 and -7.69]. Then, the trimer **35** was coupled with a dimer[38,39] with a free 5′-hydroxyl group, as in **30** [*Scheme 2* : **27** + **28** → **29** → **30**], giving a fully protected pentamer **36** in 75 % yield. One of the important features which was considered important in the design of the *fully protected* **36** was that the O^4 function of uridine residue, connecting to the 3′- of the branch-point adenosine residue (A*), was protected with 2-nitrophenyl group; such *fully-protected* pentamer **36** could be therefore easily converted to either uracil or cytosine moiety depending upon the exact condition of the deprotection procedure as we have reported before[10,14]. Using these procedures, we deprotected **36** in two different ways, giving two fully deprotected pentamers **37** [U as the 3′-residue at A*] and **38** [C as the 3′-residue at A*] in 26 % and 25 % yields respectively. The structures of both pentamers were verified by the ^{1}H- and ^{31}P-NMR spectroscopy *(vide infra)*. We subsequently removed the 5′-O-Mpx group from the fully protected pentamer **36** using 0.05 M trichloroacetic acid at 0 °C to release its 5′-hydroxyl group to give the compound **39** in 80 % yield. Compound **39** was then condensed with a 5′-protected dinucleotide **34** [*Scheme 3* : **31** + **32** → **33** → **34**] in presence of 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole [MSNT] using a phosphotriester methodology[36] to produce the *fully protected* heptamer **40** in 86 % yield. Compound **40** was deprotected in the usual way and purified by DEAE-Sephadex ion exchange chromatography to give the pure branched

heptamer **41** in 25.2 % yield. The ^{1}H-NMR and ^{31}P-NMR spectroscopic study provided unambiguous evidence regarding the structural integrity of the target compound **41** *(vide infra)*.



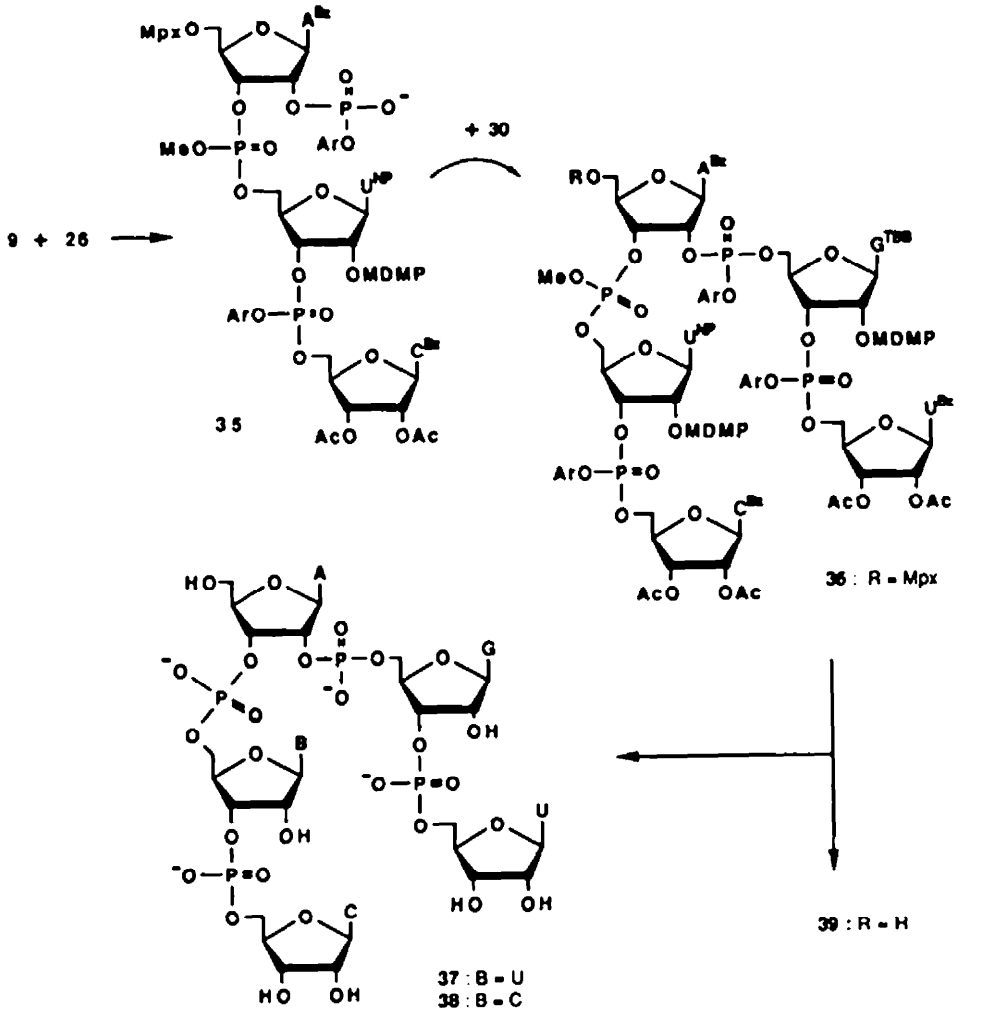
SCHEME 1

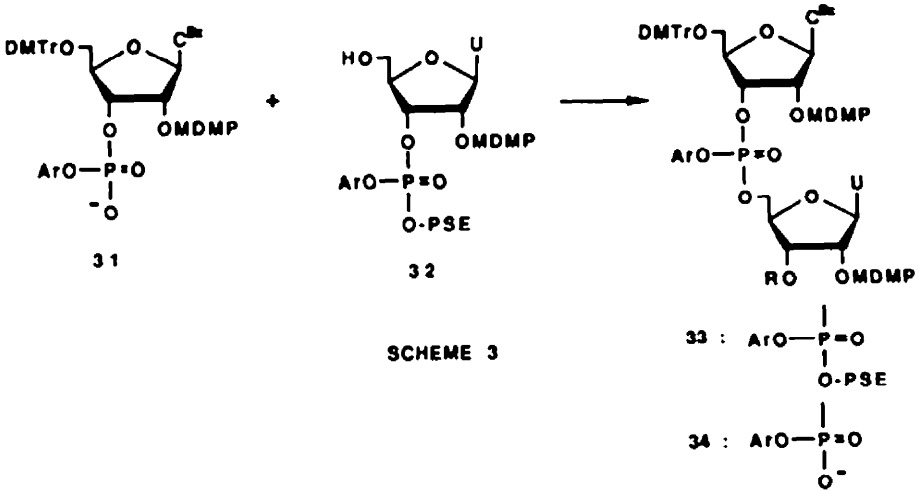


SCHEME 2

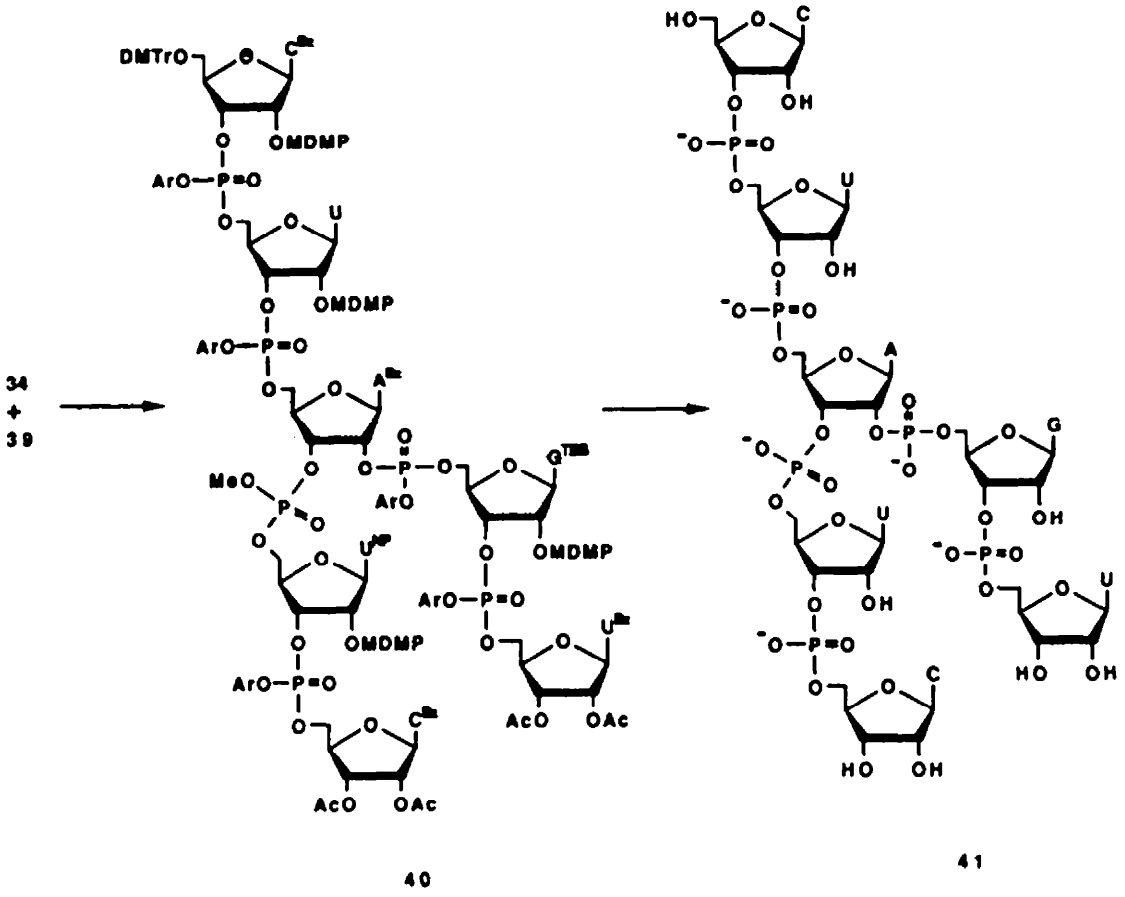
Characterization of the branched oligonucleotides by NMR spectroscopy.

