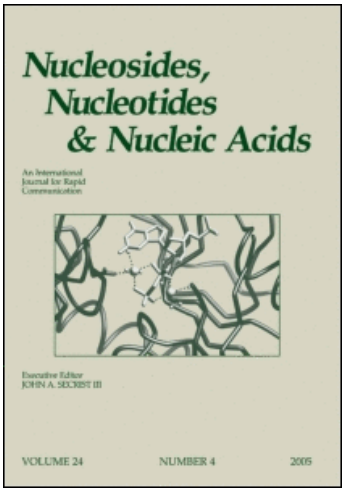

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Pyrophosphoryl Derivatives of 1-(2-Deoxy-3-*O*-Phosphonomethyl-β-and-α-D-Erythro-Pentofuranosyl)Thymine: Synthesis and Substrate Properties Towards Some DNA Polymerases

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NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 19(10–12), 1885–1909 (2000)

PYROPHOSPHORYL DERIVATIVES OF 1-(2-DEOXY-3-*O*-PHOSPHONOMETHYL-β- AND -α-D-*erythro*-PENTOFURANOSYL)THYMINE: SYNTHESIS AND SUBSTRATE PROPERTIES TOWARDS SOME DNA POLYMERASES‡

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ABSTRACT: The synthesis of 1-(2-deoxy-3-*O*-phosphonomethyl-β-D-*erythro*-pentofuranosyl)thymine (**17**) and its α-anomer **18** is described. Attempts to prepare 1-[2-deoxy-3-*O*-(pyrophosphoryl)phosphonomethyl-β-D-*erythro*-pentofuranosyl]thymine (**19**) by an activation of the respective phosphonate **17** with 1,1'-carbonyldiimidazole (Im_2CO) resulted in the quantitative formation of the corresponding pyrophosphonate derivative **21** (Scheme 2). Activation of inorganic pyrophosphate with Im_2CO followed by the condensation with the phosphonates **17** and **18** afforded the desired analogues of nucleoside triphosphate **19** (35%) and its α-anomer **20** (27%) along with the respective pyrophosphonate derivatives **21** (37%) and **24** (38%) (Scheme 3). It was found that compounds **19** and **20** display (*i*) no substrate properties toward calf thymus terminal deoxynucleotidyl transferase (TDT) and AMV reverse transcriptase, and (*ii*) moderate substrate activity with *E. coli* DNA polymerase I (Klenow fragment).

INTRODUCTION

During the last decade, there has been a continuously growing interest in short oligodeoxynucleotides (ODN's) in the framework of the antisense concept (for reviews,

‡ This paper is dedicated to the memory of Prof. Alexander A. Krayevsky.

see, *e.g.*, Refs[1-4]). Antisense ODN's bind to the target mRNA through complementary Watson-Crick base-pairing, which results in an induction of RNase H activity followed by cleavage of the RNA part of an ODN-RNA duplex. Consequently, an antisense ODN inhibits an expression of a protein encoded by the target mRNA.

Unfortunately, regular ODN's are subject to rapid exo- and endonuclease digestion in serum, precluding their application as antisense molecules. The search for ODN's with enhanced resistance against nucleases, improved hybridization characteristics and an ability to support RNase H activity has led to many structural modifications. An analysis of earlier results of the structure-activity-relationship studies has led to the development of a chimeric oligonucleotide strategy (GAPmer technology).[3-5] Among these second generation antisense, chimeric ODN's consisting of a gap, *viz.*, a stretch of regular ODN or modified units supporting the RNase H activity, with several modified nucleosides at both the 5'- and 3'-terminus have proven to be more efficient antisense agents than the uniformly modified ODN's. The modifications in the flanking regions of the gap should provide a nuclease resistance, on the one hand, and should not disturb the binding affinity and specificity, on the other.

The α-D-oligonucleotides as the nuclease-resistant flanks are of interest because they are able to form stable duplexes with complementary RNA strands.[6] Alternating α,β-oligodeoxyribonucleotides with alternating (3'→3')- and (5'→5')-internucleoside phosphodiester linkages displayed some advantages over the α-D-oligonucleotides.[7] Furthermore, oligonucleotide methylenephosphonate analogues, containing a P-C bond in the phosphate bridge, attract an attention due to the resistance to degradation by nucleases.[8-10] These data prompted us to design the ODN's bearing methylenephosphonate bonds at both the 5'- and 3'-termini. The present paper describes the synthesis of the P^{β},P^{γ}-diphosphate derivatives **19** and **20** of the respective 1-(2-deoxy-3-*O*-phosphonomethyl-β- and -α-D-*erythro*-pentofuranosyl)thymine (**17**) and (**18**), and the study of their substrate properties toward some DNA polymerases (for preliminary account, see[11]).

RESULTS AND DISCUSSION

Synthesis and structural elucidation.- For the preparation of the 3'-*O*-methanephosphonates **17** and **18**, diethyl [(*p*-toluenesulfonyl)oxy]methanephosphonate

(DEMP-Ts)[12,13] was chosen first. Due to moderate yields, it was replaced by the more reactive [(trifluoromethylsulfonyl)oxy]methanephosphonate (DEMP-Tfl).[14]

The synthesis of thymine nucleosides **2**-**5** is depicted in Scheme 1. The 1-(2-deoxy-α-D-*erythro*-pentofuranosyl)thymine (α-D-thymidine; **2**) was prepared from thymidine (**1**) employing the self-anomerization[7] reported by Yamaguchi and Saneyoshi.[15] Thymidine was silylated with a mixture of chlorotrimethylsilane (TMS-Cl) and hexamethyldisilazane (HMDS) in the presence of ammonium sulfate and then heated under reflux in acetonitrile in the presence of trimethylsilyl trifluoromethanesulfonate (TMS-Tfl) to afford, after silica gel column chromatography, the α-D-thymidine (**2**) and the recovered starting thymidine (**1**) in a 26 and 8% yield, respectively.

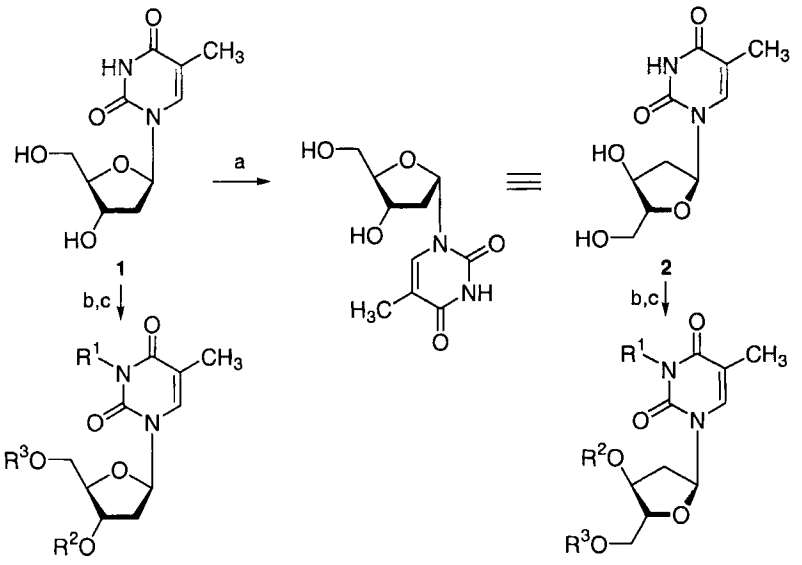
The reaction of compound **3** with DEMP-Tfl in the presence of an excess of sodium hydride (ratio of reactants 1.0:1.2:2.0, mol) in anhydrous tetrahydrofuran (THF) at 0 °C was completed within 15 min. After work-up and subsequent silica gel column chromatography, the N^3-methanephosphonate **6** and N^3,3'-*O*-bis(methanephosphonate) **8** were isolated in a 70 and 18% yield, respectively. This result is in good agreement with that previously observed by Herdewijn and coworkers[13] upon 5'-*O*-phosphonomethylation of 3'-deoxythymidine with DEMP-Ts employing a similar ratio of the reactants.

Furthermore, increasing the amount of sodium hydride to 3 molar equivalents resulted in the predominant formation of the 3'-*O*-methanephosphonate **10** in a 86% isolated yield. Compounds **6** (4%) and **8** (8%) were also isolated from the reaction mixture. In a similar way, the reaction of **4** with DEMP-Tfl in the presence of sodium hydride (1.0:1.2:3.0, mol), after standard work-up and subsequent chromatography, gave the 3'-*O*-methanephosphonate **11** in 94% yield (Scheme 1).

