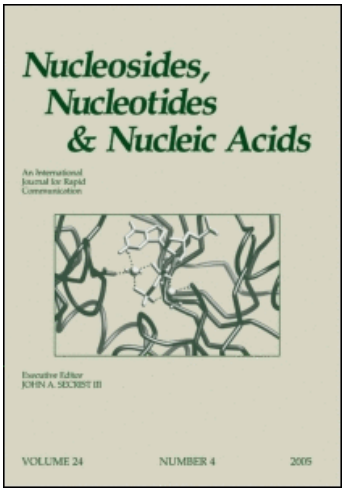

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The Stability of Trisubstituted Internucleotide Bond in the Presence of the Vicinal 2'-Hydroxyl. Chemical Synthesis of Uridyl(2'-phosphate)-(3'-5')-uridine

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THE STABILITY OF TRISUBSTITUTED INTERNUCLEOTIDE BOND IN THE PRESENCE OF THE VICINAL 2'-HYDROXYL. CHEMICAL SYNTHESIS OF URIDYL(2'-PHOSPHATE)-(3'-5')-URIDINE.

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ABSTRACT: The effect of eleven different phosphoryl center protecting groups on the stability of trisubstituted internucleotide bond of the dimers (**1a-k**), in the presence of the vicinal 2'-hydroxyl, was examined. It has been found that electronic properties of the phosphoryl center protecting groups are essential for the reactivity of the trisubstituted internucleotide bond. Those observations were applied to the chemical synthesis of the uridyl(2'-phosphate)-(3'-5')-uridine, a useful model for further pre-tRNA splicing studies.

INTRODUCTION

The modifications of nucleosides, nucleotides and oligonucleotides affect their functions and are often biologically important[1]. They can classified according to types, e.g.: methylation or their position in the molecule, e.g.: N-7 of the heterocyclic base. The 2'-O-modifications which include methylation[2], phosphorylation[3], ribosylation[4] and the addition of some RNA[5] or DNA[6] fragments (e.g.: branch RNA) represent one type of such modification. Although the chemical syntheses of various 2'-O-modified analogues have already been reported[7-10], according to my best knowledge, the chemical synthesis of 2'-O-phosphorylated oligoribonucleotides has not yet been published[8].

It was found that processing of the pre-tRNA's occurred via the 2'-O-phosphorylated intermediate[11]. Recently, it was reported that an enzyme (2'-phosphotransferase) transfers the phosphate residue from the 2'-O-phosphorylated intermediate to NAD molecule[12]. The process can be studied in detail on uridyl(2'-phosphate)-(3'-5')-uridine (**16**) as a model of pre-tRNA, and this paper presents the chemical synthesis of the model dimer. The synthesis proceeds via the 2'-hydroxyl intermediate containing a diester internucleotide

bond (**13**). The effect of eleven different protecting groups of phosphoryl center on the chemical stability of this trisubstituted internucleotide bond in the presence of vicinal 2'-hydroxyl is also described.

The chemical instability of the triester internucleotide bond in the presence of vicinal 2'-hydroxyl group was previously reported. Reese and Skone[13] demonstrated several subsequent side reactions which result from the deprotection of the 2'-hydroxyl group in the presence of the internucleotide bond protected by o-chlorophenyl. In the reported paper, the intramolecular attack of 2'-hydroxyl on phosphotriester resulted in a hypothetical intermediate, 2',3'-cyclic phosphotriester (**8**). A subsequent hydrolysis of this intermediate (**8**) caused a cleavage of the internucleotide bond and the formation of (3'-5') and (2'-5') isomers. On the other hand, Tanimura et. al.[14] claim that the removal of the 2'-O-acid labile protecting group during the chemical synthesis of RNA (bearing 2-cyanoethyl at phosphoryl center) resulted in a cleavage of the internucleotide bond only. Finaly, Fourrey et. al.[15] reported the application of internucleotide methyl phosphotriester with 2'-hydroxyl in the vicinal position to the synthesis of a branched RNA fragment. Those three papers suggest that the nature of the phosphoryl center protecting group can affect the stability of the triester internucleotide bond in the presence of vicinal 2'-hydroxyl group.