The naturally occurring trimer **14** shows a ^{1}H-NMR spectrum identical to the one which was synthesized earlier by an indepen- dant method, whose ^{1}H-NMR resonances were completely assigned (Fig. 1)[10]. Similarly the analogous branched trimer **19** possesses ^{1}H resonances in the aromatic and anomeric regions which are separated enough for assignments in order to show its exact structure (Fig. 2). The ^{1}H assignment is easily done by comparison with the published ^{1}H-NMR spectra[20,21] of $A^{2'p5'G}_{3'p5'U}$ and $A^{2'p5'C}_{3'p5'G}$. In addition, 2D NMR experiments such as DOSE-SECSY (Double quantum spin-echo spectroscopy, named also DECSY) and ^{31}P / ^{1}H correlation spectra of **19** allow a clear assignment of the 2′→ 5′ and 3′ → 5′ linkages of A* as shown in Figs. 3 and 4.





SCHEME 3



The Figures 5 and 6 show the 1D spectra of the pentamers 37 and 38 (*please note the abbreviations in Scheme 4 for different sugar-base residues in 37 and 38*). The substitution of U by C at the 3′- linkage of A* in 38 allows a complete assignment of the aromatic region. Indeed H-6 has a steady coupling constant of about 7.5 Hz for C and about 8.0 Hz for U. H-2 A*, H-8 A* and H-8 G are very slightly affected by these changes and their chemical shifts are comparable to those found in branched trimers[16,20,21]. The assignment of H-1′ protons could be done in a similar way and it is further confirmed by 2D NMR experiments. Although one can clearly detect five different H-1′ protons in 37 and 38, H-1′A* and H-1′G can be only assigned unambiguously. The Figure 7 shows the TAYCOSY spectrum (Triple Relay COSY) of 37, while the connectivities between H-1′ proton and H-2′ protons of 38 are displayed in the DOSE-SECSY spectrum (Fig. 8). Finally ^{31}P / ^{1}H 2D correlation spectra of 37 and 38 (Figs. 9 and 10 respectively) established the connectivities of the phosphate linkages.

No attempt has been made to achieve a complete assignment of the ^{1}H-NMR spectrum of 41. The following spectroscopic evidence however supports the exact structure of 41. First of all, a ^{31}P NMR spectrum of 41 as its triethylammonium salt clearly shows six peaks (Fig. 11) for six internucleotide phosphodiesters. It should be noted that only 4 peaks are detected when the ^{31}P-NMR of 41 is measured as its sodium salt (Fig. 12). An inspection of the 1D spectrum of 41 (Fig. 13) reveals that the aromatic part is resolved enough to identify H-6 of C and of U. The singlets originating from H-8 and H-2 of A* and H-8 of G are also easily assigned. Note the deshielding of H-2 A* in 41 as compared with those of 14, 19, 37 or 38. This is closely identical to the features found in the branched tetramers[24]. The DQF-COSY experiment on 41 is shown in Fig. 14 which allows the detection of six cross peaks showing correlations of six H-1′ with the corresponding H-2′. We then undertook homodecoupling experiments on compound 41 by irradiating the H-2′ absorptions in order to clarify the spectral region between 5.8 and 5.7 ppm (Fig. 15), allowing the identification of its all seven H-1′ protons. These homodecoupling experiments, shown in Fig. 15, unequivocally identified three overlapped H-1′ protons and the H-5 of one of the uracil residues. These studies confirmed that the synthetic branched ribonucleotide, as shown in 41, consists of seven pentofuranose residues and associated aglycones and six phosphodiester linkages. Further 2D ^{31}P / ^{1}H studies showed that the most shielded phosphate resonance of these six phosphate linkages is 2′ → 5′ (Fig. 12).

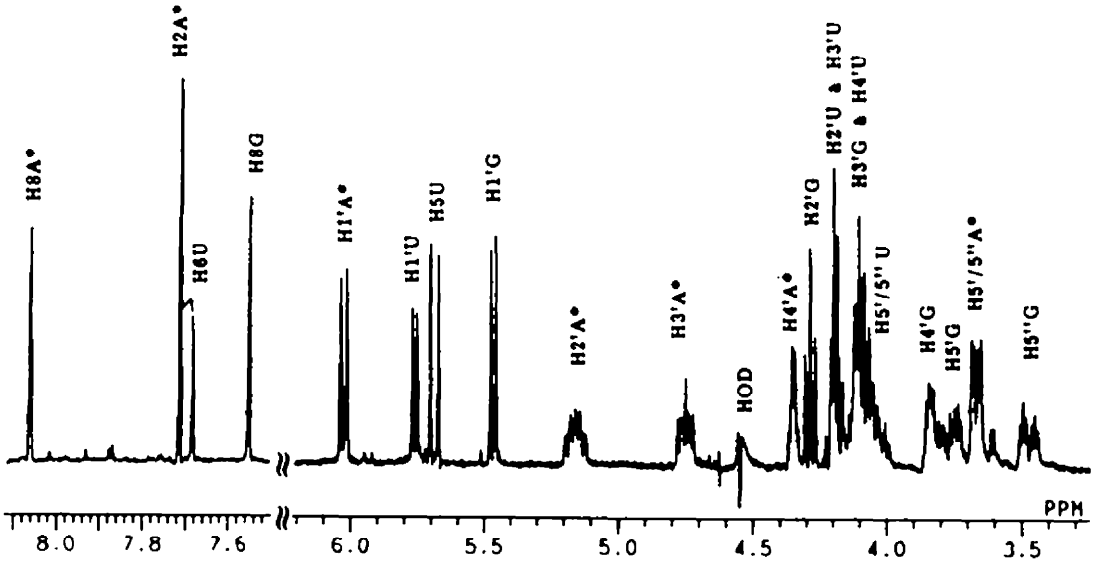


Figure 1: 270 MHz ^{1}H-NMR spectrum of **14** in ^2H_2O at 30 °C. The water peak was partly suppressed by an inversion recovery pulse sequence (WEFT).

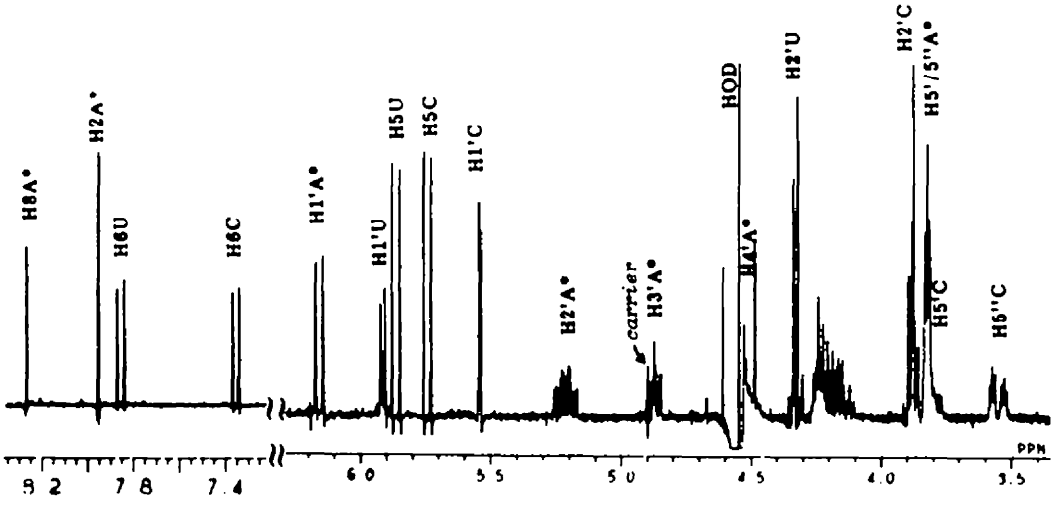


Figure 2: 270 MHz ^{1}H-NMR spectrum of **19** in ^2H_2O at 40 °C.

EXPERIMENTAL

^{1}H-NMR spectra were recorded (in δ scale) with Jeol 90 Q and JNM GX 270 spectrometer at 90 and 270 MHz respectively, using TMS (0.0 ppm) or residual HOD peak (set at 4.55 ppm at 30 °C) as the internal standards. ^{31}P-NMR spectra were recorded (in δ scale) at 36 MHz and 108 MHz in the same solvent using 85 % phosphoric acid as the external standard. TLC was carried out using Merck-pre-coated silica gel F_{254} plates in the following solvent systems: (A) Methanol-dichloromethane (9.5: 0.5, v/v), (B) methanol-dichloromethane (9:1, v/v), (C) methanol-dichloromethane (8:2, v/v), (D) ethylacetate-hexane-triethylamine (6:2:2, v/v/v). The column chromatographic separations were carried out using Merck G60 silica gel. DEAE-Sephadex A-25 from Pharmacia was used for the ion exchange chromatography.