Phosphonate esters **6**, **8** and **10** were readily converted into the corresponding acids **13**, **15** and **17** by treatment with TMS-Br in acetonitrile at room temperature.[16] Under these reaction conditions, both the *t*-butyldimethylsilyl group and the monomethoxytrityl group were also removed.

In contrast to the reaction of the β-anomers **3** and **4** with DEMP-Tfl under optimum conditions for the formation of the 3'-*O*-methanephosphonates, the same reaction with the α-anomer **5** as the starting compound led to the N^3-methanephosphonate **7** and N^3,3'-

Scheme 1



β	R^1	R^2	R^3	α
3	H	H	Si	
4	H	H	MTr	**5**
6	$-CH_2PO(OEt)_2$	H	Si	
	$-CH_2PO(OEt)_2$	H	MTr	**7**
8	$-CH_2PO(OEt)_2$	$-CH_2PO(OEt)_2$	Si	
	$-CH_2PO(OEt)_2$	$-CH_2PO(OEt)_2$	MTr	**9**
10	H	$-CH_2PO(OEt)_2$	Si	
11	H	$-CH_2PO(OEt)_2$	MTr	**12**
13	$-CH_2PO(OH)_2$	H	H	**14**
15	$-CH_2PO(OH)_2$	$-CH_2PO(OH)_2$	H	**16**
17	H	$-CH_2PO(OH)_2$	H	**18**
19	H	$-CH_2PO(OH)OP^{\beta}P^{\gamma}$	H	**20**

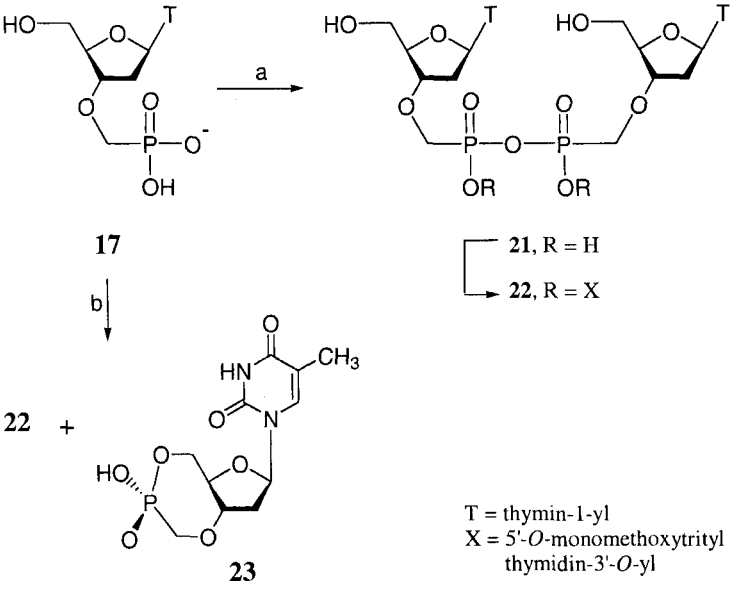
DEMP-Tfl = diethyl [(trifluoromethylsulfonyl)oxy]methanephosphonate $[(EtO)_2PO(CH_2OSO_2CF_3)]$; **Si** = *t*-butyldimethylsilyl; **MTr** = *p*-monomethoxytrityl.

(a) TMS-Cl/HMDS, $(NH_4)_2SO_4$, reflux, 1.5 h; MeCN, TMS-Tfl, reflux, 1.5 h (**2**, 26%; **1**, 8%); (b) Si-Cl/imidazole/DMF, 4 °C, 20 h (83%); (c) MTr-Cl/Py, 20 °C, 16 h (**4**, 89%; **5**, 83 %); (d) (*1*) **3**/NaH/DEMP-Tfl (1.0:2.0:1.2, mol), THF, 0 °C, 15 min (**6**, 70%; **8**, 18%); (*2*) **3**/NaH/DEMP-Tfl (1.0:3.0:1.2, mol), THF, 0 °C, 15 min (**10**, 86%, along with **6** (4%) and **8** (8%); **4** → **11**, 94%; (*3*) **5**/NaH/DEMP-Tfl (1.0:4.5:1.2, mol), THF, 0 °C, 15 min (**12**, 61%; **7**, 6%; **5**, 11%; (e) **6**-**12**/TMS-Br (1.0:10.0, mol), MeCN, 20 °C, 1.5 h (**13**, 43%; **15**, 28%; **17**, 81% from **10** and 70% from **11**; **14**, 70%; **16**, 55%; **18**, 76%); (f) tributylammonium pyrophosphate/Im_2CO/ DMF, 20 °C, 16 h; **17** or **18**, 20 °C, 20 h [**19** (35%) + **21** (37%; **20** (27%) + **24** (38%)].

O-bis(methanephosphonate) **9** as the principal products isolated as a mixture (≈3:1; according to ^{1}H NMR) in 58% yield. The desired 3'-*O*-methanephosphonate **12** was also isolated from the reaction mixture in a yield of 10%; 18% of the starting nucleoside **5** was recovered. The course of the reaction was further studied by increasing the amount of sodium hydride. The satisfactory yield of the desired 3'-*O*-methanephosphonate **12** (61%) was achieved using a molar ratio of the α-nucleoside **6**, sodium hydride and DEMP-Tfl of 1.0:4.5:1.2 (Scheme 1). Under these reaction conditions, the N^3-methanephosphonate **7** was isolated in a 6% yield as well as unconsumed starting nucleoside **5** (11%). Treatment of the fully blocked phosphonates **7**, **9** and **12** with TMS-Br in acetonitrile at room temperature, followed by column chromatography on DEAE-cellulose (HCO_3^- -form) afforded the corresponding 3'-*O*-methanephosphonic acids **14** (70%), **16** (55%) and **18** (76%).

Attempts to prepare the P^{β},P^{γ}-diphosphate derivative **19** according to Hoard and Ott[17] by an activation of the respective phosphonate **17** with 1,1'-carbonyldiimidazole (Im_2CO) resulted in the quantitative formation of the pyrophosphonate derivative **21** (*cf.*[18]). It was somewhat unexpected because Imbach and coworkers[19] have employed this method for the preparation of the P^{β},P^{γ}-diphosphate derivative of 2',3',5'-trideoxyuridine-5'-methylphosphonic acid and have not observed the formation of the related pyrophosphonate (*cf.*[13,20]). Moreover, activation of 5'-*O*-phosphonomethyl analogues of nucleoside-5'-phosphates with morpholine in the presence of *N,N'*-dicylohexylcarbodiimide (DCC) led to the quantitative formation of the corresponding morpholidates.[12] On the other hand, Rosenberg and Holy[21] have reported the ease of formation of the six-membered cyclic phosphonate derivatives on treatment of 9-(*S*)-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA) and its isomeric 3'-*O*-phosphonylmethyl ether with morpholine and DCC in pyridine (reflux for 8 h; 91%) or aqueous *t*-butyl alcohol (reflux for 6 h; 89%). In the case of phosphonate **17**, the formation of the seven-membered cyclic phosphonate **23** (Scheme 2) is evidently less favoured. However, condensation of the phosphonate **17** with 5'-*O*-monomethoxytritylthymidine (**4**) in the presence of triisopropylbenzenesulfonyl chloride (TPS-Cl) in pyridine affords a mixture of pyrophosphonate derivative **22** and cyclophosphonate **23**, isolated by silica gel column chromatography in yields of 64 and 15%, respectively. The former was also prepared by condensation of the pyrophosphonate **21** with **4** under the same reaction conditions (Scheme 2).

