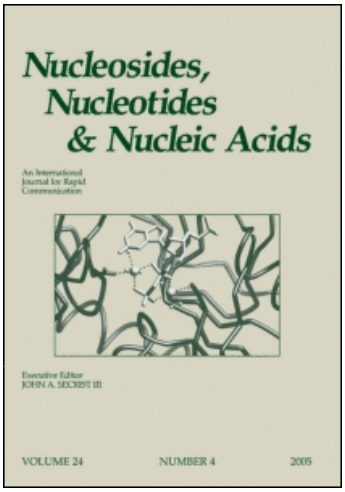

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Synthesis and Properties of Phosphorylated 3′-*O*-β-D-Ribofuranosyl-2′-deoxythymidine

Ekaterina V. Efimtseva,[1] Alexandra A. Shelkunova,[1] Sergey N. Mikhailov,[1] Koen Nauwelaerts,[2] Jef Rozenski,[2] Eveline Lescrinier,[2] and Piet Herdewijn[2,*]

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ABSTRACT

A strategy was developed for the synthesis of 3′-*O*-β-D-ribofuranosyl 2′-deoxythymidine derivatives using three different protecting groups, which allows the synthesis of a phosphoramidite building block for oligonucleotide synthesis. Likewise the 5′-*O*- and 5″-*O*-phosphorylated analogues were synthesized and their conformation was determined using NMR spectroscopy.

Key Words: Disaccharide nucleosides; Synthesis; NMR.

1. INTRODUCTION

Recently a general method for the preparation of 2′-*O*-β-D-ribofuranosylnucleosides and some other disaccharide nucleosides and their incorporation into

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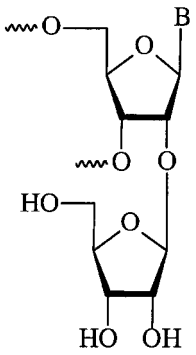
oligodeoxynucleotides (ONs) were developed.[1–4] T_m measurements have demonstrated that modified ONs form stable duplexes with complementary RNA ($\Delta Tm = 0°C$).[3] In order to determine the influence of this modification the solution structure of the self-complementary decaribonucleotide 5′-r(GCGXAUUCGC)-3′ (X-2′-*O*-β-D-ribofuranosyadenosine residue, unit I) was studied using high-resolution NMR spectroscopy and restrained molecular dynamics.[5] Incorporation of such voluminous substituent into 2′-*O*-position has no profound effect on the thermal stability of the duplex. It was found that the duplex RNA maintains an A-type helical geometry with extra 2′-*O*-ribose moiety located in the minor groove.[5] It should be also noted that 2′-*O*-β-D-ribofuranosyladenosine was found in yeast initiator tRNA in stem region of T domain.[6]

Oligodeoxynucleotides containing 2′-*O*-β-D-ribofuranosyladenosine (unit I) were prepared and used as modified primers in RNA-templated DNA synthesis catalyzed by HIV reverse transcriptase.[7] It was shown that the additional 2′-ribofuranose residue in specific position (3–4) of primer prevents its elongation. The primer may be elongated effectively if the modification is shifted further from 3′-end.[7]

The presence of additional functionalities in ONs opens new possibilities for postsynthetic functionalization. The cis-diol group in ONs containing 2′-*O*-β-D-ribofuranosylnucleosides (unit I) may be readily oxidized by periodate to give reactive dialdehyde groups (unit II). Such modified ONs were used successfully for the affinity labeling of different enzymes of nucleic acids biosynthesis.[8,9]

Molecular modeling reveals that a disaccharide nucleoside with a ribose moiety linked to the 3′-position might be elongated in the 5″ direction. Therefore, we decided to prepare ONs containing 3′-*O*-β-D-ribofuranosyl-2′-deoxythymidine (unit III) and to investigate their properties. In this manuscript we describe the synthesis of the protected disaccharide nucleoside and its NMR analysis.



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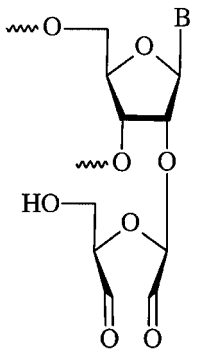

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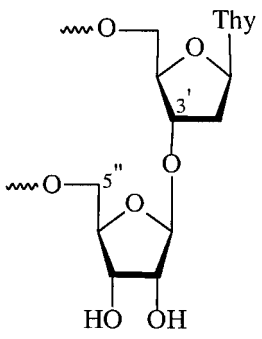

III

2. RESULTS AND DISCUSSION

3′-*O*-β-D-Ribofuranosyl-2′-deoxythymidine nucleosides can be prepared[10] by condensation of blocked nucleoside **1** and 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (**2a**) followed by deprotection (Sch. 1). However, this scheme is not useful