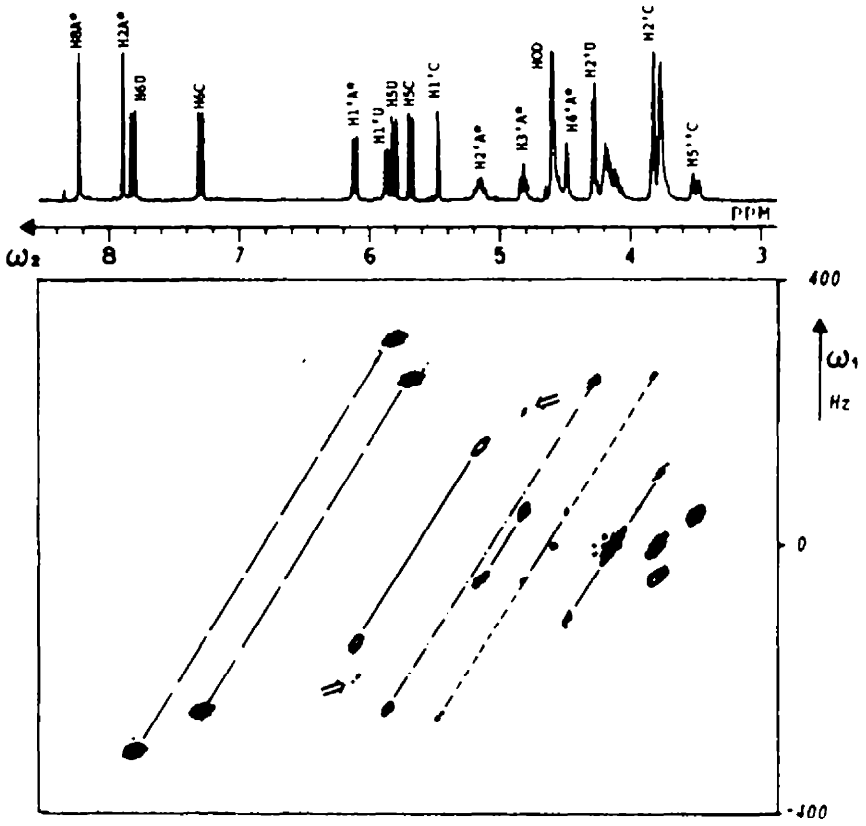


Figure 3: DOSE-SECSY 2D spectrum of 19 at 30 °C. The full J network is shown for A* by solid lines. Connectivities between H-6 and H-5, H-1′ and H-2′ of the pyrimidine residues are also shown. The double quantum coherence was created by a spin-echo with a delay of 35 ms. Note the ^4J coupling between H-1′A* and H-3′A* (⇒).

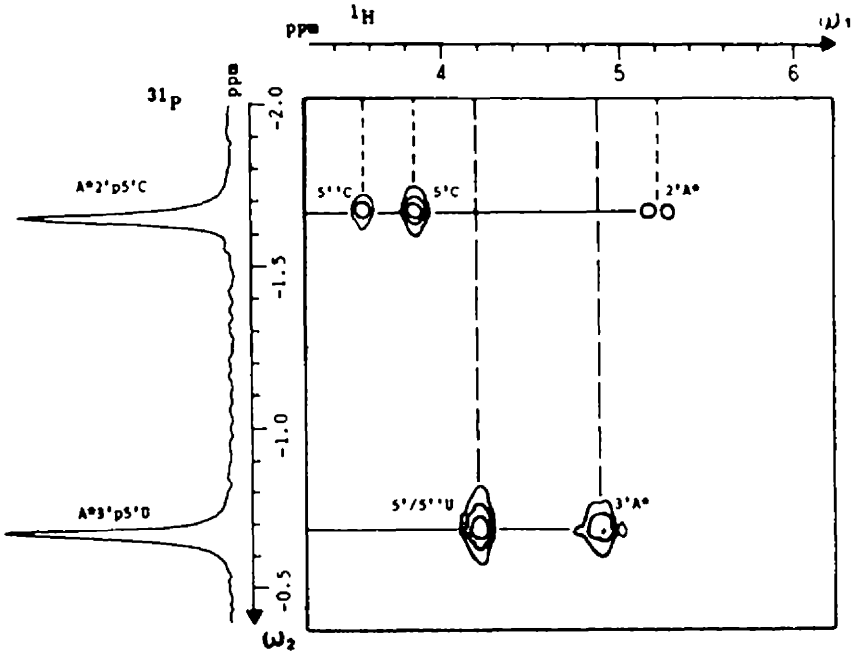


Figure 4: The 2D ^{31}P / ^1H correlation spectrum of 19 at 30 °C.

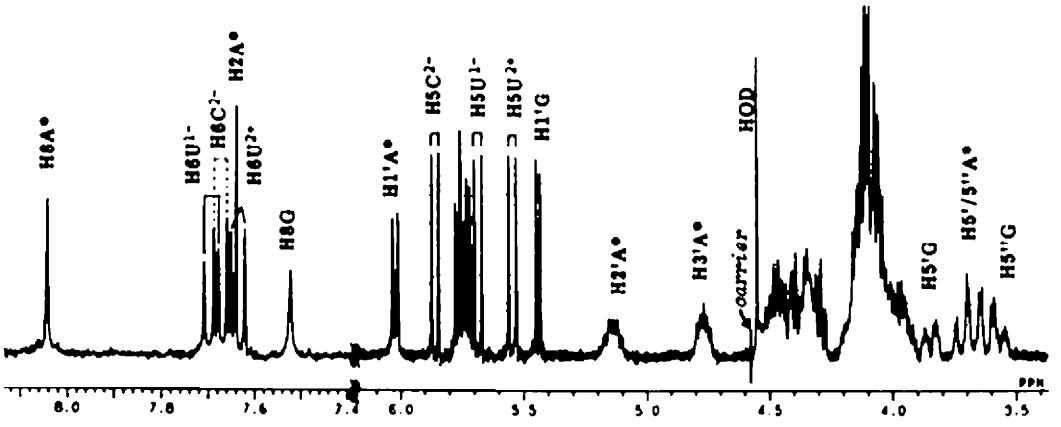


Figure 5: 270 MHz ^{1}H-NMR spectrum of 37 in ^2H_2O at 30 °C using WEFT. The numbering of the base residue is shown in scheme 4.

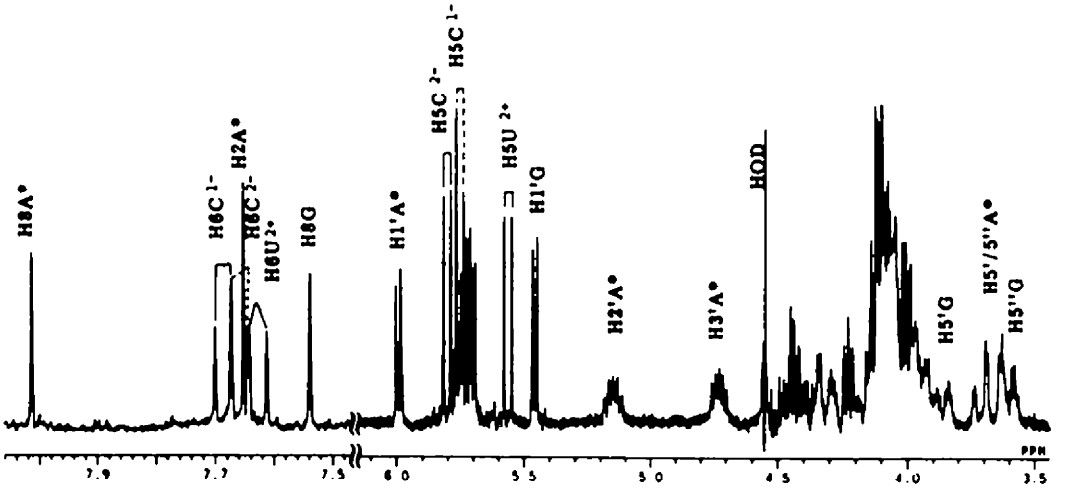
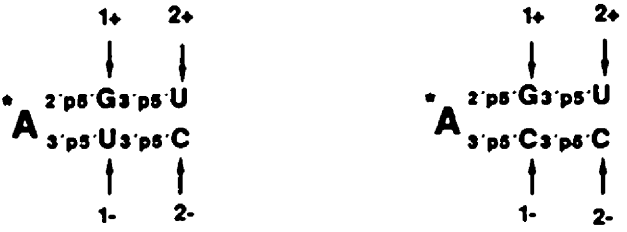


Figure 6: 270 MHz ^{1}H-NMR spectrum of 38 in ^2H_2O at 30 °C using WEFT. The numbering of the base residues are shown in scheme 4.