Scheme 2

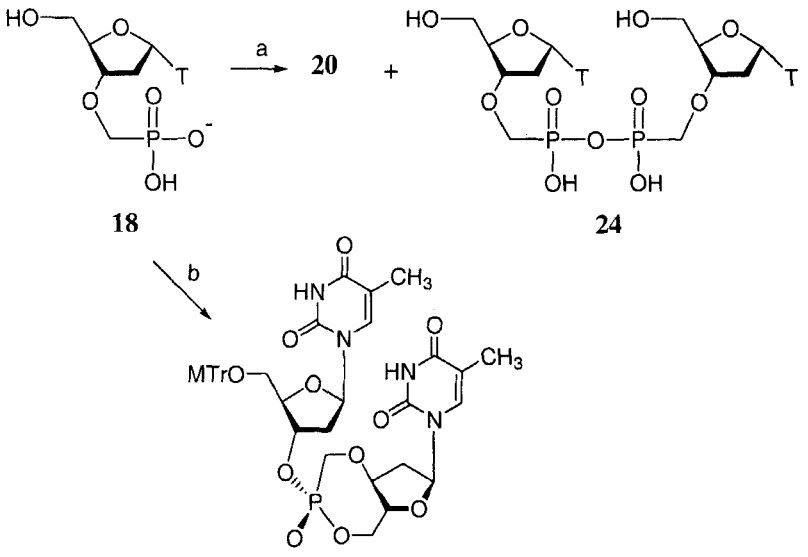


(a) **17**/Im_2CO/DMF, room temperature, 16 h (**21**, 94%); (b) **17** + **4**/TPS-Cl/pyridine, room temperature, 16 h (**22**, 64%; **23**, 15%); (c) **21** + **4**/TPS-Cl/pyridine, room temperature, 16 h (**22**, 34%)

The desired P^{β},P^{γ}-diphosphate derivatives **19** and **20** were synthesized by an activation of inorganic pyrophosphate with Im_2CO followed by condensation with phosphonates **17** and **18**. Ion-exchange chromatography on DEAE-cellulose using a linear gradient (0.001→0.4 M) of TEAB buffer gave individual pyrophosphoryl derivatives **19** and **20** in yields of 35 and 27%, respectively. The triethylammonium salts of the products were transformed into the sodium form by precipitation with NaI according to Moffatt.[23] It is noteworthy, that even in this case both phosphonates **17** and **18** demonstrated a propensity to the formation of the respective pyrophosphates **21** and **24**, which were isolated from the corresponding reaction mixtures in a 37 and 38% yield. The reaction of the α-phosphonate **18** with **4** in the presence of TPS-Cl in pyridine furnished the cyclophosphonate derivative **25** as the principal product, which was isolated by silica gel column chromatography in a yield of 67% (Scheme 3). Attempts to hydrolyze selectively the 5'-*O*-monomethoxytritylthymidine residue of **25** in order to prepare the α-D-anomer of the cyclophosphonate **23** have failed.

The structure of all the synthesized methanephosphonates was unambiguously proved by the ^{1}H and ^{13}C NMR data using the described characteristic features of the *N*- and *O*-isomers.[13] The ^{1}H NMR spectra of the *N*-isomers **13** and **14** reveal doublets of the CH_2-P fragment at 4.25 ppm with $^{2}J_{P,H}$ of 12.5 and 12.0 Hz, respectively, whereas in the case of the *O*-isomers **17** and **18** this signal appears within 3.60-3.70 ppm ($^{2}J_{P,H}$ = 9.0-9.5 Hz). The ^{13}C NMR spectra display the carbon resonance of the N-CH_2-P methylene group at 39.6 ppm ($^{1}J_{P,C}$ = 144.0-145.0 Hz), while that of the O-CH_2-P is at 65.8 ppm ($^{1}J_{P,C}$ = 156.2-157.6 Hz). Moreover, phosphonomethylation of the 3'-hydroxyl group resulted in the downfield shifts of the resonance of C(3') by *ca.* 11.0 ppm *vs.* the same resonance of the starting nucleoside and the N^3-isomer. In the proton-decoupled ^{13}C NMR spectrum, the resonance of C(3') of 3'-*O*-methanephosphonates is split into a doublet by phosphorus with the $^{3}J_{P,C}$ of *ca.* 12 Hz. The NMR spectra of bis-phosphonomethylated compounds display both CH_2-P signals (see TABLES 1-3 and EXPERIMENTAL).

Scheme 3



(a) tributylammonium pyrophosphate/Im_2CO/DMF, room temperature, 16 h; **18**, room temperature, 20 h (**20**, 27%; **24**, 38%); (b) **18** + **4**/TPS-Cl/pyridine, room temperature, 16 h (**25**, 67%)

TABLE 1. ^{1}H NMR Spectral Data (δ_{TMS}, ppm) of the 3'-*O*-Phosphonate Derivatives of α- and β-D-Thymidine.

Compound	Chemical shifts, δ_{TMS}, ppm										Others
	Base		Sugar								
	H-6	5-CH_3	H-1'	H-2'	H-2"	H-3'	H-4'	H-5'	H-5"	CH_2-P, Ha	CH_2-P, Hb	
17 (D_2O & CD_3OD)	7.69 br.s	1.93 br.s	6.32 dd	2.34 m	2.56 ddd	4.32 m	4.21 m	3.88 dd	3.82 dd	3.74 dd	3.66 dd	
18 (D_2O & CD_3OD)	7.82 d	1.95 br.s	6.31 dd	2.74 ddd	2.35 br.dt	4.27 dm	4.62 m	3.74 dd	3.65 dd	3.63 d		
21 (D_2O)	7.70 br.s	1.92 br.s	6.31 br.t	2.37 br.m	2.59 br.m	4.35 br.m	4.24 br.m	3.87 br.m				
22 (D_2O)	7.56 & 7.54 (2s) 7.00 & 6.99 (2s)	1.45 & 1.41 (2s) 1.96 & 1.94 (2s)	6.52 & 6.48 (2t) 6.03 & 5.81 (2dd)	2.4-2.8 m		5.41 & 5.29 (2br.t) ~4.35 (m)	~4.40 (m) ~4.1 ~4.5 (m)	~4.5 3.54 dd	~4.3 ~3.46 br.dd	3.91 br.d	3.74 br.d	9.56, 9.42, 9.31 & 920 (4s, N^3**H**); 7.20-7-50 (24H) & 6.89, 6.86 (2s, 4H, arom.); 3.82, 3.81 (2s, OMe)
23 (D_2O)	7.33 br.s	1.80 br.s	6.12 dd	2.47 center of m		4.28 center of m			~3.70 m	3.85 dd	3.57 br.d	
24 ($CDCl_3$)	7.83 br.s	1.95 s	6.31 dd	2.78 ddd	2.28 br.d	4.29 m	4.62 m	3.72 dd	3.66 dd	3.82 dd	3.76 dd	
25 ($CDCl_3$)	7.57 & 7.54 (2s) 7.26 & 7.25 (2s)	2.00 & 1.98 (2s) 1.44 (s, 6H)	6.49 & 6.42 (2dd) 6.26 & 6.08 (2dd)	2.61 m ~2.92 m ~2.82 m	~2.46 m ~2.20 m ~2.28 m	5.38 & 5.28 (2br.t) *~4.35* & *~4.52* 2m	~4.30 m ~3.65 m	~4.5 ~3.52 m	~3.42 m	a)	a)	9.25 (br.s, 4H N^3**H**); 7.30-7-50 (24H) & 6.89, 6.89 and 6.86 (2s, 4H, arom.); 3.82 (2s, OMe)

[a] Not detected owing to overlap.

TABLE 2. Coupling Constants (J, Hz) for the ^{1}H NMR Data of the 3'-*O*-Phosphonate Derivatives of α- and β-D-Thymidine

Compound	Coupling constants, J Hz											Others
	1',2'	1',2"	2',2"	2',3'	2",3'	3',4'	4',5'	4',5"	5',5"	P,Ha	P,Hb	
17	7.5	6.5	14.7	7.8	3.0	~3.0	3.0	3.5	11.5	2.0	2.0	6.6 ($^{gem}J_{Ha,Hb}$)
18	7.5	2.5	15.0	6.5	2.5	2.5	4.5	~6.0	12.0	9.5		1.2 ($^4J_{H6,CH3}$)
21	7.8	6.0	14.4	6.0	2.4	a)	a)	a)	b)	b)	b)	
22	5.65 5.65 8.17 8.48	5.65 5.65 3.77 2.52	n.d.	5.81 5.97	5.81 5.97	6.61 7.20 n.d.	n.d.	n.d.	n.d.	<2.0	<2.0	14.77 ($^{gem}J_{Ha,Hb}$)
23	8.01	3.61	c)	c)	c)	d)	d)	e)	e)	9.1	<1.0	14.44 ($^{gem}J_{Ha,Hb}$)
24	7.5 2.5	2.5 7.5	15.0	6.5 1.5	1.5 6.5	2.0	3.9	4.5	13.0	2.9	3.9	12.40 ($^{gem}J_{Ha,Hb}$)
25	8.05 8.63 6.23 8.15	5.75 5.75 6.23 7.58	13.25	f)	f)	f)	f)	f)	f)	f)	f)	

[a] Not determined owing to broadening of the resonances of H-3' and H-4'.
[b] Not determined owing to an overlap of the resonances of H-5', H-5", Ha and Hb.
[c] Not determined owing to an overlap of the resonances of H-2' and H-2".
[d] Not determined owing to an overlap of the resonances of H-3', H-4' and H-5'.
[e] Not determined owing to an overlap of the resonances of H-5" and Ha and Hb.
[f] Not determined owing to overlap.