RESULTS AND DISCUSSION

The stability of trisubstituted internucleotide bond in the presence of vicinal 2'-hydroxyl.

To complete the above mentioned observations, a series of eleven model dimers (**1a-k**) was synthesized and analyzed for their stability in acidic conditions (Figure 1). The tested phosphoryl center protecting groups can be classified in to four categories: *O-alkyl* [methyl (Me), 2-cyanoethyl (EtCN), 2,2,2-trichloroethyl (TCE), n-propyl (nPr) and isopropyl (iPr)], *O-aryl* [phenyl (Phe), o-chlorophenyl (oClPhe) and p-methoxyphenyl (pMeOPhe)], *N-alkyl*[n-butyl (nBu) and di-n-butyl (nBu_2)] and *H-phosphonate*. The dimers were treated with 1% trifluoroacetic acid in dichloromethane at room temperature for 15 min. for completion of 5'- and 2'-protecting group removal. Reaction mixtures were analyzed by TLC on silica. Qualitative and quantitative results of the transformations observed are collected in Table 1. It is clear that the type of the phosphoryl center protecting group is essential for the reactivity of trisubstituted internucleotide bond in the presence of vicinal 2'-hydroxyl group. Three kinds of rearrangements on the phosphorus center were observed: (i) removal of 2'-hydroxyl and phosphoryl center protecting groups *with cleavage* of the internucleotide bond (to **4** and **5**), (ii) removal of 2'-hydroxyl and phosphoryl center protecting groups *without cleavage but with isomerization* of the

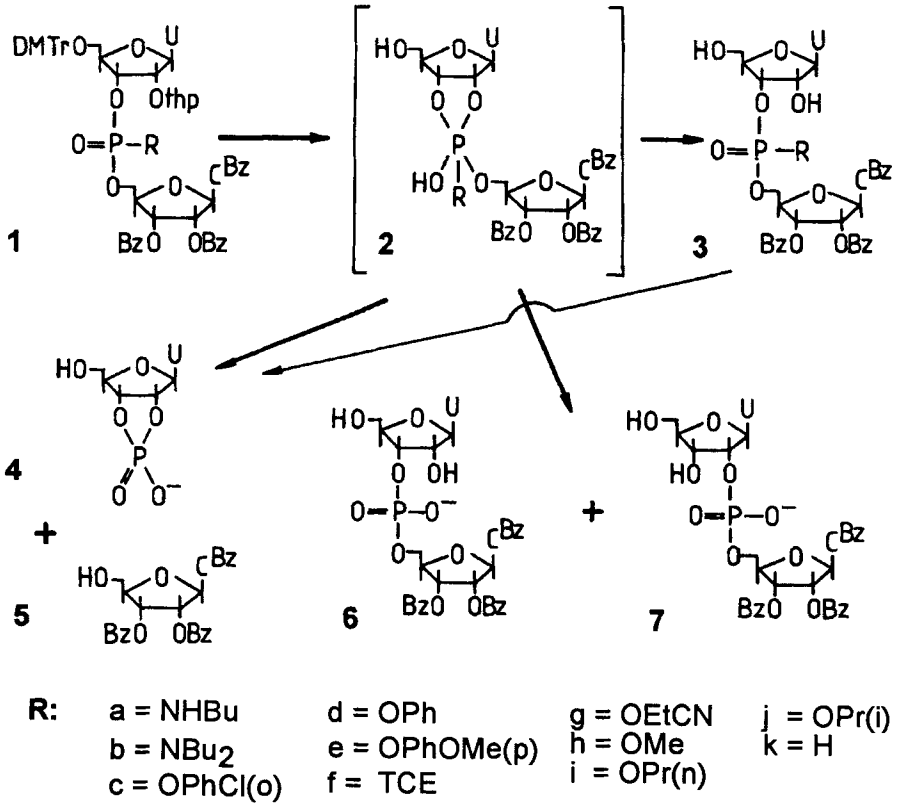

FIGURE 1.