Schematic representations of compounds 37 and 38

Scheme 4

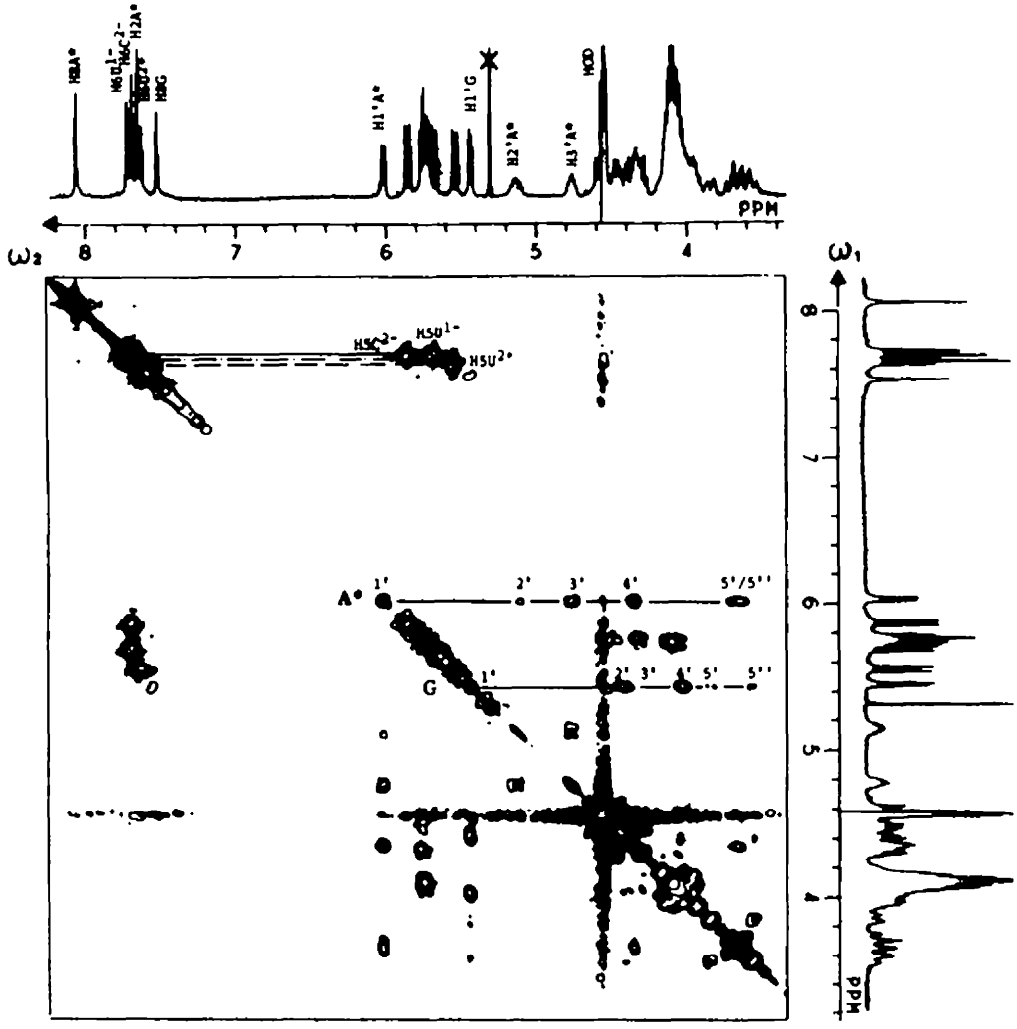


Figure 7: TAY COSY 2D spectrum of 37 at 30 °C. The delays for the relays were 120 ms each. The subspectra of A* and G were clearly detected.

After purification on DEAE-Sephadex column, the ammonium counterions of 14, 19, 37, 38 and 41 were replaced with Na^+ by passing the compounds through a Dowex (Na^+ form) column. Samples were dried from ^2H_2O and then were dissolved in 0.5 ml of ^2H_2O "100 atom % D" (Aldrich). The pH was found to be about 7.5.

The 2D NMR experiments *i.e.* triple relay COSY (TAYCOSY), double quantum filtered COSY (DQF-COSY) and ^{31}P / ^1H correlation have been performed using the reported conditions[14,24]. Regarding the double quantum coherence experiment (DOSE-6ECSY), the pulse sequence used was already described as DECSY[41]. However it needs a phase shift of 45° which was not available in our spectrometer, therefore the quadrature detection is not achieved in the ω_1 direction. Nevertheless the transmitter could be placed at the center of the spectrum, the possible artefacts being removed by symmetrization. This pulse sequence is very convenient for assignment purposes (high resolution is not needed), using a small 2D matrix size. It should be noticed that the COSY-type also exists[42], but a simple symmetrization is not possible leading to artefacts.

Synthesis of 6-N-benzoyl-3',5'-O-(tetraisopropyl-1,3-disiloxane-1,3-diyl)-2'-O-(o-chlorophenyl) phosphate (2): To the solution of 1 (4.87 g, 8 mmol) in dry pyridine (80 ml) was added the 0.25 M solution of o-chlorophenylphosphoro-bis-(1,2,4-triazolide) in acetonitrile (80 ml, 20 mmol). The reaction mixture was kept at room temperature for 25 min, and then poured into the 0.3 M triethylammonium hydrogen carbonate solution, and extracted with dichloromethane two times. The organic phase was washed with water and dried *in vacuo* . Yield: 6.97 g (96 %). ^1H-NMR ($CDCl_3$): 8.67 (s, 1H) H - 8; 8.11 (s, 1H) H-2; 8.07 - 6.92 (m, 9H) Bz and o-ClPh; 6.33 (s, 1H) H-1'; 5.41 (m, 1H) H-2'; 5.24 (m, 1H) H-3'; 4.25 (m, 1H) H-4'; 4.09 (m, 2H) H-5' and 5''; 2.96 (m, 6H) Et_3NH; 1.20 (t, 9H) Et_3NH; 1.07 and 1.00 (m, 28H) tetraisopropyldisilyl. ^{31}P-NMR ($CDCl_3$): -5.52.

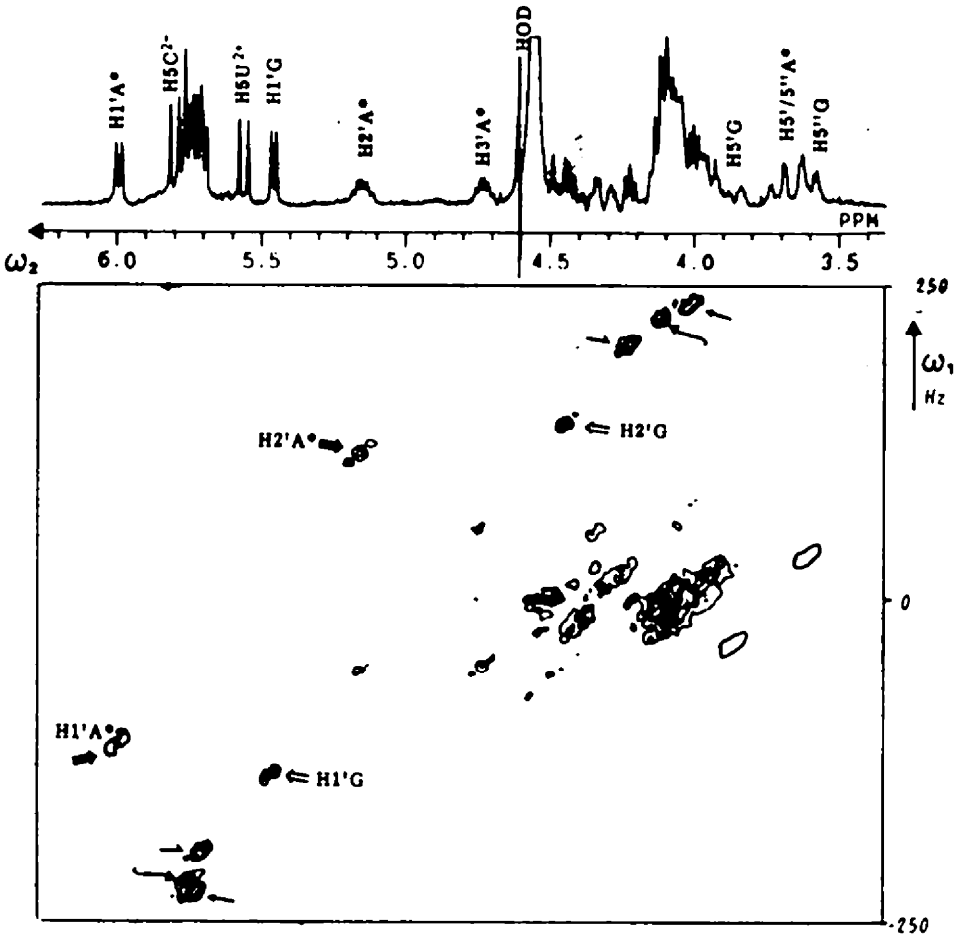


Figure 8: DQSE-SECSY 2D spectrum of 38 at 30 °C. The aromatic part is not displayed. The arrows represent the five different H-1′ and H-2′ protons from five constituent pentofuranose residues.

Synthesis of the fully protected monomer (3): Compound 2 (4.54 g, 5 mmol) and 2-phenylsulfonylethanol (1.86 g, 10 mmol) were dissolved in dry pyridine (50 ml). To this solution was added MSNT (4.4 g, 15 mmol). After 30 min, the reaction mixture was poured into a saturated sodium hydrogen carbonate (150 ml), and extracted with dichloromethane. The organic phase was washed with water two times, and dried *in vacuo* . The product was subsequently purified by short silica gel column chromatography. Yield: 4.1 g (84 %). ^{1}H-NMR ($CDCl_3$) (mixture of two diastereomers): 8.72 and 8.68 (2 x s, 1H) H-8; 8.28 (s; 1H) H-2; 8.11-7.11(m, 14H) Bz, o-ClPh and PSE; 6.21 and 5.99 (2 x s, 1H) H-1′; 5.47 (m, 1H) H-2′; 5.07 (m, 1H) H-3′; 4.67 (m, 2H) PSE; 4.08 (m, 3H) H-4′and H-5′, 5′′; 3.60 (m, 2H) PSE; 1.05 (m, 28H) tetraisopropyldisilyl; ^{31}P-NMR ($CDCl_3$): -7.45 and -8.01.