TABLE 3. Selected ^{13}C NMR Spectral Data of the 3'-*O*-Phosphonate Derivatives of α- and β-D-Thymidine.[a)]

Com-pound	Base				Sugar						Others
	C-4	C-2	C-6	C-5	C-1'	C-2'	C-3'	C-4'	C-5'	3'-O-$\underline{C}H_2$-P	
17	166.8	152.0	137.9	111.8	85.7	36.3	81.5 d ($^3J_{P,C}$ 11.9)	85.0	62.0	65.7 d ($^1J_{P,C}$ 157.6)	^{31}P: 16.05
18	167.1	152.2	138.8	111.4	86.8 [b)]	37.2	81.6 d ($^3J_{P,C}$ 12.0)	86.8 [b)]	62.3	65.8 d ($^1J_{P,C}$ 156.2)	^{31}P: 16.38
21	166.9	152.0	138.0	111.7	85.0 [b)]	36.2	81.4 "t" ($^3J_{P,C}$ 5.45 & 6.66)	85.5 [b)]	61.9	65.9 d ($^1J_{P,C}$ 168.9)	^{31}P: 9.00
22 [b)]	163.4 (1); 163.1 (3)	152.1 149.9 149.4 149.3	134.5 (2); 133.9 133.9	111.4 111.3 111.1 110.7	83.7 83.5 84.9 87.0	36.8; 36.8; 38.7 (d, $^3J_{P,C}$ 10.0); 38.7 (d, $^3J_{P,C}$ 6.89)	84.1 (d, $^3J_{P,C}$ 4.23); 84.0 (d, $^3J_{P,C}$ 6.06) *ca.* 76-77 overlap by $CDCl_3$	81.3 & 80.2 (2s) 81.9 (d, $^2J_{P,C}$ 7.30)	65.8 & 65.9 (2s) 65.2 (2s) 62.6 ($2^{13}C$)	65.4 d ($^1J_{P,C}$ 147.7) 65.0 d ($^1J_{P,C}$ 140.8)	^{31}P: 25.45 & 24.86
23	166.8	151.8 {$^3J_{C2,H6}$ 7.48; $^3J_{C2,H1'}$ *2.33*}	138.4 (180.5) {$^3J_{C6,H1'}$ & $^3J_{C6,C\underline{H}3}$ 5.22}	112.0 {$^2J_{C5,H6}$ & $^2J_{C5,C\underline{H}3}$ 6.4}	85.1 (170.8)	36.5 (132.6 & 138.6)	81.1 [b)] (148.2) ($^3J_{P,C}$ 1.21)	81.7 [b)] (150.4) $^3J_{P,C}$ <1.0	64.3 (151.3) ($^2J_{P,C}$ 4.84)	66.8 d (141.9) {$^3J_{\underline{C}H2,H3'}$ 2.23} ($^1J_{P,C}$ 142.3)	^{31}P: 20.58 br.s
24	166.9	152.0	138.7	111.4	86.6 [b)]	37.5	81.5 "t" ($^3J_{P,C}$ 5.91)	86.5 [b)]	62.1	65.9 d ($^1J_{P,C}$ 168.8)	^{31}P: 8.71
25 [b)]	163.5 163.4 163.4 163.3	150.4 150.3 150.2 150.1	134.3 134.2 134.1	112.0 111.7 111.6	84.1; 83.2; 84.0 (2)	37.5; 37.0; 39.10 "t" ($^3J_{P,C}$ 3.03 & 2.42)	84.4 (d, $^3J_{P,C}$ 6.06); 84.4 (d, $^3J_{P,C}$ 3.02) ca. 77.3; 77.8 overlap by $CDCl_3$)	80.3; 79.9; 83.0 d ($^3J_{P,C}$ 2.0); 82.4	66.5 (d, $^2J_{P,C5'}$ 6.66); 64.9 (d, $^2J_{P,C5'}$.3.63) 63.1 (d, $^4J_{P,C5'}$ 2.0); 62.9 (d, $^4J_{P,C5'}$.2.4)	67.0 d ($^1J_{P,C}$ 150.8) 65.9 d ($^1J_{P,C}$ 142.3)	^{31}P: 25.22 & 24.22

[a)] The ^{13}C resonances of the 5-methyl groups were within 11.10-12.42 ppm; the ^{13}C resonances of the monomethoxytrityl groups were at (ppm): tertiary atom ^{13}C – 86.9-87.6; phenyl – 126.9-127.5 (*para*), 127.8-128.4 (*meta*), 127.8-128.1 (*ortho*), 143.4-143.9 (*ipso*); *p*-methoxyphenyl - 54.7-55.3 ($O\underline{C}H_3$), 158.3-159.0 (*para*), 112.8-113.4 (*meta*), 129.8-130.4 (*ortho*), 134.9-135.0 (*ipso*).

[b)] The data (δ and *J*) may be interconvertible.

Analysis of the ^{1}H, ^{13}C and ^{31}P NMR spectra of the pyrophosphonates **22** and **25** *vs.* the parent respective phosphonates **17** and **18** pointed to the close similarity of both compounds, but did not lead to an unambiguous elucidation of their structures (TABLES 1-3). The assignments of the ^{1}H and ^{13}C resonances of compounds **22** and **25** was performed on the basis of the 2D $^{1}H,^{13}C$ NMR correlation spectra as well by homonuclear decoupling experiments. The only indication of the pyrophosphonate structure essentially is the ^{31}P chemical shifts at 9.00 and 8.71 ppm (*cf.*, *e.g.*, the data for the related phosphonates[22]). The correctness of structural elucidations was finally substantiated by means of the electrospray ionization mass-spectrometry.

The structure of the P^{β},P^{γ}-diphosphate derivatives **19** and **20** was proved by the ^{31}P NMR spectroscopy and matrix-assisted laser desorption-ionization time of flight (MALDI-TOF) mass-spectrometry. Thus, in the ^{31}P NMR spectrum of **19** were observed the resonances at δ_{H3PO4} (ppm) 8.8 (P^{α}, $^{2}J_{P\alpha,P\beta}$ = 28.0 Hz), –22.0 (P^{β}) and –9.5 (P^{γ}, $^{2}J_{P\alpha,P\beta}$ = 21.0 Hz); the corresponding resonances in the ^{31}P NMR spectrum of **20** are at the 9.0 ($^{2}J_{P\alpha,P\beta}$ = 24.0 Hz), –23.3 and –9.9 ($^{2}J_{P\alpha,P\beta}$ = 22.0 Hz) ppm. These data are in good agreement with those published for the closely related P^{β},P^{γ}-diphosphate derivatives of phosphonates.[19,22,24]

Biochemical studies.- Single-stranded M13*mp*10 phage DNA was isolated from the culture medium of the recipient *E. coli* K12XL1 strain as described.[25] The tetradecanucleotide primer (FIG. 1) was labeled at the 5'-terminus using [γ-^{32}P]-ATP and T4 polynucleotide kinase according to the data[26] with subsequent enzyme inactivation (10 min, 65°C). The template-primer complex was prepared by incubation of the 5'-labeled primer and the phage DNA in the buffer (10 mM Tris-HCl, pH 8.0 and 5 mM $MgCl_2$) at 65°C for 10 min and then cooling to 30°C for 60 min. The template-primer complex and the ^{32}P-labeled primer were separated by filtering through the Biogel A-1.5m column (1 ml) in 10 mM Tris-HCl, pH 7.6 containing 1 mM EDTA, and was stored at -20°C.

The results of the calf thymus terminal deoxynucleotidyl transferase (TDT) assays in the presence of **19** and **20** are presented in FIG. 2. As can be seen, the tested compounds were not substrates for the template-independent enzyme (lanes *4-6* and *7-9*)

```
          1                    15                   30
3'-d(.....GGGTCAGTGCTGCAACATTTTGCTGCCGGTCACGGTTCGA....)
5'-d([32P]CCCAGTCACGACGT)
```

FIG. 1. Template-primer complex.