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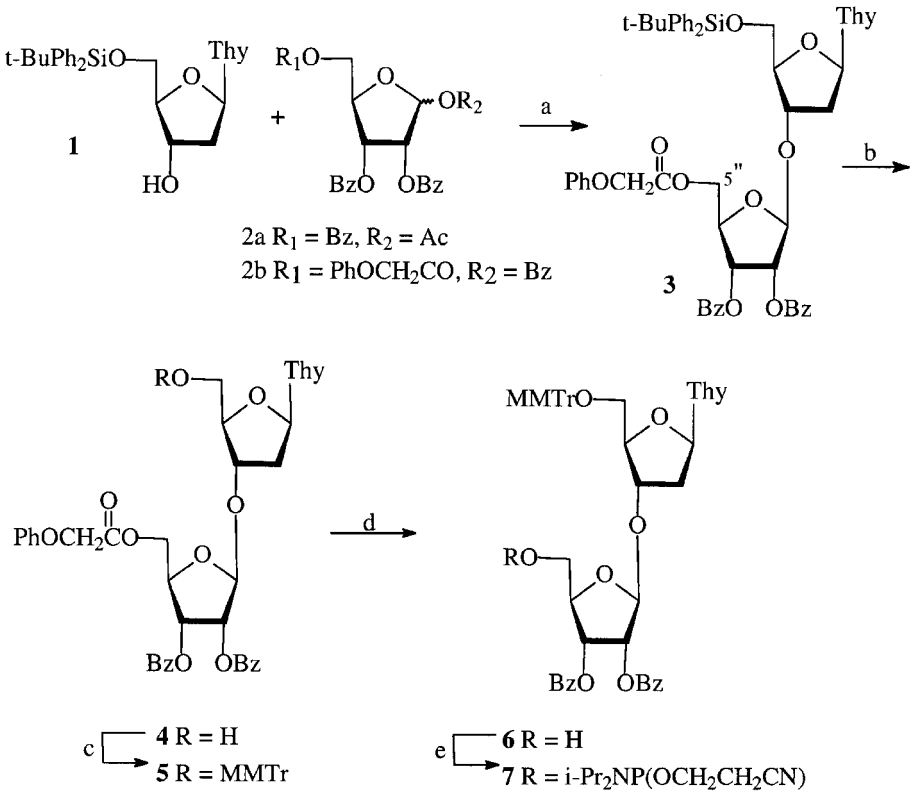


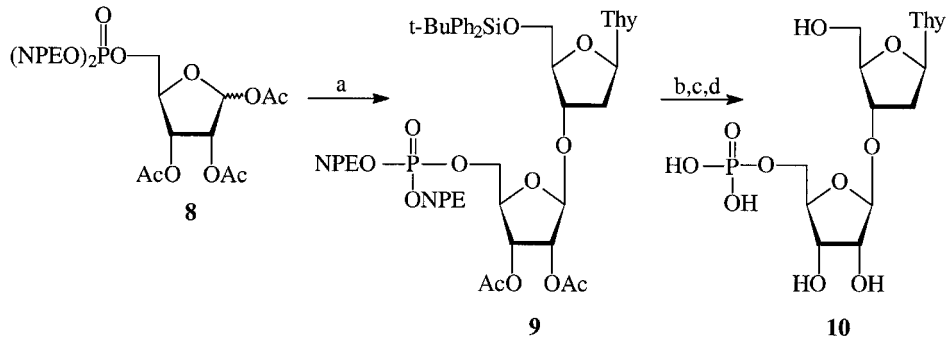
a. $SnCl_4/ClCH_2CH_2Cl$, 0°C; b. Bu_4NF/THF; c. MMTrCl/Py; d. 0.1 M $NaHCO_3$ in MeOH; e. $(i\text{-}Pr_2N)_2P(OCH_2CH_2CN)$.

Scheme 1.

for incorporation of this nucleoside in oligonucleotides as no selective deprotection of the 5″ position is possible. Very recently the blocked sugar namely 1,2,3-tri-*O*-benzoyl-5-*O*-phenoxyacetyl-D-ribofuranose (**2b**) was successfully used for the preparation of tRNA minor nucleoside *O*-β-D-ribofuranosyl-(1″-2′)-guanosine-5″-*O*-phosphate.[11] Condensation of nucleoside **1** with sugar **2b** in the presence of tin tetrachloride proceeded stereospecifically with the formation of β-anomer **3** in 65% yield. After desilylation, disaccharide nucleoside **5** was monomethoxytritylated. Selective removing of phenoxyacetyl group with 0.1 M $NaHCO_3$ in methanol gave **6** in good overall yield. Nucleoside **6** was then converted to the corresponding phosphoramidite **7** (Sch. 1).

Preparation of Model Compounds for NMR Studies

Two model phosphorylated 3′-*O*-β-D-ribofuranosyl-2′-deoxythymidines (**10** and **12**) were prepared for NMR studies. For the preparation of 5″-phosphate **10** we decided to use sugar phosphate synthon (**8**), which was recently used for the



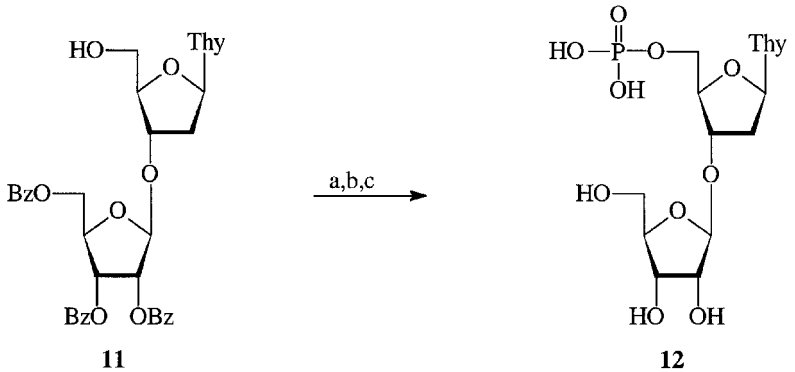
a. **1**, $SnCl_4/ClCH_2CH_2Cl$, 0°C; b. DBU/Py; c. NH_3/MeOH, d. Bu_4NF/THF

Scheme 2.

synthesis of 5′-nucleotides.[12] Condensation with **1** gave **9** in 35%. After deblocking, **10** was obtained in overall good yield (Sch. 2).