Compound 4: Compound 3 (4 g, 4.1 mmol) was dissolved in dioxane (82 ml), and to this solution was added 0.2 M aqueous hydrochloric acid (57 ml), resulting an opalescent solution. A few drops of this 0.2 M aqueous HCl solution were added frequently to keep the reaction mixture opalescent. After 2h, the reaction was quenched by pouring into the saturated sodium hydrogen carbonate solution (200 ml), and extracting with dichloromethane, The organic phase was dried and purified by silica gel column chromatography. Yield: 3.33g (82 %) ^{1}H-NMR ($CDCl_3$)(mixture of diastereomers): 8.74 and 8.67 (2 x s, 1H) H-8; 8.24 and 8.15 (2 x s, 1H) H-2; 7.99-7.14 (m, 14H) Bz, ClPh and PSE; 6.22 (m, 1H) H-1′; 5.65 (m, 1H) H-2′; 5.05 (m, 1H) H-3′; 4.37 (m, 3H) H-4′ and PSE; 3.98 (m, 2H) H-5′ and 5′′; 3.35 (m, 2H) PSE; 1.06 (m, 28H) tetraisopropyldisilyl. ^{31}P-NMR ($CDCl_3$): -8.34 and -8.59.

Compound 5: To the solution of 4 (1.42 g, 1.47 mmol) in dry pyridine (28 ml) was added triethylamine (3.9 ml, 28.6 mmol). After 45 min, the reaction mixture was dried *in vacuo* , and purified by short silica gel columnm chromatography. Yield: 1.07 g (90 %). ^{1}H-NMR ($CDCl_3$ + CD_3OD): 8.71 (s, 1H) H-8; 8.04 (s, 1H) H-2; 8.15 - 6.72 (m, 14H) Bz, PSE and o-ClPh; 6.06 (d, J = 7.6 Hz , 1H) H-1′; 5.52 (m, 1H) H-2′; 4.93 (d, J = 4.6 Hz, 1H) H-3′; 4.29 (m, 1H) H-4′; 3.87 (m, 2H) H-5′and 5′′; 2.85 (m, 6H) Et_3NH; 1.08 (m, 37 H) Et_3NH and tetraisopropyldisilyl. ^{31}P-NMR ($CDCl_3$ + CD_3OD) : -7.32.

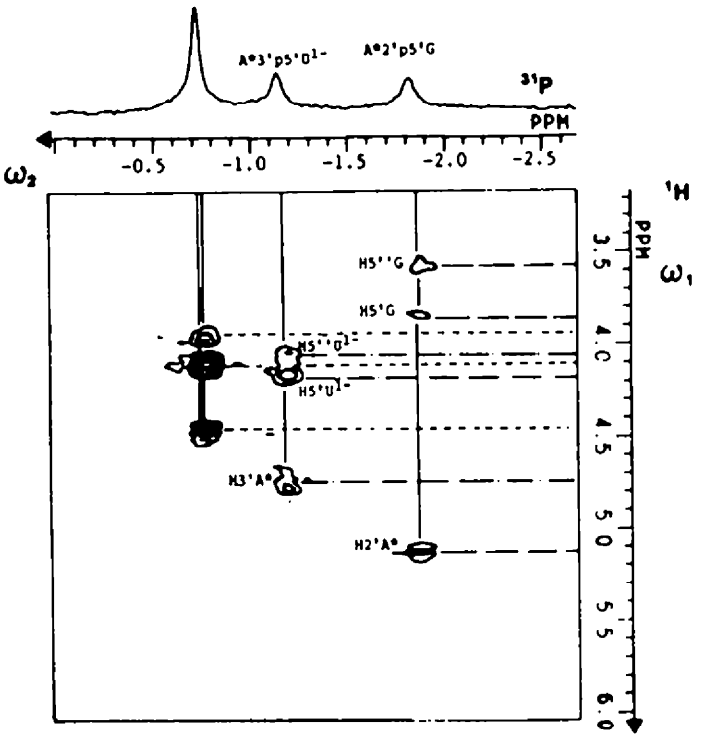


Figure 9: The 2D ^{31}P / ^{1}H correlation spectrum of **37** at 30 °C.

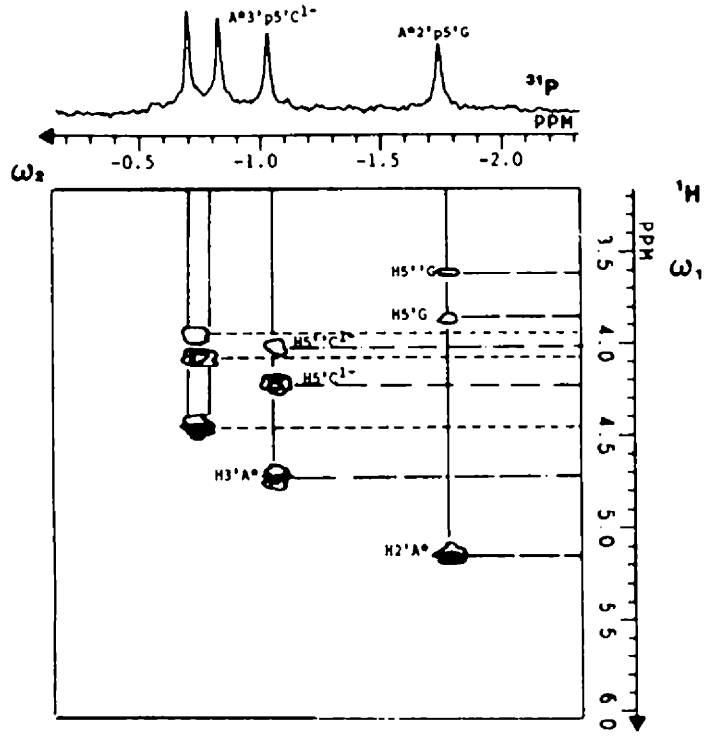


Figure 10: The 2D ^{31}P / ^{1}H correlation spectrum of **38** at 30 °C. Note that the resolution in the ω_2 direction is not good enough to distinguish amongst the most deshielded peaks.

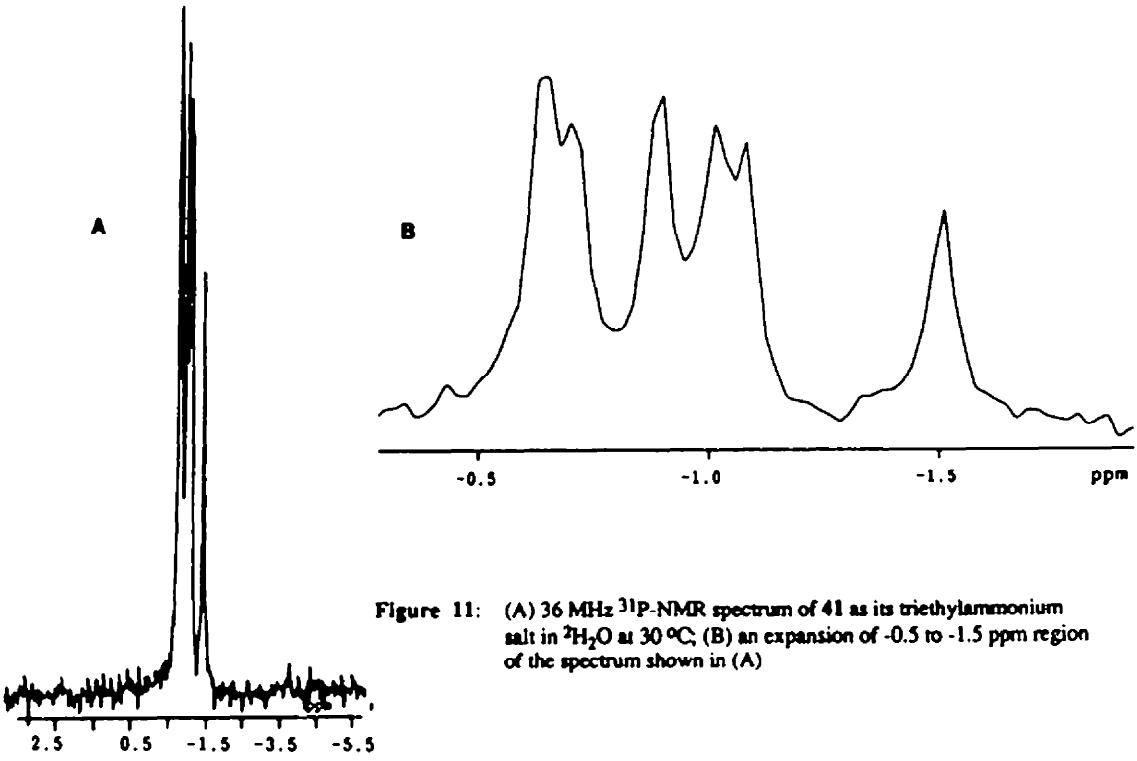


Figure 11: (A) 36 MHz ^{31}P-NMR spectrum of **41** as its triethylammonium salt in $^{2}H_2O$ at 30 °C; (B) an expansion of -0.5 to -1.5 ppm region of the spectrum shown in (A)

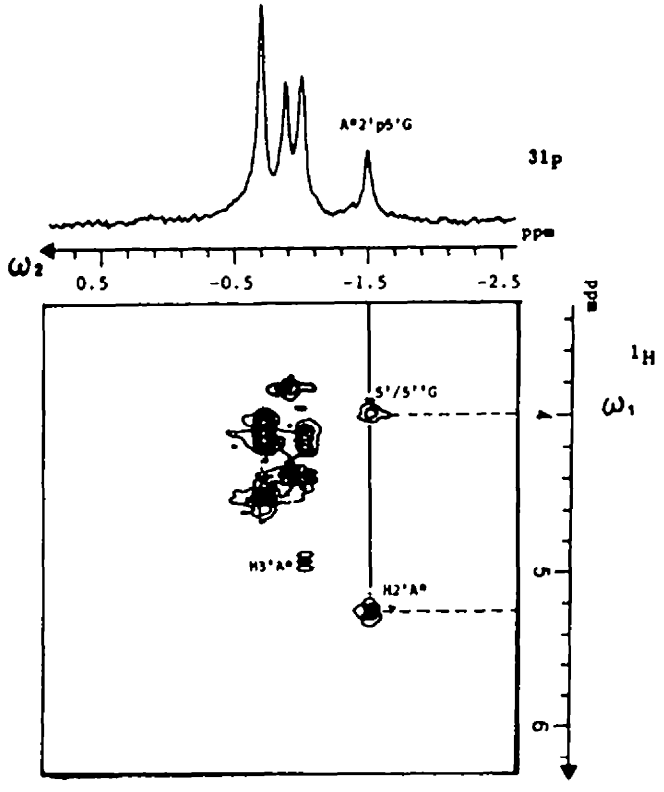


Figure 12: The 2D ^{31}P / ^{1}H correlation spectrum of **41** at 30 °C. The phosphate resonance of A*2'p5'G residue is the most shielded.