FIG. 2. Experiments with TDT. Lane *1* is primer + enzyme (control); lane *2* as in lane *1* + 10 μM ddTTP; lane *3* as in lane *1* + 10 μM dTTP; lanes *4-6* as in lane *1* + **19** 10 μM (*4*), 100 μM (*5*) and 1 mM (*6*); lanes *7-9* as in lane *1* + **20** 10 μM (*7*), 100 μM (*8*) and 1 mM (*9*), respectively.

even at the 1 mM concentration. Similar results were obtained for avian myeloblastosis virus reverse transcriptase (FIG. 3, Series B, lanes *5-7* and *8-10*). Bacterial DNA polymerase I (Klenow fragment) was capable of incorporating into the DNA chain one nucleotide residue of both analogues. It is evident from FIG. 3, Series A, that only one **19** (lanes *3-5*) or **20** (lanes *6-8*) unit was weakly incorporated into the primer. It is worth noting that the formation of the elongated primer was increased with an increase in the concentration of the tested compounds from 10 μM to 100 μM and then decreased.

The efficiency of the primer extension depended on the substrate concentration in a bell-like mode, probably due to the 3',5'-exonuclease hydrolysis. Indeed, the concentration-dependent enhancement of the formation of the shorter oligonucleotides than the initial primer was observed (FIG. 3, Series A, lines *3-5* and *6-8*). This observation harmonises with the finding that the misincorporation (non-Watson-Crick) into the primer of natural substrate or the incorporation of modified weak substrate results in an enhancement of the 3'-5'exonuclease activity of the Klenow fragment of *E. coli* DNA polymerase I[27], probably due to the switch from polymerization to exonuclease activity of the enzyme.

The lack of substrate properties of the P^{β},P^{γ}-diphosphate derivatives **19** and **20** toward calf thymus TDT and AMV reverse transcriptase was somewhat unexpected. Indeed, the closely related P^{β},P^{γ}-diphosphate derivatives of phosphonates have been shown to be substrates of these enzymes.[28,29] On the other hand, it was shown that P^{β},P^{γ}-diphosphates of 2',3',5'-trideoxyuridine-5'-methylphosphonic acid[19] and 2'-deoxy-5'-*O*-phosphonomethylnucleosides[30] are not substrates of HIV-1 reverse transcriptase. The former compound is an isosteric, but not an isoelectronic[31] analogue of the natural 2'-deoxynucleoside-5'-triphosphate (dNTT-5'), the latter, containing an additional methylene group between the 5'-oxygen atom and the triphosphate function, are neither isosteric, nor isoelectronic analogues. Moreover, the P^{β},P^{γ}-diphosphates of 2'-deoxy-5'-*O*-phosphonomethylnucleosides are also not substrates of AMV reverse transcriptase and *E. coli* DNA polymerase I (Klenow fragment).[30]

The P^{β},P^{γ}-diphosphate **20** may be considered as an isosteric (but not an isoelectronic[31]) mimic of 2'-deoxythymidine-5'-triphosphate (dTTP). However, the absence of substrate properties toward DNA polymerases employed in this study could

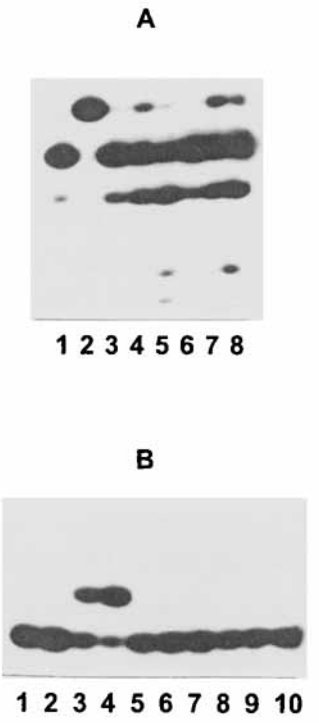


FIG. 3. Primer extension catalyzed by DNA polymerase I (Series A) and AMV reverse transcriptase (Series B). Series A, lane *1* - primer-template complex + enzyme (control); lane *2* as in lane *1* + 1 μM dTTP (*2*); lanes *3-5* as in lane *1* + **19** 10 μM (*3*), 100 μM (*4*) and 1 mM (*5*); lanes *6-8* as in lane *1* + **20** 10 μM (*6*), 100 μM (*7*) and 1 mM (*8*), respectively. Series B, lane *1* - primer-template complex + enzyme (control); lanes *2-4* as in lane *1* + dTTP 0.1 μM (*2*), 1 μM (*3*) and 10 μM (*4*); lanes *5-7* as in lane *1* + **19** 10 μM (*5*), 100 μM (*6*) and 1 mM (*7*); lanes *8-10* as in lane *1* + **20** 10 μM (*8*), 100 μM (*9*) and 1 mM (*10*), respectively.

stem from several causes. The simplest explanation consists in that the 4'-CH_2OH group of **20** caused an unfavorable steric hindrance with critical residues in an enzyme active site. The other reason may be connected with stereochemical peculiarities of **20** *vs.* dTTP. It was suggested that the pentofuranose ring of dNTT-5' is not involved in binding to the polymerases, but properly orientate the heterocyclic base and the triphosphate residue in a polymerization complex.[29] The sugar moieties of nucleosides are involved in a two-state $N \leftrightarrow S$ pseudorotational equilibrium, which is driven by the relative strength of various *gauche* and anomeric stereoelectronic effects (for a review, see Ref.[32]). It was shown that

dTTP predominantly adopts in solution the *S*-conformation (C-3'-*exo*/C-2'-*endo*; ${}_3T^2$).[32] The conformational analysis of the furanose ring of the analogues **19** and **20** was performed by the PSEUROT (version 6.2) program, which calculates the best fits of five experimental ^3J(H,H) coupling constants ($^3J_{H\text{-}1',H\text{-}2'}$, $^3J_{H\text{-}1',H\text{-}2''}$, $^3J_{H\text{-}2',H\text{-}3'}$, $^3J_{H\text{-}2'',H\text{-}3'}$, and $^3J_{H\text{-}3',H\text{-}4'}$) to the five conformational parameters (*P* and ψ_m for both *N*- and *S*-type conformers and corresponding mole fractions).[33,34] Similar to dTTP, the sugar moieties of the β-D-isomer **19** is in a two-state $N \leftrightarrow S$ pseudorotational equilibrium in the ratio of 31:69. The conformational behavior of the α-D-isomer **20** is also very similar with the predominant population of the *S*-conformation (77% mole fraction; P_S 165.3; P_N 9.8, $^2E \leftrightarrow {}^3E/{}_2T^3$). However, considering the α-D-isomer **20** as a mimic of dTTP, the most populated ^2E conformation will simulate the ${}_0E$ (O4'-*exo*) conformation of dTTP. Thus, the $N \leftrightarrow S$ pseudorotational equilibrium of the α-D-isomer **20** and dTTP displays essential differences, which may decrease the substrate properties of the former.

EXPERIMENTAL

General.- Column chromatography was performed on silica gel 60 (70-230 mesh ASTM) (*Merck*, Germany) and DEAE-cellulose 23-SS (*Serva*, Germany). Kieselgel $60F_{254}$ (*Merck*, Germany) plates were used for the thin-layer chromatography (TLC). The following solvent systems were used for TLC: $CHCl_3$-MeOH, 15:1 (A), i-PrOH-NH_4OH-H_2O, 7:1:2 (B), i-PrOH-NH_4OH-H_2O, 11:7:2 (C). The UV spectra were recorded with a *Specord M-400* spectrometer (*Carl Zeiss*, Germany). ^1H and ^{13}C NMR Spectra were measured at 200.13 and 50.325 MHz, respectively, at 23° on an *AC-200* spectrometer equipped with an Aspect 3000 data system (*Bruker*, Germany), and at 500.14 and 125.76 MHz on an AMX-500 spectrometer (*Bruker*, Germany); δ values in ppm downfield from internal $SiMe_4$ (^1H, ^{13}C)(s = singlet; d = doublet; t = triplet; m = multiplet; br.s = broad signal); assignments of proton resonances were confirmed, when possible, by selective homonuclear decoupling experiments. ^{31}P NMR spectra were recorded at 101.25 MHz on an AC-250 spectrometer (*Bruker*, Germany); δ values are given in ppm relative to H_3PO_4. The solvent employed for recording the NMR spectra was $CDCl_3$, unless otherwise stated. Mass-spectra were recorded using either electrospray ionization ES (*Bruker*, Germany) or matrix-assisted laser desorption-ionization time of flight (MALDI-TOF) (Compact MALDI-4 spectrometer, *Kratos Analytical*, USA).