The other nucleotide **12** was prepared according to the Sch. 3 by phosphorylation of **11**.[10]

The structures of the obtained compounds were proved by mass- and NMR spectroscopy. Most of the chemical shifts and coupling constants may be calculated directly from NMR spectra. In some cases comparison with the published spectra of disaccharide nucleosides and ^1H-^{13}C correlation and COSY spectra were used for the assignment.



a. $NC(CH_2)_2OPO_3H_2$/DCC/Py; b. NH_3/MeOH; c. 1M NaOH.

Scheme 3.

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Table 1. Measured chemical shifts (ppm) in the ribose and nucleoside moieties.

	Compound **12** (rTP)		Compound **10** (PrT)	
Nucleus	Ribose moiety	Nucleoside moiety	Ribose moiety	Nucleoside moiety
H1′	5.22 d	6.36 dd	5.17 d	6.30 dd
H2′	4.16 dd	2.58 ddd	4.17 dd	2.61 ddd
H2″		2.44 ddd		2.49 ddd
H3′	4.30 dd	4.64 ddd	4.37 dd	4.51 ddd
H4′	4.11 ddd	4.34 ddd	4.19 ddd	4.17 ddd
H5′a	3.90 dd	4.11 dd	4.09 ddd	3.87 dd
H5′b	3.74 dd	4.11 dd	3.98 ddd	3.81 dd
H6	–	7.839[e]	–	7.669[e]
HMe	–	1.95 d	–	1.93 d
C1′	106.00 s	84.58 s	106.34 s	85.27 s
C2′	74.01 s	36.83 s	74.35 s	37.03 s
C3′	70.17 s	77.77 s	70.56 s	76.98 s
C4′	82.30 s	83.30 d[a]	81.67 d[c]	84.30 s
C5′	62.24 s	63.94 d[b]	65.52 d[d]	61.13 s
C2		166.03 s	–	166.46 s
C4		151.23 s	–	151.04 s
C5		111.25 s	–	111.37 s
C6		136.93 s	–	137.63 s
CMe-5		11.10 s	–	11.46 s
P		1.99	1.46	–

[a] $J_{C4'\text{-}P} = 7.8$ Hz.
[b] $J_{C5'\text{-}P} = 5.1$ Hz.
[c] $J_{C4'\text{-}P} = 8.2$ Hz.
[d] $J_{C5'\text{-}P} = 5.3$ Hz.
[e] $J = 1.2$ Hz.

Conformational Study

The conformation of a five membered ring can be completely described by two independent parameters: phase angle and puckering amplitude. Since the puckering amplitude always stays within a narrow range and the pseudorotational angle can adopt values ranging from 0 to 360 degrees, the different conformations are mostly visualized using the pseudorotational wheel.

It is widely accepted that furanose rings, which are flexible entities, will show a high-speed equilibrium between two low energy conformations in solution. One of those conformations is situated in the northern hemisphere of the pseudorotational wheel (the so called N conformer) and the other in the southern hemisphere (the S conformer).

A complete description of this system now requires five independent parameters. Four parameters describing the conformers and one parameter describing the fraction of one of the conformers.

The two conformers and their relative abundance can be resolved by NMR spectroscopy, through measurement of the $^3J_{H,H}$ coupling constants. Because

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Table 2. Measured J coupling constants (Hz) in the ribose and nucleoside moieties.

	Compound **12** (rTP)		Compound **10** (PrT)	
Nucleus	Ribose moiety	Nucleoside moiety	Ribose moiety	Nucleoside moiety
$J_{H1'\text{-}H2'}$	1.5	8.2	1.6	7.8
$J_{H1'\text{-}H2''}$		5.7		6.2
$J_{H2'\text{-}H3'}$	5.0	5.6	4.5	5.4
$J_{H2''\text{-}H3'}$		3.2		3.5
$J_{H2''\text{-}H2'}$		−13.9		−14.1
$J_{H3'\text{-}H4'}$	7.8	2.3	6.7	2.7
$J_{H4'\text{-}H5'a}$	6.4	4.3	6.0	4.1
$J_{H4'\text{-}H5'b}$	3.0	4.3	4.9	4.7
$J_{H5'a\text{-}H5'b}$	−12.6		−12.6	−12.4
$J_{P\text{-}H5'a}$		5.2	4.8	
$J_{P\text{-}H5'b}$		5.2	5.9	

the equilibrium, mentioned above, can be considered as fast on NMR time scale, average coupling constants and average chemical shifts will be observed. Assuming the two state model, described above, it is possible to find the conformations and their populations that best fit the experimentally observed $^3J_{H,H}$ coupling constants.[13,14]

A 2D TOCSY experiment[15,16] was performed for the assignment of all signals (Table 1). The presence of a phosphate group was confirmed by ^{31}P spectroscopy and its correct attachment to the molecule was evident from characteristic downfield shift of H5′a and H5′b and their couplings with phosphorous ($J_{H,P}$). The correct glycosidic linkage of C1′ of the ribose moiety with C3′ of the nucleoside moiety could be confirmed by a NOE-contact between H1′ of the ribose and H3′ of the nucleoside.

Table 3. Ring conformers of the ribose and nucleoside moieties.