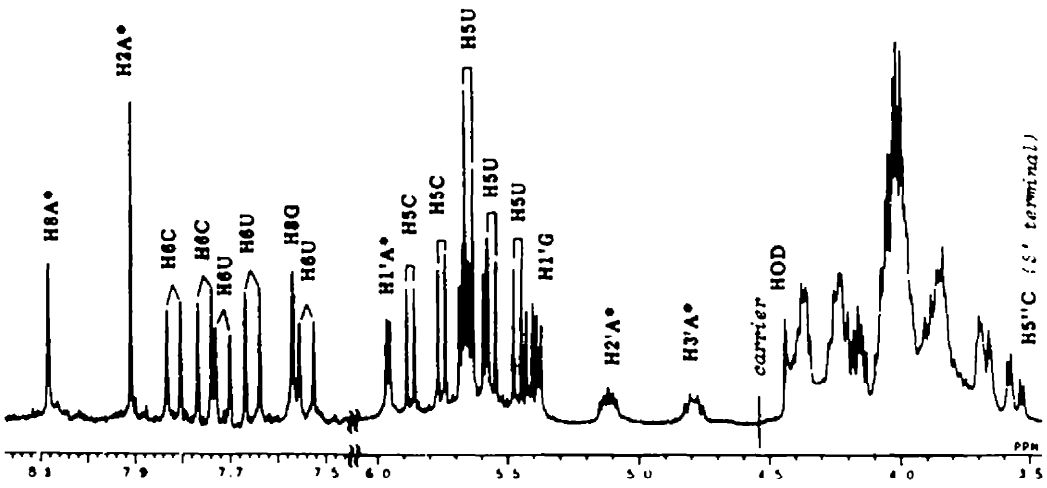


Figure 13: 270 MHz ^{1}H-NMR spectrum of 41 in ^2H_2O at 30 °C using WEFT.

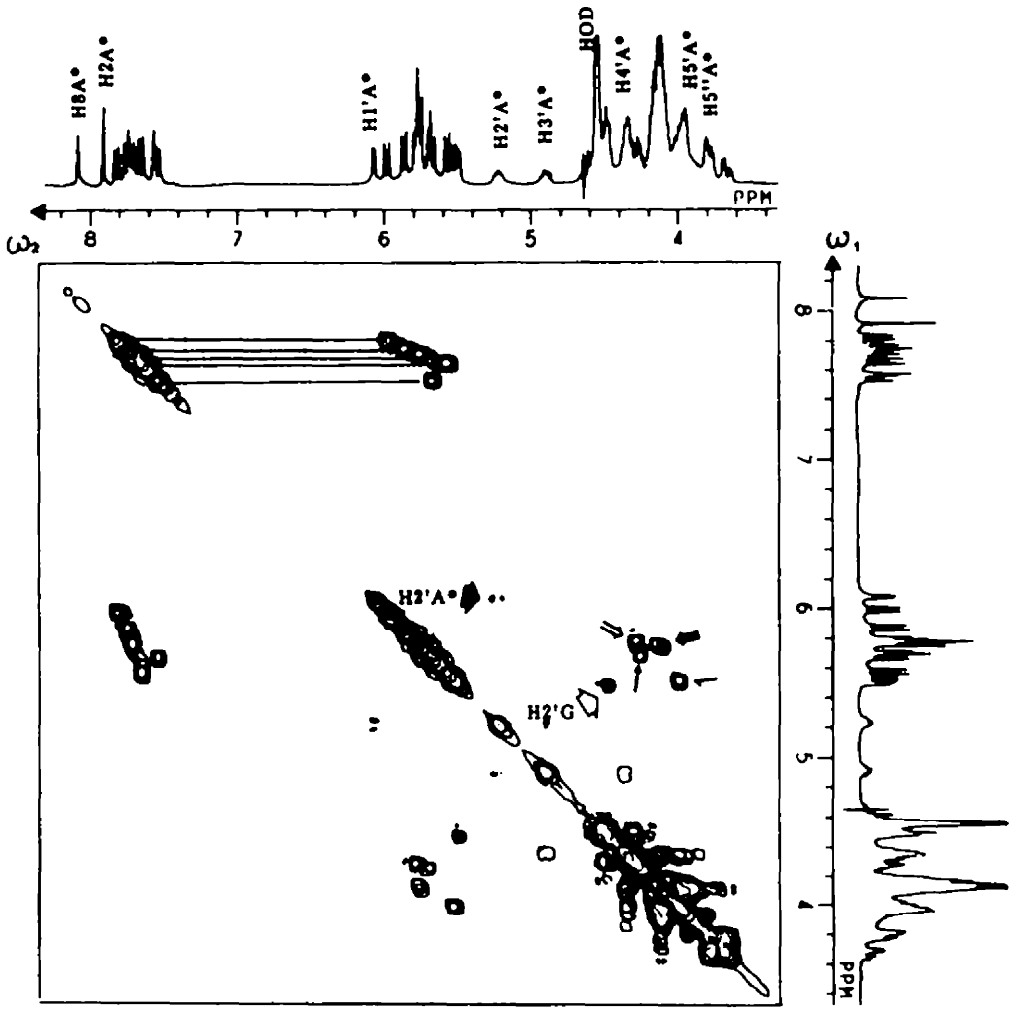


Figure 14: DQF-COSY spectrum of 41 at 30 °C. The solid lines show the correlation of H-6 and H-5 protons of the five constituent pyrimidine residues. The arrows indicate six different H-2′ protons amongst the seven expected H-2′ absorptions from seven constituent pentofuranose residues.

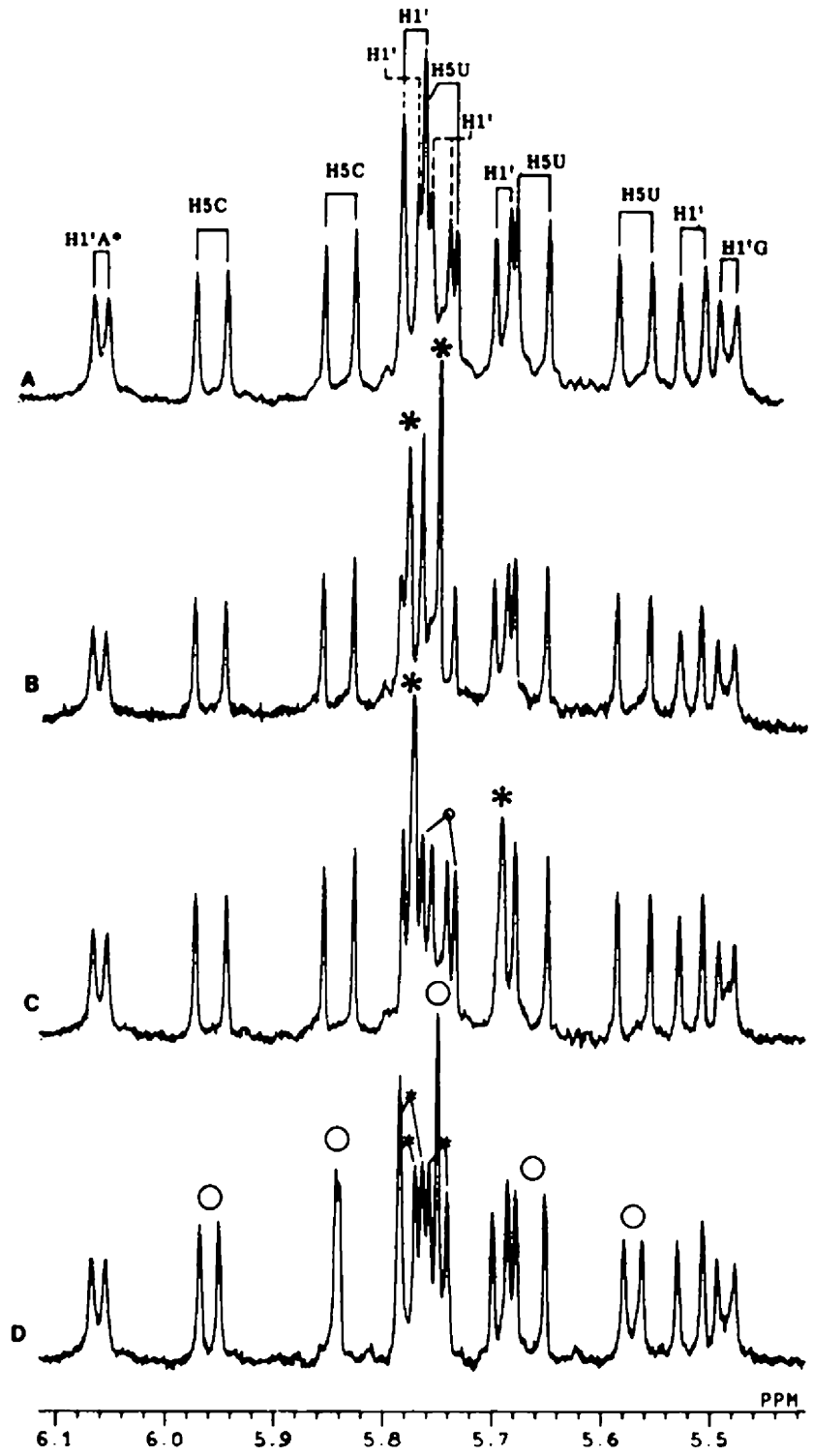


Figure 15: Homodecoupling experiments of the anomeric region of ^{1}H-NMR spectrum of **41** at 30 °C.
(A) a normal spectrum in which five H-5 protons and seven H-1′ protons could be detected.
(B) a selective decoupling at 4.11 ppm : two H-1′ protons became a singlet (✱).
(C) a selective irradiation at 4.30 ppm : two H-1′ protons became a singlet (✱). H-5 of one of the U residues was easily detected (o).
(D) a selective irradiation at 7.71 ppm : the H-5 proton detected in (C) became a singlet (○). Note that the other H-5 protons were also affected (○) owing to the off-resonance effect; three H-1′ protons in the region from 5.7 to 5.8 ppm were detected as doublets (*).