TABLE 1. Quantitative stability analysis of the model dimers (**1a-j**) in acidic conditions.

Phosphorus protecting groups at internucleotide bond (1)	2',3',4Ntribenzoylcytidine (hydrolysis)	Up(R)2',3',4NBz$_3$C (phosphotriester)	Up(O$^-$)2',3',4NBz$_3$C (isomerization)
NH-n-butyl	**0%**	**0%**	**100%**
N-di-n-butyl	**8.4%**	**0%**	**91.4%**
O-(o)-chlorophenyl	**66.4%**	**0%**	**33.6%**
O- phenyl	**47.3%**	**13.8%**	**38.9%**
O-(p)-methoxyphenyl	**52.0%**	**6.0%**	**42.0%**
O-2,2,2-trichloroethyl	**72.3%**	**6.7%**	**21.0%**
O-2-cyanoethyl	**43.2%**	**6.0%**	**50.8%**
O-methyl	**15.4%**	**16.8%**	**67.8%**
O-n-propyl	**13.7%**	**81.0%**	**5.3%**
O-isopropyl	**13.4%**	**85.4%**	**1.2%**
H (H-phosphonate)	**100%**	**0%**	**0%**

All silica gel plates were developed three times: in system B followed by system C followed by n-butanol/acetic acid/water (5:3:2v/v). The last two runs were done on limited distance. The proper spots were scratched out and the material was eluted with methanol. Data concerning uridine-2',3'-cyclic phosphate and uridine-2'(3')-phosphate formation in the reaction mixture are not included.

internucleotide bond (to **6** and **7**), (iii) removal of 2'-hydroxyl protecting group *without any further transformations* of the triester internucleotide bond (to **3**).
For most of the discussed dimer models all the transformations occurred parallelly. The results suggest that the electronic character of the phosphoryl center protecting group (electron withdrawing or donating) changes the electrophility of the phosphorus atom and causes the transformation of this center. The electron donating phosphorus substituents increase the stability of the trisubstituted phosphoryl center towards intramolecular cyclization, whereas the electron withdrawing protecting groups destabilize it. However, the best correlations occur within one type of the phosphoryl protecting group, e.g.: O-alkyl. These observations are in agreement with previously reported studies on the stability of o-chlorophenyl[13] and methyl[15] phosphotriester as well as H-phosphonate[16] in the presence of vicinal 2'-hydroxyl group.

The enzymatic and spectroscopic analyses of the "diester products" (**6** and **7**) prove the presence of (2'-5') and (3'-5') isomers in the reaction mixture (obtained in 92% from N-di-n-butyl derivative **1b**). The enzymatic hydrolysis of this diester dimer by ribonuclease A and ribonuclease "One" caused a cleavage of ca. 50% of the initial material. A mixture of uridine-2',3'-cyclic phosphate, uridine-2'(3')-phosphates and 2',3',4N-tribenzoylcytidine was obtained as the product of hydrolysis. However, the hydrolysis of the same sample with snake venom phosphodiesterase (SVPD), over 24 h at 37°C, was completed yielding uridine and 2',3',4N-tribenzoylcytidine-5'-phosphate. This pattern of enzymatic hydrolysis proves the isomerization of the internucleotide bond and is similar to that reported for an enzymatic hydrolysis of (2'-5) and (3'-5') oligoribonucleotides[17-19]. Moreover, ^{31}P NMR spectra confirm the isomerization of the internucleotide bond. The phosphorus spectrum of the diester dimer (obtained from N-di-n-butyl derivative **1b**) showed the presence of two signals at -3.11 and -4.07 ppm, whereas a (3'-5') model dimer gave only one signal, at -3.11 ppm. Enzymatic and spectroscopic analyses as a proof of isomerization were done for N-di-n-butyl dimer only, but this observation seems to be general and can be extended to all diester-type products of the transformation of dimers (**1a-j**). This was also the conclusion drawn by Reese and Skone[13], who reported isomerization of the internucleotide bond of the dimer protected with p-chlorophenyl.