	Compound **12** (rTP)		Compound **10** (PrT)	
	Ribose moiety	Nucleoside moiety	Ribose moiety	Nucleoside moiety
P_N[a]	18	−20.2	2.2	2.4
N[b]	^3E	$_2E$	$^3T_2$	$^3T_2$
X_N[c]	0.89	0.26	0.87	0.28
P_S[a]	162	160	178	171
S[b]	^2E	$^2T_3$	$^2T_3$	$^2T_3$
X_S[c]	0.11	0.74	0.13	0.72
RMSD[d]	0.151	0.125	0.130	0.098

[a]Phase angle of pseudorotation (degrees).
[b]Type of conformer.
[c]Molar fraction of the conformer (%).
[d]Root Mean Square Deviation. The puckering amplitude was kept 38 degrees in all cases.

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A high-resolution 1D ^{1}H spectrum was used to measure the coupling constants. The $^{3}J_{H,H}$ coupling constants of both ribose moieties, measured from spectra recorded at 292 K, are presented in Table 2. Analysis of these data using Pseurot 6.2,[17] generated the results as shown in Table 3.

It was shown that the additional ribose moiety of both compounds will appear predominantly in the N conformation, which we could expect because of stereo electronic effects due to the 2′OH.[18] The deoxyribose rings of the nucleoside prefer the S conformer (74% and 72%) as could be expected.

The presence of a phosphate group on the C5′ of the deoxyribose and ribose moieties causes some conformational changes. The phosphate causes a slight shift towards the S conformer. (deoxyribose 0.72 *vs.* 0.74 by 5′-*O*-phosphorylation; ribose 0.11 *vs.* 0.13 after 5″-*O*-phosphorylation) and a change of pseudorotational angle of the N conformer (deoxyribose −20.2 *vs.* 2.4; ribose: 18 *vs.* 2.2). In the deoxyribose moiety, where the effect is more pronounced, the phosphate group is then located closer to the heterocyclic base.

To probe the *O*- and *N*-glycosidic bond populations, NOESY-spectra were recorded with 150, 300 and 500 ms mixing times respectively. No relevant data could be obtained, most likely due to fast rotation around these bonds in a fast equilibrium between several low energy conformations in solution. To obtain more experimental information on the relative populations of these bond conformations, experiments on a ^{13}C labeled sample will be necessary.

Observation of intermediate values of $J_{H5'\text{-}P}J_{H5''\text{-}P}$ $J_{H5''\text{-}H4'}$ and $J_{C5'\text{-}P}$ indicate free rotation around the C4′-C5′ and C5′-O5′ bonds. This can also be confirmed by the identical chemical shifts of H5′ and H5″ in the nucleoside moiety of rTP.

CONCLUSIONS

An efficient method is developed for the synthesis of protected 3′-*O*-β-ribofuranosyl-2′-deoxythymidine, which will allow the incorporation of this disaccharide nucleoside in oligonucleotides. NMR analysis reveals that phosphorylation of the disaccharide nucleoside in the 5′-*O*-position or in the 5″-*O*-position leads to a shift in the pseudo rotational phase angle of about 20°.

EXPERIMENTAL PART

General

Column chromatography (CC): silica gel (0.06–0.20 mm). TLC: Kieselgel 260 F (*Merck*); eluents: $CHCl_3$ (A); $CHCl_3$/MeOH 98:2 (B); $CHCl_3$/MeOH 95:5 (C); isopropanol/NH_4OH/H_2O 7:1:2 (D); detection by UV light. NMR Spectra: *Bruker AMX* 400 and *Varian Unity* 500 NMR spectrometers; at 300 K; chemical shifts δ in ppm were measured relative to the solvent signals (^{1}H and ^{13}C) and relative external ref. = H_4PO_3 (capil.) (^{31}P); coupling constants *J* in Hz. The signals were assigned using double resonance techniques and COSY experiments. Mass spectrometry and exact mass measurements: quadruple/orthogonal-acceleration time-of-flight tandem

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mass *spectrometer* (*Q-Tof-2, Micromass*, Manchester, UK) equipped with a standard electrospray ionization (ESI) interface.

NMR Spectra

Samples of **10** and **12** were prepared with 6 mg of both compounds, dissolved in 250 μL D_2O.pD was adjusted to 7.2. Spectra were recorded using a Varian 500 Unity spectrometer at 292 K. Chemical shifts were measured relative to the water signal. The coupling constants were measured in Hz. The signals were assigned using double resonance techniques and TOCSY-experiments.[16,17] The TOCSY consisted of 2048 data points in t_2 and 256 increments in t_1. The data were apodized with a shifted sine-bell square function in both dimensions and processed to a 2K × 1K matrix.

Analysis of the couplings was performed with Pseurot 6.2.[17] This program uses the Altona-Sundaralingam formalism[13,14] to describe the ring puckering and a generalized Karplus equation developed by Diez et al. in collaboration with Donders et al.[20] to describe the relation between coupling and dihedral angle. RMS was minimized, using a Newton Raphson Minimization.

NOESY-spectra[21] were recorded with 150, 300 and 500 ms mixing times respectively. The NOESY consisted of 2048 data points in t_2 and 256 increments in t_1. The data were apodized with a shifted sine-bell square function in both dimensions and processed to a 2K × 1K matrix.