Sodium hydride (60% suspension in mineral oil) was purchased from *Fluka* (Switzerland); in all protocols, the quantity of NaH employed in a reaction is indicated. Bromotrimethylsilane was purchased from *Lancaster* (UK). Diethyl hydroxymethylphosphonate was purchased from *Aldrich* (USA). Diethyl[(trifluoromethylsulfonyl)oxy]-methanephosphonate (DEMP-Tfl) was prepared according to the data.[14]

Following enzymes were used: *E. coli* DNA polymerase I Klenow fragment (*Boehringer Mannheim*, Germany), AMV reverse transcriptase, calf thymus terminal deoxynucleotidyltransferase (TDT), and T4 polynucleotide kinase (*Amersham*, England).

In all reactions, freshly distilled anhydrous tetrahydrofuran (THF), acetonitrile, pyridine and dimethylformamide (DMF) were used. Solutions of compounds in organic solvents were dried with anhydrous Na_2SO_4 for 4 h. Reactions were carried out at room temperature, unless stated otherwise.

1-(2-Deoxy-5-*O*-monomethoxytrityl-α-D-*erythro*-pentofuranosyl)thymine (5). α-D-Thymidine (**2**) (0.5 g; 2.06 mmol) was coevaporated with pyridine (2x5 mL) and dissolved in anh pyridine (6 mL) under an argon atmosphere. To this solution, monomethoxytrityl chloride (0.76 g; 2.47 mmol) was added, the reaction mixture was stirred for 16 h, then methanol (3 mL) was added and the mixture was evaporated to dryness *in vacuo*. The residue was dissolved in chloroform (100 mL) and extracted with water (40 mL). The organic phase was dried and evaporated. The residue was purified by silica gel (3x12 cm) column chromatography; nucleoside **5** was eluted with chloroform-methanol mixture (95:5), product contained fractions were combined and evaporated. The residue was dissolved in chloroform and precipitated in hexane to give **5** as an amorphous powder (0.88 g; 83%). TLC: R_f 0.56 (A). UV (MeOH): λ_{max} 267 nm.

1-[2-Deoxy-5-*O*-(*tert*-butyldimethylsilyl)-β-D-*erythro*-pentofuranosyl]-3-*N*-[(diethylphosphono)methyl]thymine (6) and **1-[2-deoxy-3-*O*-(diethylphosphono)-methyl-5-*O*-(*tert*-butyldimethylsilyl)-β-D-*erythro*-pentofuranosyl]-3-*N*-[(diethylphosphono)methyl]thymine (8).** To the stirred solution of 5'-*O*-(*tert*-butyldimethylsilyl)-thymidine[35] (**3**) (0.5 g; 1.40 mmol) in THF (35 mL) cooled to 4 °C, NaH (0.07 g; 2.80 mmol) and, after 15 min, DEMP-Tfl (0.36 mL; 1.68 mmol) were added. The reaction mixture was stirred at the same temperature for additional 15 min, neutralized with acetic

acid (0.17 mL, 2.80 mmol) and evaporated *in vacuo*. The residue was purified by silica gel (2.5x28 cm) column chromatography (elution with a linear methanol gradient in chloroform; 1→5%, v/v; 1 L) to give the N^3-isomer **6** (0.50 g; 70%) as an oil. TLC: R_f 0.56 (A). UV (MeOH): λ_{max} 269 nm. Bisphosphonate **8** (oil-like product; 0.17 g; 18%) was also isolated. TLC: R_f 0.67 (A). UV (MeOH): λ_{max} 269 nm.

1-[2-Deoxy-3-*O*-(diethylphosphono)methyl-5-*O*-(*tert*-butyldimethylsilyl)-β-D-*erythro*-pentofuranosyl]thymine (10). To the stirred solution of 0.50 g (1.40 mmol) of 5'-O-(*tert*-butyldimethylsilyl)thymidine[35] (**3**) in 35 mL of THF cooled to 4 °C, NaH (0.10 g; 4.20 mmol) and, after 15 min, DEMP-Tfl (0.36 mL; 1.68 mmol) were added. The stirring was continued for additional 15 min at the same temperature, then the reaction mixture was neutralized with acetic acid (0.25 mL; 4.20 mmol) and evaporated. Pure compound **10** (oil-like product; 0.57 g; 80%) was isolated by silica gel column chromatography as described for the compounds **6** and **8**. TLC: R_f 0.60 (A). UV (MeOH): λ_{max} 268 nm. Compounds **6** (30 mg; 4%) and **8** (70 mg; 8%) were also isolated.

1-[2-Deoxy-3-*O*-(diethylphosphono)methyl-5-*O*-monomethoxytrityl-β-D-*erythro*-pentofuranosyl]thymine (11) was obtained from the nucleoside **4** (0.29 g; 0.56 mmol), NaH (0.04 g, 1.68 mmol) and DEMP-Tfl (0.14 mL; 0.67 mmol) in THF (14 mL) as described for the phosphonate **10**, and isolated by silica gel column chromatography in a 94% yield (0.35 g). TLC: R_f 0.59 (A). UV (MeOH): λ_{max} 268 nm.

1-[2-Deoxy-5-*O*-monomethoxytrityl-α-D-*erythro*-pentofuranosyl]-3-*N*-[(diethylphosphono)methyl]thymine (7) and **1-[2-deoxy-3-*O*-(diethylphosphono)-methyl-5-*O*-monomethoxytrityl-α-D-*erythro*-pentofuranosyl]-3-*N*-[(diethylphosphono)methyl]thymine (9)**. To the stirred solution of nucleoside **5** (0.17 g; 0.33 mmol) in THF (8.3 mL) cooled to 4 °C, NaH (0.024 g; 0.99 mmol) and, after 15 min, DEMP-Tfl (0.12 g; 0.40 mmol) were added. The reaction mixture was stirred for additional 15 min at 4 °C, neutralized with acetic acid (0.06 mL; 1.00 mmol) and evaporated to dryness. The residue was purified by silica gel (2x32 cm) column chromatography; the elution was performed by chloroform and then by chloroform-methanol mixture (98:2). The title compounds **7** and **9** were isolated as a mixture (0.14 g; 3:1 according to ^{1}H NMR). TLC: R_f 0.89 (A). The individual 3'-*O*-(diethylphosphono)methyl derivative **12** was also isolated in a 10% yield (0.02 g) as well as 0.03 g (18%) of starting nucleoside **5**.

1-[2-Deoxy-3-*O*-(diethylphosphono)methyl-5-*O*-monomethoxytrityl-α-D-*erythro*-pentofuranosyl]thymine (12) was obtained from 0.23 g (0.45 mmol) of nucleoside **5**, 0.05 g (2.03 mmol) of NaH and 0.12 mL (0.54 mmol) of DEMP-Tfl in THF (11.2 mL) as described above for the compounds **7** and **9**, and isolated by silica gel column chromatography in a 61% yield (0.18 g). TLC: R_f 0.70 (A). UV (MeOH): λ_{max} 268 nm. 3-*N*-(Diethylphosphono)methyl derivative **7** (18 mg; 6%) and an unconsumed nucleoside **5** (25 mg; 11%) were also isolated.

1-(2-Deoxy-3-*O*-phosphonomethyl-β-D-*erythro*-pentofuranosyl)thymine sodium salt (17). A. To a solution of the diethyl phosphonate **10** (0.28 g; 0.55 mmol), coevaporated previously with benzene (2x10 mL), in acetonitrile (4.5 mL) TMS-Br (0.72 mL; 5.53 mmol) was added; the reaction mixture was kept for 1.5 h, evaporated *in vacuo*, then dissolved in 20 mL of 0.2 M TEAB-ethanol mixture (1:1), kept for 30 min and evaporated to dryness. The residue was dissolved in water (100 mL) and washed with chloroform (2x30 mL). Crude phosphonate **17** was purified by DEAE-cellulose (3x17 cm) column chromatography (elution with a linear TEAB gradient, 0.001→0.25 M, 1.0 L). Fractions contained compound **17** were combined and concentrated; the residue was evaporated with ethanol (3x10 mL). The triethylammonium salt of the desired product thus obtained was dissolved in methanol and treated with concentrated solution of sodium iodide in acetone[23], affording compound **17** in form of sodium salt as an amorphous powder (0.17 g, 81%). TLC: R_f 0.16 (B). UV (H_2O): λ_{max} 267 nm.