Electron withdrawing protecting groups at internucleotide bond also affect the reactivity of the phosphoryl center. The derivatives, e.g.: **1i** and **1j**, can be subsequently deprotected at 5'- and 2'-positions without any transformation of the O-alkyl phosphotriester. The strong electron donating character of the protecting groups of the phosphoryl center reduces the electrophilicity of the phosphorus atom so, the released vicinal 2'-hydroxyl

group does not attack the phosphoryl center. The triester character of those products was confirmed by TLC and ^{1}H, and ^{31}P NMR. In ^{31}P NMR spectrum the n-propyl, the 5'-deprotected dimer (**1i**) gave two signals at -1.07 and -1.40 ppm, whereas subsequently the 2'-deprotected one showed only one signal at 1.13 ppm. The presence of one signal for the second derivative suggests isomeric purity of the sample. However, the appearance of two signals (due to a chiral character of phosphotriester) was expected. In addition, the presence of an n-propyl chain on the phosphoryl center was confirmed by 1D and 2D ^{1}H NMR spectra. The phosphotriesters were found to be stable during acidic deprotection. However, under natural conditions, hydrolysis (ca. 80%) to uridine-2',3'-cyclic phosphate (**4**) and 2',3',4N-tribenzoylcytidine (**5**) was observed. This limits the application of such derivatives to chemical syntheses. A similar instability of methyl phosphorus triester in the presence of 2'-hydroxyl group was previously reported[15].

The above presented experiments support the following conclusions: (i) the electronic character of the substituent at internucleotide bond affects the susceptibility of the phosphoryl center to a nucleophilic, intramolecular attack by vicinal 2'-hydroxyl group, (ii) electron withdrawing protecting groups activate the phosphoryl center, whereas the electron donating ones stabilize it, (iii) the consequence (for most models **1**) of the deprotection of the 2'-hydroxyl group vicinal to trisubstituted internucleotide bond are two simultaneous processes: (*a*) nucleophilic attack of 2'-hydroxyl group on the trisubstituted internucleotide bond, a subsequent isomerization of that bond (to (2'-5')(**7**) and (3'-5')(**6**) isomers) and a partial hydrolysis of the internucleotide bond (to **4** and **5**) and/or (*b*) removal of the 2'-protecting group without transformation of the phosphotriester internucleotide bond (to **3**), (iv) the type of phosphoryl center protecting groups influence the proportion in which the products of cleavage and isomerization of the internucleotide bond occur.

From the last remark one can conclude that the intermediate in the hydrolysis of the internucleotide bond is different from the previously suggested 2',3'-cyclic phosphotriester[13], since in the intermediate (**8**) type of phosphoryl center protecting group should not affect the proportion between the isomerization and cleavage of internucleotide bond products (Figure 2). Oxyphosphorane (**9** and/or **10**) seems to be more likely as a transition stage, since its hydrolysis would be affected by the nature of the phosphorus atom protecting group. As can be seen from Table 1, the correlations were observed experimentally. In oxyphosphorane (**9** and/or **10**) the selection between isomerization and/or hydrolysis of the internucleotide bond depends on the competition between O5' and the oxygen (or nitrogen) protecting group of the phosphoryl center to be protonated,

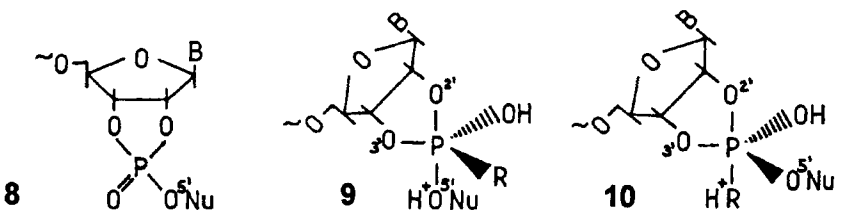

FIGURE 2.