1-[5-*O*-tert-Butyldiphenylsilyl-3-*O*-(2,3-di-*O*-benzoyl-5-*O*-phenoxyacetyl-β-D-ribofuranosyl)-2-deoxy-β-D-ribofuranosyl]thymine (3). To a cooled solution (0°C) under nitrogen of 1,2,3-tri-*O*-benzoyl-5-*O*-phenoxyacetyl-D-ribofuranose (**2**) (1.15 g, 1.93 mmol) in 1,2-dichloroethane (20 mL), $SnCL_4$ (0.27 mL, 2.32 mmol) was added and the solution was kept at 0°C for 10 min. After addition of 5′-*O*-tert-butyldiphenylsilyl-2′-deoxythymidine (**1**) (774 mg, 1.61 mmol), the resulting solution was kept at 0°C for 1.5 h. The reaction mixture was diluted with $CHCl_3$ (20 mL), 10% aqueous solution of sodium bicarbonate (20 mL) was added and the suspension was stirred at 20°C for 20 min. The suspension was filtered through *Hyflo Super Cel*, the organic layer was separated, washed with water (20 mL), dried over Na_2SO_4 and evaporated to dryness. The residue was purified by CC (silica gel 50 g). The column was washed with system A and then elution with system 0.5% MeOH/$CHCl_3$ gave **3** as a foam. Yield 1.0 g (65%). R_f 0.51 (C). ESI MS (pos.): 955.3470 ($[C_{53}H_{54}N_2O_{13}Si + H]^+$; calc. 955.3473).

^{1}H NMR ($CDCl_3$): 8.63 brs (1H, NH), 8.01–7.89 m (4H, Bz), 7.67–7.23 m (19H, Bz, Ph, H-6), 6.95 t (1H, $J = 7.5$, Ph), 6.90 d (2H, $J = 7.8$, Ph), 6.34 dd (1H, $J_{1',2'a} = 5.6$, $J_{1',2'b} = 8.1$, H-1′ Thd), 5.67 dd (1H, $J_{3',2'} = 5.0$, $J_{3',4'} = 6.8$, H-3′ Rib), 5.59 d (1H, H-2′ Rib), 5.29 s (1H, H-1′ Rib), 4.71 s (2H, PhO*CH*$_2$), 4.66–4.51 m (4H, H-3′ Thd, H-4′,5′a,5′b Rib), 4.12 m (1H, H-4′ Thd), 3.96 dd (1H, $J_{5'a,4'} = 3.1$, $J_{5'a,5'b} = -11.5$, H-5′a Thd), 3.84 dd (1H, $J_{5'b,4'} = 2.8$, H-5′b Thd), 2.66 ddd (1H, $J_{2'a,3'} = 1.9$, $J_{2'a,2'b} = -13.7$, H-2′a Thd), 2.18 ddd (1H, $J_{2'b,3'} = 6.5$, H-2′b Thd), 1.63 d (3H, $J_{5,6} = 1.2$, Me-5), 1.08 s (9H, t-Bu).

^{13}C NMR ($CDCl_3$): 168.70, 165.23, 165.11 (C=O), 163.46 (C-4), 157.79 (Ph), 155.04 (C-2), 135.52 (C-6), 135.33, 133.26, 133.48, 130.11, 129.77, 129.74, 129.51,

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128.51, 128.40, 127.99, 127.95 (Bz, Ph), 121.74, 114.76 (Ph), 111.06 (C-5), 105.16 (C-1′ Rib), 84.66 (C-1′ Thd, C-4′ Thd), 79.32 (C-3′ Thd), 78.62 (C-4′, Rib), 75.73 (C-2′, Rib), 71.88 (C-3′, Rib), 65.29, 65.01 (C-5′ Thd, C-5′ Rib), 63.95 (CH_2), 38.61 (C2′ Thd), 26.98 (Me_3), 19.31 (Si*C*Me_3), 12.04 (Me-5).

1-[3-*O*-(2,3-Di-*O*-benzoyl-5-*O*-phenoxyacetyl-β-D-ribofuranosyl)-2-deoxy-β-D-ribofuranosyl]thymine (4). To a solution of **3** (1.7 g, 1.78 mmol) in tetrahydrofuran (4 mL), a solution of tetrabutylammonium fluoride trihydrate (674 mg, 2.14 mmol) in tetrahydrofuran (4 mL) was added. After 20 min at 20°C water (2 mL) and Dowex-50 (Na^+-form) (2 mL) were added and the mixture was stirred for 20 min at 20°C. The resin was filtered off and washed with ethyl acetate (100 mL). The filtrate was washed with water (2×30 mL). The organic layer was dried over Na_2SO_4, evaporated to dryness and applied to CC (silica gel (30 g). The column was washed with system A, and eluted with system B to give **4** as a foam. Yield 950 mg (75%). R_f 0.25 (C). ESI MS (pos.): 717.2297 ($[C_{37}H_{36}N_2O_{13} + H]^+$; calc. 717.2296).

^{1}H NMR ($CDCl_3$): 8.87 brs (1H, NH), 8.01–7.89 m (4H, Bz), 7.61–7.25 m (9H, Bz, Ph, H-6), 6.97 t (1H, $J = 7.4$, Ph), 6.89 d (2H, $J = 8.1$, Ph), 6.02 t (1H, $J_{1',2'a} = J_{1',2'b} = 6.9$, H-1′ Thd), 5.66 dd (1H, $J_{3',2'} = 5.0$, $J_{3',4'} = 6.5$, H-3′ Rib), 5.60 d (1H, H-2′ Rib), 5.37 s (1H, H-1′ Rib), 4.72 s (2H, PhO*C*H_2), 4.65–4.51 m (4H, H-3′ Thd, H-4′, 5′a, 5′b Rib), 4.12 m (1H, H-4′ Thd), 3.91 dd (1H, $J_{5'a,4'} = 2.8$, $J_{5'a,5'b} = -12.1$, H-5′a Thd), 3.77 dd (1H, $J_{5'b,4'} = 2.8$, H-5′b Thd), 2.67 brs (1H, HO-5′), 2.52 m (2H, H-2′a,2′b Thd), 1.87 d (3H, $J_{5,6} = 1.2$, Me-5).