B. In a similar way, starting from (diethylphosphono)methyl derivative **11** (0.20 g; 0.30 mmol) and TMS-Br (0.39 mL; 3.00 mmol) in acetonitrile (2.5 mL), 0.08 g (70%) of phosphonate **17** was obtained.

1-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-3-*N*-(phosphonomethyl)thymine sodium salt (13) was obtained from the phosphonate **6** (0.13 g; 0.26 mmol) by the action of TMS-Br (0.33 mL; 2.57 mmol) in acetonitrile (2 mL) in a 43% yield (42 mg) as described for the compound **17**. TLC: R_f 0.14 (B). UV (H_2O): λ_{max} 268 nm; ^{1}H NMR (D_2O), δ_{TMS}, ppm (*J*, Hz): 7.66 (br.s, H-6), 6.37 (t, $J_{1',2'} = J_{1',2''} = 6.5$; H-1'), 4.49 (dt, $J_{3',4'} = 4.0$; H-3'), 4.05 (m, H-4'), 4.26 (d, $^2J_{C\underline{H}2,P} = 12.5$; N-C$\underline{H}_2$-P), 3.87 (dd, $J_{4',5'} = 3.5$; $J_{5',5''} = 12.5$; H-5'), 3.79 (dd, $J_{4',5''} = 5.0$; H-5"), 2.40 (dd, $J_{2',3'} = 5.5$; H-2' and H-2"), 1.96 (s, 5-CH_3); ^{13}C NMR (D_2O), δ_{TMS}, ppm (*J*, Hz): 165.0 (C-4), 151.6 (C-2), 135.5 (C-6), 110.7

(C-5), 86.9 (C-4'), 86.3 (C-1'), 70.7 (C-3'), 61.5 (C-5'), 39.0 (C-2'), 39.6 ($^1J_{\underline{C}H2,P}$ = 145.0; N-$\underline{C}H_2$-P), 12.8 (5-CH_3).

1-(2-Deoxy-3-*O*-phosphonomethyl-β-D-*erythro*-pentofuranosyl)-3-*N*-(phosphonomethyl)thymine sodium salt (15) was prepared in a similar way, starting from bis(methanephosphonate) **8** (0.23 g; 0.35 mmol) and TMS-Br (0.68 mL; 5.25 mmol) in acetonitrile (2.7 mL) in a 28% yield (47 mg): TLC: R_f 0.04 (C). UV (H_2O): λ_{max} 269 nm; ^{1}H NMR (D_2O), δ_{TMS}, ppm (*J*, Hz): 7.80 (br.s, H-6), 6.38 (m, H-1'), ~4.30 (m, H-3'), 3.90-3.60 (m, H-4', H-5' and H-5"), 4.22 (d, $^2J_{CH2,P}$ = 12.0; N^3-CH_2-P), 3.58 (d, $^2J_{CH2,P}$ = 9.0; $O^{3'}$-CH_2-P), 2.60-2.30 (m, H-2' and H-2"), 1.96 (s, 5-CH_3).

1-(2-Deoxy-α-D-*erythro*-pentofuranosyl)-3-*N*-(phosphonomethyl)thymine sodium salt (14) and 1-(2-deoxy-3-*O*-phosphonomethyl-α-D-*erythro*-pentofuranosyl)-3-*N*-(phosphonomethyl)thymine sodium salt (16). The mixture of phosphonates **7** and **9** (0.30 g; 3:1) was coevaporated with benzene (2x10 mL), dissolved in acetonitrile (5.8 mL) and TMS-Br (0.75 mL) was added. After 1.5 h, the solution was evaporated *in vacuo*, the residue was dissolved in 20 mL of 0.2 M TEAB-ethanol (1:1) mixture and after 30 min evaporated to dryness. The residue was dissolved in water (100 mL), extracted with chloroform (2x30 mL) and applied to a DEAE-cellulose column (3x21 cm) eluted with a linear TEAB gradient (0.001→0.3 M; 1.5 L). Fractions contained individual phosphonates **14** and **16** were collected separately, concentrated *in vacuo* and coevaporated with ethanol. After conversion into sodium salt[23], 90 mg of compound **14** was obtained as well as 26 mg of compound **16**.

N^3-Methanephosphonate **14**: TLC: R_f 0.15 (B). UV (H_2O): λ_{max} 270 nm; ^{1}H NMR (D_2O), δ_{TMS}, ppm (*J*, Hz): 7.75 (br.s, H-6), 6.24 (dd, $J_{1',2'}$ = 7.0; $J_{1',2"}$ = 3.0; H-1'), 4.45 (m, H-3', H-4'), 4.25 (d, $^2J_{C\underline{H}2,P}$ = 12.0; N-C$\underline{H}_2$-P), 3.76 (dd, $J_{4',5'}$ = 3.5; $J_{5',5"}$ = 12.5; H-5'), 3.66 (dd, $J_{4',5"}$ = 5.0; H-5"), 2.77 (ddd, $J_{2',3'}$ = 7.0; $J_{2',2"}$ = 14.5; H-2'), 2.24 (dt, $J_{2",3'}$ = 3.0; H-2"), 1.98 (s, 5-C$\underline{H}_3$); ^{13}C NMR (D_2O), δ_{TMS}, ppm (*J*, Hz): 165.1 (C-4), 151.4 (C-2), 135.9 (C-6), 109.8 (C-5), 89.0 (C-4'), 88.1 (C-1'), 71.0 (C-3'), 61.9 (C-5'), 41.0 (C-2'), 39.5 ($^1J_{\underline{C}H2,P}$ = 143.93; N-$\underline{C}H_2$-P), 12.9 (5-CH_3).

N^3,$O^{3'}$-Dimethanephosphonate **16**: TLC: R_f 0.05 (B). UV (H_2O): λ_{max} 269 nm; ^{1}H NMR (D_2O), δ_{TMS}, ppm (*J*, Hz): 7.77 (br.s, H-6), 6.34 (dd, $J_{1',2'}$ = 7.5; $J_{1',2"}$ = 2.0; H-1'), 4.63 (m, H-3', H-4'), 4.25 (d, $^2J_{C\underline{H}2,P}$ = 12.0; N^3-C$\underline{H}_2$-P), 3.74 (dd, $J_{4',5'}$ = 9.0; $J_{5',5"}$ =

16.5; H-5'), ≈3.64 (dd, $J_{4',5''}$ = 5.0; H-5''), 3.60 (d, $^2J_{C\underline{H}2,P}$ = 9.5; $O^{3'}$-C$\underline{H}_2$-P), 2.72 (ddd, $J_{2',3'}$ = 7.0; $J_{2',2''}$ = 15.0; H-2'), 2.39 (dt, $J_{2'',3'}$ = ≈2.0; H-2''), 1.96 (s, 5-CH_3); ^{13}C NMR (D_2O), δ_{TMS}, ppm (*J*, Hz): 165.2 (C-4), 151.6 (C-2), 136.3 (C-6), 110.2 (C-5), 87.8 (C-1'), 86.8 (C-4'), 81.7 ($^3J_{C3',P}$ = 12.9; C-3'), 65.6 ($^1J_{\underline{C}H2,P}$ = 156.9; $O^{3'}$-$\underline{C}H_2$-P), 62.2 (C-5'), 37.2 (C-2'), 39.5 ($^1J_{\underline{C}H2,P}$ = 145.10; N^3-$\underline{C}H_2$-P), 12.9 (5-CH_3)].

1-(2-Deoxy-3-*O*-phosphonomethyl-α-D-*erythro*-pentofuranosyl)thymine sodium salt (18) was prepared from the phosphonate **12** (0.17 g; 0.26 mmol) by the action of TMS-Br (0.33 mL; 2.57 mmol) in acetonitrile (2 mL) in a 76% yield (0.074 g) as described for the compound **17**. TLC: R_f 0.20 (B). UV (H_2O): λ_{max} 268 nm.