Compound 6: Compound 5 (135 mg, 0.2 mmol) was reacted with 4-methoxytrityl chloride (126 mg, 0.4 mmol) in pyridine (3 ml) for 18 h at 20 °C. After work up with 0.3 M aqueous triethylammonium bicarbonate solution, the product was isolated by silica gel column chromatography. Yield : 165 mg (98 %). ^{1}H-NMR ($CDCl_3$): 8.96 (s, 1H) NHBz; 8.62 (s, 1H) H-8; 8.08 (s, 1H) H-2; 8.86 - 6.73 (m, 23 H) Bz, MMTr and o-ClPh; 6.33 (d, J = 6.6 Hz, 1H) H-1′; 5.65 (m, 1H) H-2′; 5.02 (m, 1H) H-3′; 4.31 (m, 1H) H-4′; 3.76 (s, 3H) OMe; 3.42 (m, 2H) H-5′and 5′′; 2.86 (m, 6H) Et_3NH; 1.02 (m, 37H) Et_3NH and tetraisopropyldisilyl. ^{31}P -NMR ($CDCl_3$) : -6.62.

Compound 7: To a solution of 6 (118 mg, 0.1 mmol) in dry THF (2.8 ml) , was added n-tetrabutylammonium fluoride in dry THF (1 M, 0.7 ml, 0.7 mmol). After 20 min, the reaction was evaporated and purified by short silica gel column chromatography. Yield: 89 mg (95 %). ^{1}H-NMR: ($CDCl_3$ + CD_3OD) : 8.65 (s, 1H) H-8; 8.17 (s, 1H) H-2; 8.15 - 6.73 (m, 23H) Bz, MMTr and o-ClPh; 6.27 (d, J = 5.9 Hz, 1H) H-1′; 5.43 (m, 1H) H-2′; 4.76 (t, J = 4.2 Hz, 1H) H-3′; 4.33 (dd, J = 7.8 Hz, 1H) H-4′; 3.42 (m, 2H) H-5′and 5′′; 2.75 (m, 6H) Et_3NH; 1.21 (m, 9H) Et_3NH. ^{31}P-NMR ($CDCl_3$ + CD_3OD): -6.37.

Compound 8: To the solution of 5 (740 mg, 0.8 mmol) in pyridine (10 ml) was added 0.25 M solution of 9-chloro-9-(p-anisoyl)xanthane in dichloromethane (4.8 ml, 1.2 mmol). After reaction for 30 min, the mixture was worked up with 0.3 M triethylammonium bicarbonate solution (pH 7.5) and purified by silica gel column chromatography. Yield 1.07 g (89 %). ^{1}H-NMR ($CDCl_3$ + pyridine-d_5): 9.01 (s, 1H) NHBz; 8.61 (s, 1H) H-8; 8.16 (s, 1H) H-2; 8.05 -6.72 (m, 21H) Bz, Mpx and o-ClPh; 6.32 (d, J = 7.5 Hz, 1H) H-1′; 5.65 (m, 1H) H-2′; 4.95 (dd, J = 4.7 Hz, 1H) H-3′; 4.24 (m, 1H) H-4′; 3.70 (s, 3H) OMe; 3.25 (m, 2H) H-5′and 5′′; 2.87 (m, 6H) Et_3NH; 1.13 (m, 27H) Et_3NH and tetraisopropyldisilyl. ^{31}P-NMR ($CDCl_3$ + pyridine-d_5) : -6.69.

Compound 9: Compound 8 (847 mg, 0.76 mmol) was treated with n-tetrabutylammonium fluoride (4.9 mmol) in dry THF (24.5 ml) in a similar way as for the preparation of 7. Yield: 600 mg, (89 %). ^{1}H-NMR ($CDCl_3$ + pyridine-d_5): 9.10 (s, 1H) NHBz; 8.64 (s, 1H) H-8; 8.16 (s, 1H) H-2; 8.08 - 6.64 (m, 21H) Bz, Mpx and o-ClPh; 6.24 (d, J = 6.1 Hz, 1H) H-1′; 5.38 (m, 1H) H-2′; 4.73 (dd, J = 4.6 Hz, 1H) H-3′; 4.25 (m, 1H) H-4′; 3.71 (s, 3H) OMe; 3.29 (m, 2H) H-5′and 5′′; 3.02 (m, 6H) Et_3NH; 1.31 (t, 9H) Et_3NH. ^{31}P-NMR ($CDCl_3$ + pyridine-d_5): -6.27.

Compound 11: Compounds 7 (75 mg, 0.08 mmol), 10 (428 mg, 0.7 mmol) and 1,2,3,4-tetrazole (490 mg, 7 mmol) were dissolved in dry acetonitrile (5 ml). After reaction for 30 min, 0.1 M iodine in mixture of THF : water : pyridine (8:1:1, v/v/v) was added to the reaction mixture until the iodine color did not fade. After 20 min, the reaction was worked up with 0.3 M triethylammonium bicarbonate solution (pH 7.5) and dichloromethane, and purified by short silica gel column chromatography. Yield: 82 mg, (70 %), R_f: 0.55 (Solvent C) ^{31}P-NMR ($CDCl_3$): -0.66, -1.07, -6.81 and -7.30.

Compound 13: Compounds 11 (67 mg, 0.05 mmol) and 12 (53 mg, 0.1 mmol) and MSNT (104 mg, 0.35 mmol) were dissolved in dry pyridine (3 ml). After reaction for 1 h and usual work up, the product was isolated by silica gel column chromatography. Yield: 81 mg, (92 %). R_f: 0.5 (Solvent B). ^{31}P-NMR ($CDCl_3$) : -0.15,-0.51, -0.56, -1.03, -8.18, -8.40, -8.62 and -8.86.

Compound 14: Compound 13 (53 mg, 0.03 mmol) was dissolved in Dioxane : water (8:2, v/v) (9 ml) solution. To the solution, *syn*-4-nitrobenzaldoxime (104 mg, 0.63 mmol) and 1,1,3,3-tetramethylguanidine (0.078 ml, 0.6 mmol) were added. After 20 h, concentrated ammonia (40 ml, d = 0.9) was added. The reaction mixture was kept for 6 days, and then evaporated *in vacuo* . The residue was treated with 80 % acetic acid (30 ml) for 5 h. After evaporation, the residue was dissolved in water, extracted with dichloromethane. The aqueous phase was dried *in vacuo* , and then dissolved in water and applied to DEAE-Sephadex A-25 column (HCO_3^- form). The column was eluted with a linear gradient of 0 - 0.3 M triethylammonium bicarbonate solution (1000 ml / 1000 ml, pH 7.5). Appropriate fractions were pooled, dried and coevaporated with distilled water for a few times to remove salt to give 14. Yield: 502 A_{260} units (48 %).

Compound 16: Compound 7 (65 mg, 0.07 mmol) was condensed with 15 (247 mg, 0.49 mmol) in acetonitrile (5 ml) in presence of tetrazole (343 mg , 4.9 mmol) in the similar way as described for the preparation of 13. Yield: 71 mg, (78 %). ^{31}P-NMR: ($CDCl_3$): -0.54, -0.66, -1,46, -1.64, -5.86, -6.20, -6.32 and -6.59.

Compound 18: Compounds 16 (65 mg, 0.05 mmol) and 17 (43 mg, 0.1 mmol) were coupled in pyridine (3 ml) in presence of MSNT (1.3 mg, 0.35 mmol) by usual phosphotriester method. Yield: 50 mg, (63 %). ^{31}P-NMR ($CDCl_3$): -0.54, -0.61, -1.22, -1.29, -7.10, -7.22, -7.47, -7.62 and -8.01.