^{13}C NMR ($CDCl_3$): 168.80, 165.35, 165.26 (C = O), 163.58 (C-4), 157.71 (Ph), 150.28 (C-2), 137.65 (C-6), 133.63, 133.56, 129.79, 129.75, 129.56, 129.01, 128.78, 128.54, 128.44 (Bz, Ph), 121.87, 114.68 (Ph), 110.93 (C-5), 105.48 (C-1′ Rib), 88.11 (C-1′ Thd), 85.00 (C-4′ Thd), 79.50 (C-3′ Thd), 78.84 (C-4′, Rib), 75.73 (C-2′, Rib), 71.91 (C-3′, Rib), 65.25, 65.05 (C-5′ Thd, C-5′ Rib), 62.55 (CH_2), 37.61 (C2′ Thd), 12.34 (Me-5).

1-[5-*O*-Monomethoxytrityl-3-*O*-(2,3-di-*O*-benzoyl-β-D-ribofuranosyl)-2-deoxy-β-D-ribofuranosyl]thymine (6). Following co-evaporation with anhydrous pyridine, nucleoside **4** (890 mg, 1.24 mmol) was dissolved in pyridine (15 mL) and monomethoxytrityl chloride (460 mg, 1.49 mmol) was added. The mixture was kept for 16 h at 20°C. MeOH (0.2 mL) was added and after 30 min the mixture was concentrated. The residue was dissolved in $CHCl_3$ (70 mL), washed with 10% aqueous solution of sodium bicarbonate (20 mL) and water (2×20 mL). The organic layer was dried over Na_2SO_4, evaporated in vacuo, co-evaporated with toluene (2×10 mL) to give *1-[5-O-monomethoxytrityl-3-O-(2,3-di-O-benzoyl-5-O-phenoxyacetyl-*β*-D-ribofuranosyl)-2-deoxy-*β*-D-ribofuranosyl]thymine* (**5**). R_f 0.53 (C). ESI MS (pos.): 1011.3301 ($[C_{57}H_{52}N_2O_{14} + Na]^+$; calc. 1011.3316).

^{1}H NMR ($CDCl_3$): 8.62 brs (1H, NH), 8.02–7.87 m (4H, Bz), 7.61–7.19 m (21H, Bz, Ph, H-6), 6.97–6.82 m (5H, Ph), 6.36 dd (1H, $J_{1',2'a} = 5.9$, $J_{1',2'b} = 7.8$, H-1′ Thd), 5.65 dd (1H, $J_{3',2'} = 5.0$, $J_{3',4'} = 6.8$, H-3′ Rib), 5.57 d (1H, H-2′ Rib), 5.27 s (1H, H-1′ Rib), 4.70 s (2H, PhO*C*H_2), 4.64–4.50 m (4H, H-3′ Thd, H-4′, 5′a, 5′b Rib), 4.15 m (1H, H-4′ Thd), 3.75 s (3H, OMe), 3.50 dd (1H, $J_{5'a,4'} = 3.4$, $J_{5'a,5'b} = -10.6$,H-5′a Thd), 3.35 dd (1H, $J_{5'b,4'} = 2.8$, H-5′b Thd), 2.66 ddd (1H, $J_{2'a,3'} =$

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2.5, $J_{2'a,2'b} = -13.7$, H-2′a Thd), 2.32 ddd (1H, $J_{2'b,3'} = 6.5$, H-2′b Thd), 1.48 d (3H, $J_{5,6} = 1.2$, Me-5).

^{13}C NMR ($CDCl_3$): 168.71, 165.33, 165.22 (C = O), 163.50 (C-4), 158.86, 157.76 (Ph), 150.07 (C-2), 137.62 (C-6), 135.45, 134.85, 133.62, 133.56, 130,32, 129.78, 129.73, 129.55, 129.21, 128.52, 128.39, 127.99, 127.84, 127.27 (Bz, Ph), 121.73, 114.75, 113.33 (Ph), 111.07 (C-5), 105.05 (C-1′ Rib), 87.28 (C-O), 84.99 (C-1′ Thd), 83.72 (C-4′ Thd), 79.49 (C-3′ Thd), 78.67 (C-4′, Rib), 75.72 (C-2′, Rib), 71.89 (C-3′, Rib), 65.24, 65.03 (C-5′ Thd, C-5′ Rib), 63.48 (CH_2), 55.19 (OMe) 38.71 (C2′ Thd), 12.35 (Me-5).

Nucleoside **5**, without further purification, was dissolved in MeOH (5 mL), 0.1 M $NaHCO_3$ in MeOH (15 mL) was added. A solution was kept for 40 min at 20°C. Following neutralization with 10% acetic acid in MeOH, the mixture was concentrated in vacuo to dryness and the residue was partitioned between ethyl acetate (100 mL) and 10% aqueous solution of sodium bicarbonate (30 mL), the organic layer was washed with water (30 mL), dried over Na_2SO_4 and evaporated to dryness. The residue was applied to CC (silica gel 30 g). The column was washed with system A and then eluted with system B affording **6** as a foam. Yield 650 mg (61%). R_f 0.27 (C). ESI MS (pos.): 877.2923 ($[C_{49}H_{46}N_2O12 + Na]^+$; calc. 877.2948).