Reaction of the phosphonate 17 with inorganic pyrophosphate. A. To the solution of the tributylammonium salt of the phosphonate **17** (1800 A_{268} units, 0.187 mmol), previously coevaporated with DMF (3x2 mL), in DMF (1.9 mL), the solution of Im_2CO (150 mg; 0.935 mmol) in DMF (1.9 mL) was added in an argon atmosphere. The reaction mixture was stirred for 24 h, then methanol (61 μL; 0.748 mmol) and, after 30 min, a 0.1 M solution of the tributylammonium pyrophosphate in DMF (9.35 mL) were added. The stirring was continued for additional 24 h, then the mixture was diluted with methanol (12 mL) and, after 30 min, evaporated to dryness *in vacuo*. The residue was purified by DEAE-cellulose (3x17 cm) column chromatography (elution with a linear TEAB gradient; 0-0.4 M; 2 L). Product contained fractions were combined, evaporated and coevaporated with ethanol. After precipitation with NaI[23], the pyrophosphonate **21** (1730 A_{268} units, 96%) was obtained as an amorphous powder. R_f 0.75 (C). UV (H_2O): λ_{max} 268 nm.

B. Similar reaction of the tributylammonium salt of the phosphonate **17** (1800 A_{268} units, 0.187 mmol) with Im_2CO (150 mg; 0.935 mmol) in DMF (4.0 mL) furnished, after analogous work-up and chromatography, the pyrophosphonate **21** as an amorphous powder (Na^+-salt; 1690 A_{268} units, 94%).

1-[2-Deoxy-3-*O*-(pyrophosphoryl)phosphonomethyl-β-D-*erythro*-pentofuranosyl]thymine sodium salt (19). To a 0.2 M solution of bis(tributylammonium) pyrophosphate in DMF (2.25 mL), Im_2CO (0.36 g; 2.25 mmol) was added and the reaction mixture was stirred for 16 h. Then the reaction was quenched with methanol (0.11 mL; 2.70 mmol) and, after 30 min, a solution of the

tributylammonium salt of the phosphonate **17** (860 A_{268} units, 0.09 mmol) in DMF (3.6 mL) was added. After stirring for 20 h, the mixture was taken up *in vacuo*, the residue was dissolved in water (50 mL) and applied to a DEAE-cellulose column (3x17 cm). Elution was performed by a linear TEAB gradient (0.001-0.4 M, 2 L). Fractions contained individual products were collected, concentrated *in vacuo* and coevaporated with ethanol. The products were converted from the triethylammonium salts to the sodium forms by the action of sodium iodide in acetone on the triethylammonium salt solutions of the products in methanol[23], to yield:

(a) the 300 A_{268} units (35%) of the (pyrophosphoryl)phosphonomethyl derivative **19** [TLC: R_f 0.37 (C). UV (H_2O): λ_{max} 267 nm; HPLC: column Nova-Pak C18 (3.9x300 mm), isocratic elution with 0.1 M K-phosphate buffer in water-acetonitrile (95:5, vol), 0.65 mL/min, R_t = 3.343 min];

(b) the 320 A_{268} units (37%) of compound **21** [TLC: R_f 0.75 (C). UV (H_2O): λ_{max} 267 nm]; ES MS of Na^+-salt (full scan negative): m/z 675.2 $(M+Na-H)^-$ and m/z 653.3 $(M-H)^-$ ($C_{22}H_{32}N_4O_{15}P_2$ requires 654.13); m/z 527.39 [$(M-H)^-$-126 (thymine)]; m/z 335.2 (phosphonate **17**, $(M-H)^-$, $C_{11}H_{16}N_2O_8P$ requires 335.06); m/z 125.1 (thymine: $(M-H)^-$, $C_5H_5N_2O_2$ requires 125.04).

1-[2-Deoxy-3-*O*-(pyrophosphoryl)phosphonomethyl-α-D-*erythro*-pentofuranosyl]thymine sodium salt (20) was obtained from the phosphonate **18** (960 A_{268} units; 0.10 mmol) in a 27% yield (260 A_{268} units) as described for the compound **19** [TLC: R_f 0.43 (C). UV (H_2O): λ_{max} 268 nm; HPLC: column Nova-Pak C18 (3.9x300 mm), isocratic elution with 0.1 M K-phosphate buffer in water-acetonitrile (95:5, vol), 0.65 mL/min, R_t = 3.347 min]. Compound **24** was also isolated from the reaction mixture (360 A_{268} units; 38%); TLC: R_f 0.82 (C). UV (H_2O): λ_{max} 267 nm; ES MS of Na^+-salt (full scan negativ): m/z 675.2 $(M+Na-H)^-$ and m/z 653.3 $(M-H)^-$ ($C_{22}H_{32}N_4O_{15}P_2$ requires 654.13); m/z 527.39 [$(M-H)^-$-126 (thymine)]; m/z 335.2 (phosphonate **18**, $(M-H)^-$, $C_{11}H_{16}N_2O_8P$ requires 335.06); m/z 125.1 (thymine: $(M-H)^-$, $C_5H_5N_2O_2$ requires 125.04).

Tetranucleoside derivative 22. A mixture of triethylammonium salt of the phosphonate **17** (94 mg; 0.22 mmol), the nucleoside **4** (0.554 g; 1.08 mmol) and TPS-Cl

(0.196 g; 0.65 mmol) in pyridine (2 mL) was kept for 16 h in a darkness, then diluted with chloroform (50 mL) and extracted with 0.1 M TEAB (2x15 mL). Organic layer was dried, evaporated and coevaporated with toluene (2x2 mL). The residue was purified by silica gel (3x21 cm) column chromatography using a linear methanol gradient in chloroform (0→4%, v/v, 1.5 L) and then chloroform-methanol-triethylamine (80:19:1) mixture (0.3 L) to give:

(a) the tetranucleoside derivative **22** (0.116 g; 64%) [TLC: R_f 0.56 (A). UV (MeOH): λ_{max} 268 nm]; ES MS (full scan skimmer-nozzel-fragmentation; positive): m/z 1652.2 $(M+Na-H_2O)^+$ ($C_{82}H_{88}N_8O_{25}P_2$ requires 1646.53); m/z 837.2 $[(M+Na-H_2O)-815]^+$ and m/z 519.0 $[(M+Na-H_2O)-815-318]^+$;

(b) the cyclophosphonate **23** (14 mg; 15%); [TLC: R_f 0.73 (B). UV (MeOH): λ_{max} 268 nm]; ES MS (full scan negative): m/z 317.1 $(M-H)^-$ ($C_{11}H_{15}N_2O_7P$ requires 318.06); m/z 190.9 $[(M-H)^-$-126 (thymine)]; m/z 125.1 (thymine: $(M-H)^-$, $C_5H_5N_2O_2$ requires 125.04).

Dinucleoside cyclophosphonate derivative 25 was obtained from the phosphonate **18** (0.113 g; 0.26 mmol), the nucleoside **4** (0.663 g; 1.29 mmol) and TPS-Cl (0.234 g; 0.77 mmol) in pyridine (2.4 mL) in a 67% yield (0.14 g) as described for the compound **22**. TLC: R_f 0.61 (A). UV (MeOH): λ_{max} 268 nm. ES MS (full scan negative): m/z 813.4 $(M-H)^-$ ($C_{41}H_{43}N_4O_{12}P$ requires 814.26); m/z 317.2 [(M-H)-496] [3',5'-cyclophosphonate (an α-D-anomer of **23**), $(M-H)^-$, $C_{11}H_{15}N_2O_7P$ requires 318.06); m/z 125.1 (thymine: $(M-H)^-$, $C_5H_5N_2O_2$ requires 125.04)].

Primer Extension Assays for DNA Polymerase I (Klenow fragment), AMV Reverse Transcriptase and TDT.- For *E. coli* DNA polymerase I, the assay mixture (6 μl) contained 10 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 1 mM DTT, 0.3 unit of enzyme, 40-50 nM template-primer complex, and variable concentrations of the compounds under study or dNTP. The reaction was carried out for 15 min at 20°C. For AMV reverse transcriptase: the assay (6 μl) contained 10 mM Tris-HCl, pH 8.2, 5 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, 3 units of enzyme, 40-50 nM template-primer complex, and substrates. The mixture was incubated for 30 min at 37°C. For the TDT: the assay (6 μl) contained 100 mM sodium cacodylate, pH 7.2, 2 mM $CoCl_2$, 0.1 mM DTT, 2 units of enzyme, 20

nM 5'-labeled primer and substrates. The mixture was incubated for 30 min at 37°C. All reactions were stopped by adding 3 μl of deionized formamide containing 0.5 mM EDTA and 0.1% bromophenol blue and xylene cyanol. The products were heated at 100°C for 2 min and separated by electrophoresis in 16% polyacrylamide/7M urea sequencing gel, and the gels were autoradiographed with Kodak RX films at -20°C.

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