and which of them occupies the apical position in the transition stage (**9** or/and **10**). This oxyphosphorane transition stage is suggested in the alkaline hydrolysis of five-member cyclic triester[20] and additionally supported by theoretical calculations[21]. Moreover, the observed isomerization of 2'-O-phosphorodianilidate to 2'-O- and 3'-O-phosphorodianilidates could proceed via the oxyphosphorane transition stage[22]. Also the formation of this type of transition stage is suggested by kinetic studies of the hydrolysis of RNA dimer[23]. In addition, Brown et. al.[24] proposed oxyphosphorane as a transition stage for the hydrolysis of benzyl diester under acidic conditions.

Chemical synthesis of uridyl(2'-phosphate)-(3'-5')uridine (16).

The chemical synthesis of uridyl(2'-phosphate)-(3'-5')-uridine (**16**) was designed on the basis of the chemical properties of the trisubstituted internucleotide bond in the presence of vicinal 2'-hydroxyl group[8] (Figure 3). Due to shown and known transformations of the trisubstituted internucleotide bond (isomerization and cleavage), the diester dimer (**14**) was chosen as a substrate for the synthesis. The diester substrate (**14**) was obtained from a fully protected dimer by selective deprotection of the 2-cyanoethyl group[25] followed by the cleavage of the 2'-O-tetrahydropyranyl group (thp). The diester substrate ensures (3'-5') the orientation of the internucleotide bond, but makes TLC analysis and chromatographic purification less convenient. Moreover, it can be a potential source of side reactions[26]. The protection of the N-3 position of uracil moiety[27], not only prevents 4-O-phosphorylation but also increases the solubility of the substrate (**14**).

To select the optimal conditions for 2'-O-phosphorylation five different phosphorylation reagents were tested, namely: tri(triazole)phosphate[28], phosphorus oxychloride/triethoxyphosphate[29], tri(imidazole)phosphine[30], bis(diisopropylamino)-2-cyanoethoxy-

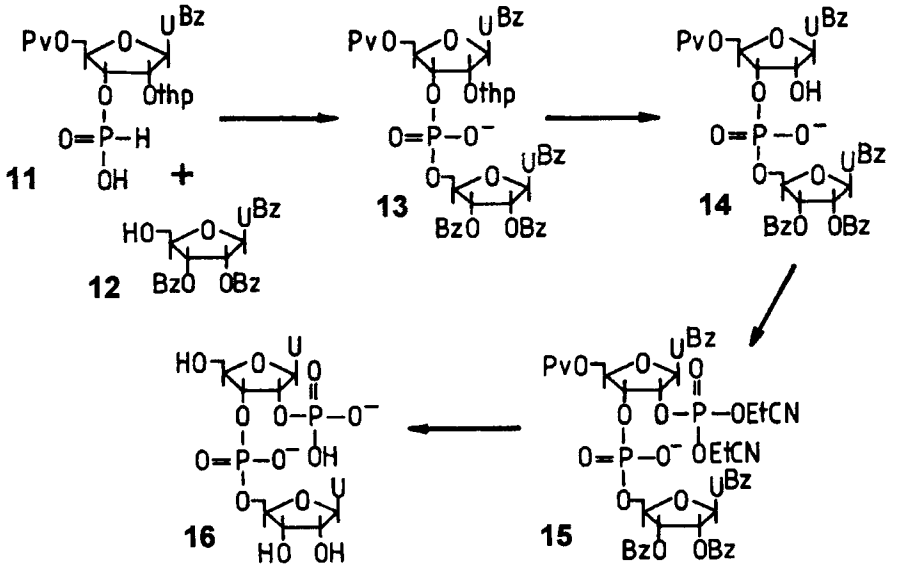

FIGURE 3.