^{1}H NMR ($CDCl_3$): 8.69 brs (1H, NH), 8.00–7.88 m (4H, Bz), 7.65–7.23 m (19H, Bz, Ph, H-6), 6.85 d (2H, $J = 8.7$, Ph), 6.38 dd (1H, $J_{1',2'a} = 5.3$, $J_{1',2'b} = 8.7$, H-1′ Thd), 5.72 dd (1H, $J_{3',2'} = 5.0$, $J_{3',4'} = 7.2$, H-3′ Rib), 5.60 d (1H, H-2′ Rib), 5.30 s (1H, H-1′ Rib), 4.50 m (2H, H-3′ Thd, H-4′ Rib), 4.49 m (1H, H-4′ Thd), 4.01 dd (1H, $J_{5'a,4'} = 3.1$, $J_{5'a,5'b} = -12.1$, H-5′a Rib), 3.88 dd (1H, $J_{5'b,4'} = 5.0$, H-5′b Rib), 3.77 s (3H, OMe), 3.53 dd (1H, $J_{5'a,4'} = 3.1$, $J_{5'a,5'b} = -10.6$, H-5′a Thd), 3.37 dd (1H, $J_{5'b,4'} = 2.8$, H-5′b Thd), 3.01 ddd (1H, $J_{2'a,3'} = 1.6$, $J_{2'a,2'b} = -13.4$, H-2′a Thd), 2.23 ddd (1H, $J_{2'b,3'} = 5.6$, H-2′b Thd), 1.49 d (3H, $J_{5,6} = 1.0$, Me-5).

^{13}C NMR ($CDCl_3$): 165.54, 165.21 (C = O), 163.77 (C-4), 158.85 (Ph), 150.75 (C-2), 135.37 (C-6), 133.44, 133.30, 130.33, 129.74, 129.70, 129.22, 129.07, 128.45, 128.36, 128.32, 127.99, 127.26 (Bz, Ph), 113.33 (Ph), 111.40 (C-5), 105.58 (C-1′ Rib), 87.32 (C-O), 85.04 (C-1′ Thd), 84.26 (C-4′ Thd), 82.36 (C-3′ Thd), 80.29 (C-4′, Rib), 76.16 (C-2′, Rib), 71.45 (C-3′, Rib), 63.65, 62.78 (C-5′ Thd, C-5′ Rib), 55.19 (OMe) 38.82 (C2′ Thd), 11.84 (Me-5).

5′-*O*-Monomethoxytrityl-3′-*O*-[5-*O*-(P-β-cyanoethyl-N,N-diisopropylaminophosphinyl)-2,3-di-*O*-benzoyl-β-D-ribofuranosyl]-thymidine (7). The monomethoxytritylated derivative **6** (822 mg, 0.96 mmol) was dissolved in 6 mL dichloromethane under argon and diisopropylethylamine (0.5 mL, 2.88 mmol) and 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (535 μL, 2.4 mmol) were added and the solution was stirred for 60 min when TLC indicated complete reaction. Water (3 mL) was added, the solution was stirred for 10 min and partitioned between CH_2Cl_2 (60 mL) and aqueous $NaHCO_3$(40 mL). The organic phase was washed with aqueous sodium chloride (3 × 40 mL) and the aqueous phases were back extracted with CH_2Cl_2 (30 mL). Evaporation of the organics left an oil which was flash purified on 45 g of silica gel (hexane: acetone: TEA, 66:33:1) to afford the product as a foam after coevaporation with dichloromethane. Dissolution in 3 mL of dichloromethane and double precipitation in 100 mL cold (−70°C) hexane afforded 840 mg (0.83 mmol, 83%) of the title product **7** as a white powder. Rf (hexane: acetone:

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TEA 49:49:2): 0.43. $C_{58}H_{64}N_4O_{13}P_1$ calcd. 1055.4207, found 1055.4204 $[M+H]^+$. ^{31}P NMR (ppm, external ref. $= H_3PO_4$ capil.) 149.86, 149.18

1-[5-*O*-tert-Butyldiphenylsilyl-3-*O*-(2,3-di-*O*-acetyl-5-*O*-bis(p-nitrophenylethyl)-phosphorylyl-β-D-ribofuranosyl)-2-deoxy-β-D-ribofuranosyl]thymine (9). Following the procedure for the preparation of **3**, condensation of 1,2,3-tri-*O*-acetyl-5-*O*-bis(p-nitrophenylethyl)phosphorylyl-D-ribofuranose (**8**) (300 mg, 0.46 mmol) in the presence of tin tetrachloride (0.064 mL, 0.55 mmol) with nucleoside **1** (250 mg, 0.52 mmol) for 4 h at 0°C gave **9** as a foam. Yield 169 mg (35%). R_f 0.37 (C).

^1H NMR ($CDCl_3$): 9.18 brs (1H, NH), 8.14 m (4H, Ph), 7.67–7.37 m (15H, Ph, H-6), 6.22 dd (1H, $J_{1',2'a} = 5.9$, $J_{1',2'b} = 8.1$, H-1′ Thd), 5.29 dd (1H, $J_{3',2'} = 5.0$, $J_{3',4'} = 5.9$, H-3′ Rib), 5.13 dd (1H, $J_{2',1'} = 1.5$, H-2′ Rib), 5.07 d (1H, H-1′ Rib), 4.47 m (1H, H-3′ Thd), 4.23 m (5H, H-4′ Rib, CH_2O), 4.08 m (2H, H-5′a,5′b Rib), 4.01 m (1H, H-4′ Thd), 3.93 dd (1H, $J_{5'a,4'} = 3.4$, $J_{5'a,5'b} = -11.5$, H-5′a Thd), 3.81 dd (1H, $J_{5'b,4'} = 2.5$, H-5′b Thd), 3.05 (4H, CH_2), 2.55 ddd (1H, $J_{2'a,3'} = 1.9$, $J_{2'a,2'b} = -13.7$, H-2′a Thd), 2.14 m (1H, H-2′b Thd, Ac), 2.12 s (3H, Ac), 2.06 s (3H, Ac), 1.60 d (3H, $J_{5,6} = 1.1$, Me-5), 1.09 s (9H, t-Bu).