Compound 19: Compound 18 (40 mg, 0.025 mmol) was deprotected in the similar way as described for the deprotection of 13, except a treatment of 80 % formic acid for 24 h for removal of the isopropylidine group[40] instead of 80 % acetic acid. Yield: 225 A_{260} units (30 %):

Synthesis of O^4-(2-nitrophenyl)-2′-O-(3-methoxy-1,5-dicarbomethoxypentan-3-yl) [MDMP] uridine (21): To the solution of 20 (4.37 g, 9.5 mmol) in dichloromethane (142 ml) was added triethylamine (7.93 ml, 57 mmol) and chlorotrimethylsilane (7.2 ml, 57 mmol). After 40 min, the reaction was worked up in the usual way. The organic phase was dried and dissolved in dichloromethane (142 ml). To the solution were added triethylamine (13.2 ml, 95 mmol), mesitylene-sulfonyl chloride (6.24 g, 28.5 mmol) and dimethylaminopyridine (232 mg, 1.9 mmol). After 1 h, 2-nitrophenol (9.3 g, 66.5 mmol) and 1,4-diazabicyclo[2.2.2]octane (211 mg, 1.9 mmol) were added. The reaction was kept for 2 h, and then worked up in the usual way. The residue was treated with 0.1 M n-tetrabutylammonium fluoride in dry THF (95 ml) for 20 min. After evaporation, the product was isolated by short silica gel column chromatography. Yield: 5.06 g, (87 %). ^{1}H-NMR ($CDCl_3$ + CD_3OD + pyridine-d_5): 8.34 (d, J = 7.3 Hz, 1H) H-6; 8.18 - 7.20 (m, 4H) NP; 6.29 (d, J = 7.3 Hz, 1H) H-5; 5.91 (d, J = 5.9 Hz, 1H) H-1′; 4.66 (dd, J = 5.9 Hz, 1H) H-2′; 4.22 (m, 1H) H-3′; 4.16 (m, 1H) H-4′; 3.83 (m, 2H) H-5′and 5′′; 3.69 and 3.68 (2 x s, 6H) MDMP; 3.06 (s, 3H) MDMP; 2.27 (m, 8H) MDMP.

Compound 22: Compound 21 (5.06 g, 8.6 mmol) was reacted with 4-octadecyloxyphenylxanthenyl chloride (12.9 mmol) in pyridine (50 ml) by the literature method[38]. Yield: 7.10 g (75 %). ^{1}H-NMR ($CDCl_3$ + CD_3OD + pyridine-d_5): 8.51 (d, J = 7.6 Hz, 1H) H-6; 8.18-6.79 (m, 16H) NP and C_{18}-px; 6.12 (d, J = 4.6 Hz, 1H) H-1'; 6.07 (d, J = 7.3 Hz, 1H) H-5; 4.61 (t, J = 4.4Hz, 1H) H-2'; 4.17 (m, 2H) H-3' and H-4'; 3.94 (t, 2H) C_{18}-px; 3.68 and 3.64 (2 x s, 6H) MDMP; 3.29 (m, 2H) H-5'and 5''; 3.17 (s, 3H) MDMP; 2.14 (m, 8H) MDMP; 1.25 and 0.88 (m, 35H) C_{18}-px.

Compound 23: Compound 22 (6.57 g, 6 mmol) was phosphorylated with o-chlorophenyl-phosphorodichloridate (15 mmol) in pyridine in the usual way. Yield: 8.1 g (99 %). ^{1}H-NMR ($CDCl_3$ + pyridine-d_5): 8.37 (d, J = 8.0 Hz, 1H) H-6; 8.13-6.76 (m, 20H) NP, C_{18}-px and o-ClPh; 6.38 (d, J = 6.6 H, 1H) H-1'; 6.05 (d, J = 8.0 Hz, 1H) H-5; 4.81 (m, 2H) H-2'and H-3'; 4.53 (m, 1H) H-4'; 3.92 (m, 2H) C_{18}-px; 3.59 and 3.53 (2 x s, 6H) MDMP; 3.15 (s, 3H) MDMP; 3.12-3.01 (m, 8H) H-5', 5''and Et_3NH ; 1.96 (m, 8H) MDMP; 1.24 (m, 44H) C_{18}-px, Et_3NH. ^{31}P-NMR ($CDCl_3$ + pyridine-d_5): -5.91.

Compound 24: Compound 23 (8.11 g, 6 mmol) and 17 (3.12 g, 7.2 mmol) were condensed in presence of MSNT (5.36 g, 18 mmol) in the usual way. Yield: 7.68 g (76 %) . ^{31}P-NMR ($CDCl_3$ + pyridine-d_5): -6.89 and -7-98.

Compound 25: Compound 24 (7 g, 4.7 mmol) was treated with 0.05 M trichloroacetic acid in chloroform : ethanol (98:2, v/v) (940 ml) at 0 °C for 2 h. After work up, the product was isolated by silica gel column. Yield: 4.65 g, (82 %). ^{31}P-NMR ($CDCl_3$ + CD_3OD): -4.54 and -7.61.

Compound 26: To the solution of 25 (3.52 g, 3 mmol) in dichloromethane (21 ml) were added N,N-diisopropylamino methoxy chlorophosphine (1.456 ml, 7.5 mmol) and N,N-diisopropylethylamine (2.62 ml, 15 mmol). After reaction for 40 min, the reaction mixture was worked up by partitioning between saturated sodium chloride and ethylacetate. The organic phase was dried and purified by silica gel column chromatography. Yield: 2.96 g (74 %) R_f: 0.3 (Solvent D). ^{31}P-NMR ($CDCl_3$ + pyridine-d_5): 150.97, 150.43, -6.84, -6.96 and -7.76.

Compound 29: Compounds 27 (4.85 g, 3.5 mmol) and 28 (1.25 g, 2.92 mmol) were condensed in presence of MSNT (3.1 g, 10.5 mmol) in pyridine by phosphotriester method. Yield: 3.87 g, (83 %). ^{31}P-NMR ($CDCl_3$ + pyridine-d_5): -7.28 and -7.40.

Compound 30: Compound 29 (3.87 g, 2.3 mmol) was treated with 0.05 M trichloroacetic acid in chloroform : ethanol (98:2, v/v) (460 ml) at 0 °C for 2 h. After work up, the product was purified from silica gel column chromatography. Yield: 2.05 g, (70 %). ^{31}P-NMR ($CDCl_3$): -6.49 and -8.49.

Compound 33: Compounds 31 (523 mg, 0.45 mmol) and 32 (291 mg, 0.35 mmol) were coupled in presence of MSNT (399 mg, 1.35 mmol) by the phosphotriester methodology. Yield: 583 mg (84 %) ^{31}P-NMR ($CDCl_3$ + pyridine-d_5): -6.12, -6.98, -7.07, -7.27, -7.39, -7,49, -7.62 and -7.71.

Compound 34: Compound 33 (396 mg, 0.2 mmol) was treated with triethylamine (0.56 ml, 4 mmol) in pyridine (6 ml) for 40 min. After evaporation, the product was purified by silica gel column chromatography. Yield: 345 mg (91 %). ^{31}P-NMR ($CDCl_3$ + pyridine-d_5): -5.49, -5.63, -6.98 and -7.03.

Compound 35: Compounds 9 (289 mg, 0.3 mmol) and 26 (1.4 g, 1.05 mmol) and tetrazole (735 mg, 10.5 mmol) were dissolved in acetonitrile (20 ml). After reaction for 20 min, the 0.1 M iodine in THF:pyridine:water (8:1:1, v/v/v) was added to the mixture until the color was not discharged. After 20 min, the reaction was worked up, and purified by silica gel column. Yield: 472 mg, (71 %). ^{31}P-NMR ($CDCl_3$ + pyridine-d_5): -0.66, -0.88, -1.02, -1.63, -6.52, -6.62, -6.78, -7.00, -7.05, -7.32, -7.57 and -7.69.

Compound 36: Compounds 35 (370 mg, 0.167 mmol) and 30 (333 mg, 0.25 mmol) were coupled in presence of MSNT (247 mg, 0.83 mmol) using the phosphotriester methodologies. Yield: 433 mg (75 %).

Compound 39: Compound 36 (242 mg, 0,07 mmol) was deblocked at its 5'-hydroxyl in similar way as described for the preparation of 25. Yield: 177 mg (80 %).

Compound 40: Compounds 39 (158 mg, 0.05 mmol) and 34 (190 mg, 0.1 mmol) were coupled in presence of MSNT (207 mg, 0.7 mmol) in pyridine using the phosphotriester methodology. Yield: 214 mg (86 %).

Deprotection of Compound 36 to 37: Compound 36 (104 mg, 0.03 mmol) was deprotected in the similar way as described for the deprotection of 13. For the elution of the DEAE-Sephadex A-25 column chromatography, a linear gradient of 0 - 0.6 M triethylammonium bicarbonate solution (1500 ml / 1500 ml, pH 7.5) was used. Yield: 387 A_{260} units (26 %).

Deprotection of Compound 36 to 38: Compound 36 (52 mg, 0.015 mmol) was treated with 0.05 M tetrabutylammonium fluoride in THF:pyridine:water (8:1:1, v/v/v) (6 ml) for 4 h, and then evaporated. The residue was dissolved in dry THF (7 ml), and liquid ammonia was added to the solution. After two days at room temperature, the mixture was evaporated and dissolved in saturated aqueous ammonia (60 ml, d = 0.9) for 7 days. After evaporation, it was treated with 80 % acetic acid (40 ml) for 5 h. The solution was evaporated *in vacuo* , and extracted with water / dichloromethane. The aqueous layer was applied to DEAE-Sephadex A-25 Column, eluted with 0 - 0.6 M triethylammonium bicarbonate solution (1200 ml / 1200ml, pH 7.5). Yield: 177 A_{260} units (25 %)

Deprotection of 40 to 41: Compound 40 (198 mg, 0.04 mmol) was deprotected to 41 in the similar way as described for the preparation of 37. For the elution of DEAE-Sephadex A-25 column, 0 - 0.7 M triethylammonium bicarbonate (pH 7.5) was used . Yield: 665 A_{260} units (25.2 %).

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