phosphine[31] and diisopropylamino-bis(2-cyanoethoxy)phosphine. Ammonia deprotection of the reaction mixtures and their TLC analysis on silica gel (system D) demonstrate that only last two reagents provided the required product. Nevertheless, some amounts of the product (ca. 5%) were obtained using tri(imidazole)phosphine. Phosphorus(V) phosphorylation reagents were found less reactive in 2'-O-phosphorylation probably due to a steric hindrance in the substrate (**14**). TLC analysis of ammonia deprotected reaction mixture indicated the presence of uridyl-(3'-5')-uridine and the products of alkaline hydrolysis: uridine, uridine-2',3'-cyclic phosphate and uridine-2'(3')-phosphates[32]. Several derivatives of unknown structure were also observed. When a five-fold excess of bis(diisopropylamino)-2-cyanoethoxyphosphine, in the presence of tetrazole as used for the phosphorylation of the substrate (**14**), the reaction was almost quantitative. Then, 2'-O-phosphoramidite was hydrolyzed to H-phosphonate[33] and oxidized[34]. Unfortunately, after ammonia deprotection, besides the expected product (UPpU)(**16**) an equivalent amount of a side product was observed in the reaction mixture. Determination of the structure of this side product was not successful. The phosphorylation agent chosen was diisopropylamino-bis(2-cyanoethoxy)phosphine, which was synthesized in the reaction of bis(diisopropylamino)-2-cyanoethoxyphosphine with 1.1 equivalent of 2-cyanoethanol in the presence of 0.5 equivalent of diisopropylammonium tetrazolide (^{31}P NMR spectrum

of diisopropylamino-bis(2-cyanoethoxy)phosphine showed a peak at 149.11 ppm). With a five-fold excess of this phosphine and a twenty-fold excess of tetrazole the 2'-hydroxyl of the substrate dimer (**14**) was phosphorylated in over 90% (^{31}P NMR spectrum of the reaction mixture: 140.88 and -6.01 ppm for phosphorus atoms in 2'-O-phosphite and in the internucleotide bond, respectively). In the next step, the reaction mixture was oxidized (^{31}P NMR spectrum of the reaction mixture: -5.37 and -5.15 ppm for phosphorus atoms in 2'-O-phosphate and in the internucleotide bond, respectively) and finally ammonia deprotected. A spectroscopic analysis of TLC purified reaction mixture revealed a 52% yield of UPpU, whereas silica gel column chromatography estimated UPpU at 41.4%.

The structure of the product, UPpU (**16**), was established by multienzymatic hydrolysis[5,12,35]. It was found that UPpU (**16**) was dephosphorylated by calf intestinal phosphatase (CIP) to UpU dimer. Hydrolysis by snake venom phosphodiesterase (SVPD) digested it to a mixture of uridine-5'-phosphate and uridine-2'-phosphate. Moreover, UPpU (**16**) was resistant to P1 nuclease treatment. The resonance signals: at 0.96 ppm (2'-O-phosphate phosphorus atom) and -0.58 ppm (internucleotide bond phosphorus atom) in ^{31}P NMR spectrum enforced the results of enzymatic hydrolyses and confirmed the structure of uridyl(2'-phosphate)-(3'-5')-uridine (**16**).

GENERAL SECTION

The solvents for the syntheses were prepared according to an earlier published procedure[36]. The syntheses of H-phosphonate of 5'-O-dimethoxytrityl-2'-O-tetrahydropyranyluridine[36,37] and 3N,3',5'-O-tribenzoylcytidine[27] were performed according to the published method. The models of dimers (**1a,b**) were synthesized from the H-phosphonate dimer (**1k**) and a respective amine[38]. The remaining dimers (**1c-j**) were prepared by condensation of diester dimer (obtained by removal of the 2-cyanoethyl group from dimer **1g**) and proper alcohols or phenols in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride and 1-methylimidazole[39]. Enzymatic hydrolyses of dimers by ribonuclease A (Boehringer), ribonuclease "One" (Promega), calf intestinal phosphatase (Promega), snake venom phosphatase (Worthington) and nuclease P1 (Boehringer) were performed under the generally recommended conditions[5,35]. Thin layer chromatography was performed on silica gel plates using the following systems: chloroform/methanol (95:5v/v, system A), chloroform/methanol (90:10v/v, system B), chloroform/methanol (85:15v/v, system C), n-propanol/ammonia/water (55:35:10v/v, system D). The reversed phase chromatography plates (RP-8 F_{254}) were developed in acetone/water (7:3v/v, system E). The 1D and 2D ^{1}H NMR spectra were recorded on Varian Unity 300 (299.94 MHz) using tetramethylsilane as the internal standard and ^{31}P NMR spectra were taken on the same apparatus (121.42 MHz) using H_3PO_4 as the external standard. The ultraviolet spectra (UV) were recorded on a Beckman DU-65 spectrophotometer.