^{13}C NMR ($CDCl_3$): 169.54, 169.40 (C=O), 163.63 (C-4), 150.14 (C-2), 144.85, 144.89 (Ph), 135.49, 135.29 (Ph), 135.06 (C-6), 132.42, 130.12, 130.07, 129.86, 127.98, 127.95 (Bz, Ph), 123.65, 114.76 (Ph), 111.10 (C-5), 105.00 (C-1′ Rib), 84.68 (C-1′ Thd), 84.60 (C-4′ Thd), 79.74 d (C-4′ Rib, J(C,P) = 7.9), 78.73 (C-3′ Thd), 74.83 (C-2′ Rib), 70.69 (C-3′ Rib), 67.30 d (CH_2O, J(C,P) = 5.1), 67.11 d (C-5′ Rib, J(C,P) = 5.6), 63.82 (C-5′ Thd,), 38.61 (C2′ Thd), 36.28, 36.21 (CH_2), 26.96 (Me_3), 20.42, 20.40 (Ac), 19.30 (Si*C*Me_3), 11.99 (Me-5). ^{31}P NMR (161.98 MHz) ($CDCl_3$): −3.61.

***O*-β-D-Ribofuranosyl-(1″-3′)-thymidine-5″-*O*-phosphate (10).** A solution of triester **9** (155 mg, 0.144 mmol) in 0.5 M DBU in dry pyridine (15 mL, 7.5 mmol) was stored for 24 h at 20°C, neutralized with acetic acid (0.43 mL, 7.5 mmol), and evaporated. The residue was dissolved in 5 M ammonia in methanol (5 mL) and kept for 2 days at 20°C and then concentrated in vacuo. The residue was dissolved in 0.5 M tetrabutylammonium fluoride in tetrahydrofuran (10 mL) and kept for 16 h at 20°C, and evaporated. The residue was dissolved in water (50 mL) and washed with $CHCl_3$ (2 × 15 mL). The aqueous layer was concentrated in vacuo and diluted with water (50 mL) and then applied to a column of DEAE-cellulose (200 mL, HCO_3^--form). The column was washed with water (500 mL), a 0.05 M solution of NH_4HCO_3 and eluted with 0.1 M solution of NH_4HCO_3. The UV-absorbing fractions were combined, evaporated in vacuo, and coevaporated with water (5 × 10 mL). The residue was dissolved in water (2 mL), applied to a column of Dowex 50 (Na^+-form) (2 mL), eluted with water and freeze-dried. Monophosphate **10** was obtained as its sodium salt. Yield 40 mg (58%). R_f 0.15 (D). ESI MS (pag.): 477.0877 $[M+Na]^+$ UV (pH 1): λ_{max} 267 nm (ε 9100); (pH 7): λ_{max} 267 nm (ε 9000); (pH 13): λ_{max} 266 nm (ε 7000).

***O*-β-D-Ribofuranosyl-(1″-3′)-thymidine-5′-*O*-phosphate (12).** A mixture of 1-[3-*O*-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-2-deoxy-β-D-ribofuranosyl]thymine (**11**) (686 mg, 1 mmol) and a 3 mL 1 M solution of β-cyanoethyl phosphate in pyridine

was evaporated in vacuo and dried by co-evaporations with dry pyridine (2×10 mL). The residue was dissolved in pyridine (10 mL), *N,N'*-dicyclohexylcarbodiimide (1,03 g, 5 mmol) was added and the mixture was stored at 20°C for 3 days. After addition of water (10 mL), the precipitated dicyclohexyl urea was filtered off and washed with of 20% aqueous pyridine (20 mL). The combined filtrates were washed with $CHCl_3$ (2×50 mL), concentrated in vacuo, evaporated with toluene (3×10 mL) and applied on a column of silica gel (30 g). The column was washed with system B (300 mL) and then eluted with $CHCl_3$/MeOH/Et_3N 95:5:5. Fractions containing the product were collected and evaporated in vacuo to dryness. The residue was dissolved in 5 M ammonia in methanol (10 mL) and kept for 3 days at 20°C and then concentrated in vacuo to dryness. The residue was partitioned between $CHCl_3$ (15 mL) and water (30 mL), the water layer was washed with chloroform (2×15 mL). The aqueous layer was concentrated in vacuo. The residue was dissolved in 1N NaOH (5 mL) and kept for 20 min at 20°C, then 10% aqueous acetic acid was added to pH 7.0. The solution was diluted with water (30 mL) and then applied to a column of DEAE-cellulose (200 mL, HCO_3^--form). The column was washed with water (500 mL), a 0.05 M solution of NH_4HCO_3 and eluted with 0.1 M solution of NH_4HCO_3. The UV-absorbing fractions were combined, evaporated in vacuo, and coevaporated with water (5×10 mL). The residue was dissolved in 20 mL of water and freeze-dried. Monophosphate **12** was obtained as its ammonium salt. Yield 177 mg (37%). R_f 0.15 (D).

ESI MS (pos.): 477.0896 $[M + Na]^+$.

UV (pH 1): λ_{max} 267 nm (ε 9200); (pH 7): λ_{max} 267 nm (ε 9100); (pH 13): λ_{max} 267 nm (ε 7100).

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