EXPERIMENTAL SECTION

Synthesis of 5'-O-pivaloyl-2'-O-tetrahydropyranyl-3N-benzoyluridine.
Synthesis of 2'-O-tetrahydropyranyl-5',3'-O-(tetraisopropyldisiloxane-1,3-diyl)uridine was performed in 7 mMol scale according to published procedure[37]. Then this derivative was dissolved in 15 mL of anhydrous pyridine and treated with 2 equivalents (1.62 mL) of benzoyl chloride for 16 h at 37°C. The reaction mixture was worked-up with 50 mL of aqueous solution of sodium bicarbonate and extracted with chloroform (3x50 mL). Combined organic layers were dried and evaporated. Silyl protection group was deprotected by triethylammonium fluoride according to published procedure[37]. The residue was coevaporated with anhydrous pyridine (2x20 mL), 1.2 equivalent (1.03 mL) of pivaloyl chloride was added dropwise and the reaction mixture was stirred for 2 h. The reaction mixture was worked-up with 50 mL of aqueous solution of sodium bicarbonate and extracted with chloroform (3x50 mL). The combined organic layers were dried, evaporated and coevaporated with toluene (3x20 mL). The mixture was purified by silica gel column chromatography and eluted with chloroform/methanol (98:2 v/v). Overall yield: 64.5% (4.50 mMol, 2.31 g).
^{1}H NMR ($CDCl_3$): 7.96-7.93 (2H, m, phenyl), 7.56-7.47 (4H, m, phenyl, H-6), 5.96-5.82 (2H, m, H-1', H-5), 4.74-3.49 (7H, m, H-2', H-3', H-4', H-5', H-5", thp, 3'-OH), 1.82-1.52 (8H, m, thp), 1.24 and 1.21 (9H, 2s, pivaloyl). UV (MeOH) λmax: 254nm, 268nm (sh), λmin: 212nm TLC, R_f: 0.52 and 0.69 (A), 0.77 and 0.90 (B), 0.36 (D).

Synthesis of 5'-O-pivaloyl-2'-O-tetrahydropyranyl-3N-benzoyluridyl-(3'-5')-3N,2',3'-O-tribenzoyluridine(13)[40].
5'-O-Pivaloyl-2'-O-tetrahydropyranyl-3N-benzoyluridine-3'-phosphonate (2.30 mMol, 1.50 g) and 3N,3',5'-O-tribenzoyluridine (2.76 mMol, 1.44 g) were coevaporated with anhydrous pyridine (2x25 mL) and dissolved in 25 mL of anhydrous pyridine. Then pivaloyl chloride (6.90 mMol, 0.84 mL) was added and left for 30 min at room temperature. Next, 15 mL of pyridine/3-hydroxypropionitrile (9:1v/v) containing 2% iodine ere added to the reaction mixture and left for 30 min at room temperature. The reaction mixture was worked-up with 50 mL of 0.5 M aqueous solution of sodium sulfite and extracted with chloroform (3x50 mL). The combined organic layers were dried, evaporated and coevaporated with toluene (3x20 mL). The reaction mixture was preliminarily purified by silica gel chromatography. The respective fractions were collected and evaporated. The residue was treated with 10 mL of pyridine/triethylamine (9:1 v/v) for 16 h at room temperature. The mixture was evaporated and coevaporated with toluene (2x20 mL) and purified by silica gel column chromatography. The column was eluted by chloroform/methanol (98:2v/v) following chloroform/methanol/triethylamine (90:9:1v/v). Overall yield: 54.4% (1.25 mMol, 1.52 g).
^{1}H NMR ($CDCl_3$): 7.99-7.38 (22H, m, phenyl, 2xH-6), 6.53-5.69 (6H, m, 2xH-1', 2xH-5, H-2', H-3'), 4.92-4.80 (8H, m, H-2', H-3', 2xH-4', 2xH-5', 2xH-5"), 1.82-1.40 (8H, m, thp), 1.21 (9H, s, thp). ^{31}P NMR ($CDCl_3$): 1.50. UV (MeOH) λmax: 241nm, 254nm, 269nm (sh), λmin: 214nm, 245nm. TLC, R_f: 0.71 (C), 0.50 (D).

Synthesis of 5'-O-pivaloyl-3N-benzoyluridine-(3'-5')-3N,2',3'-O-tribenzoyluridine (14).
5'-O-Pivaloyl-2'-O-tetrahydropyranyl-3N-benzoyluridyl-(3'-5')-3N,2',3'-O-tribenzoyluridine (1.25 mMol, 1.52 g) was dissolved in 25 mL of 80% aqueous solution of acetic acid and left for 5 h at room temperature. Then reaction mixture was evaporated and

coevaporated with toluene (3x10 mL). The oily residue was dissolved in chloroform and precipitated to n-hexanes. Yield: 89.0% (1.10 mMol, 1.27 g).
^{1}H NMR ($CDCl_3$): 7.98-7.39 (22H, m, benzoyl, 2xH-6), 6.54-5.67 (6H, m, 2xH-1', H-2', H-3', 2xH-5), 4.76-4.34 (8H, m, H-2', H-3", 2xH-4', 2xH-5', 2xH-5"), 1.21 (9H, s, pivaloyl). ^{31}P NMR ($CDCl_3$); 0.48. UV (MeOH) λmax: 242nm, 254nm, 267nm (sh), λmin: 215nm, 243nm. TLC, R_f: 0.62 (C), 0.53 (D).

Synthesis of uridyl(2'-phosphate)-(3'-5')-uridine (16) .
A round bottom flask with 0.2 mMol (0.228 g) of 5'-O-pivaloyl-3N-benzoyluridine-(3'-5')-2',3',3N-tribenzoyluridine and 4 mMol (0.28 g) of tetrazole covered with a rubber septum (with a ventilation needle in) and dried under vacuum for a few hours. Then 4 mL of anhydrous acetonitrile added following 4 mL of 0.25 M acetonitrile solution of diisopropylamino-bis(2-cyanoethoxy)phosphine and stirred for 1 h at room temperature. TLC analysis in system C demonstrates a nearly completed phosphorylation. To the reaction mixture were added 4 mL of 2% solution of iodine in pyridine/water (9:1v/v) and after 5 min the reaction mixture was worked-up with 50 mL of 0.5 M solution of sodium sulfite, and extracted with chloroform (3x30 mL). The combined organic layers were dried and evaporated. The residue was treated with 20 mL of pyridine/saturated aqueous ammonia (1:4v/v) for 20 h at 55°C. The mixture was evaporated and purified by silica gel column chromatography. The column was eluted with a mixture of isopropanol/ammonia/water (90:5:5v/v) with a progressively increasing amount of water (up to 35%) in the solvent mixture. Yield: 41.4% (0.082 mMol, 55 mg)
^{1}H NMR (D_2O): 7.90 (1H, d, J=8.4Hz, H-6), 7.81 (1H, d, J=8.4Hz, H-6), 6.03-5.85 (4H, m, 2xH-1', 2xH-5), 4.35-4.10 (8H, m, 2xH-2' ,2xH-3', 2xH-4', H-5', H-5"), 3.85-3.77 (2H, m, H-5', H-5"). ^{31}P NMR (D_2O): -0.58 and 0.98 ppm. UV (MeOH) λmax: 260nm, λmin: 231nm. TLC, R_f: 0.32 (D).

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