Towards Nucleotide Prodrugs Derived from 2,2-Bis(hydroxymethyl)malonate and Its Congeners: Hydrolytic Cleavage of 2-Cyano-2-(hydroxymethyl)-3-methoxy-3-oxopropyl and 3-(Alkylamino)-2-cyano-2-(hydroxymethyl)-3-oxopropyl Protections from the Internucleosidic Phosphodiester and Phosphorothioate Linkages

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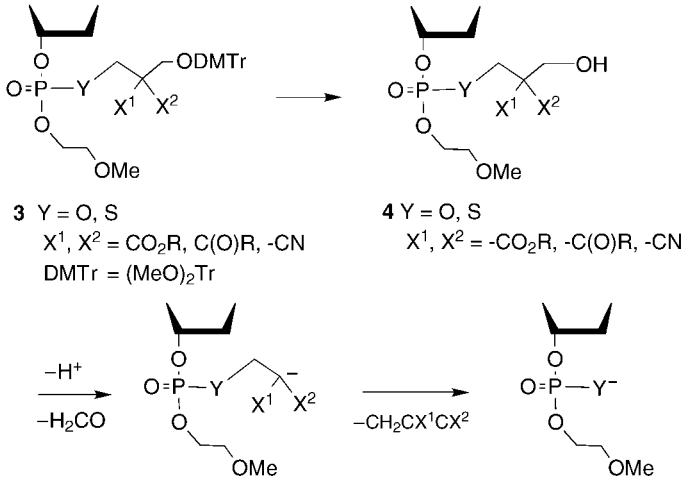
Dedicated to Prof. *Wolfgang Pfleiderer* on the occasion of his 75th birthday

Thymidylyl-(3′ → 5′)-thymidine (TpT) and its stereoisomeric phosphoromonothioate analogs [*P*(*R*)]- and [*P*(*S*)]-Tp(s)T having the phosphate or thiophosphate linkage protected with a 2-cyano-2-{[(4,4′-dimethoxytrityl)oxy]methyl}-3-methoxy-3-oxopropyl group (see **5a**,**b**), as well as [*P*(*R*)]-Tp(s)T bearing a *S*-(2-cyano-2-{[(4,4′-dimethoxytrityl)oxy]methyl}-3-oxo-3-[(2-phenylethyl)amino]propyl) protection (see **5c**), were prepared. The kinetics of the cleavage of the protecting group from the corresponding detritylated compounds **6a**–**c** was studied over a pH range from 2 to 7. All compounds undergo a hydroxide-ion-catalyzed reaction that releases the unprotected TpT (**7a**) or Tp(s)T (**7b**), in all likelihood by departure of the hydroxymethyl group as formaldehyde and concomitant elimination of the phosphodiester or phosphorothioate from the resulting carbanion. The half-life for the deprotection of **6a** and **6b** is *ca*. 6 s at pH 7 and 25°, and that of **6c** *ca*. 600 s. The reasonably fast release of Tp(s)T from **6c** offers a novel method for temporary intrachain attachment of peptides to oligonucleotides to enhance the cellular uptake.

1. Introduction. – Masking of the negative charge of nucleoside monophosphates with biodegradable protecting groups has received considerable interest as a potential prodrug strategy for antiviral nucleotide analogs [1]. The compounds introduced for this purpose include several nucleoside phosphotriesters, such as bis[(pivaloyloxy)-methyl] [2], bis{[(isopropoxy)carbonyloxy]methyl} [3], bis[2-(acylthio)ethyl] [4], [2-(acylthio)ethyl] aryl [5], and cyclosaligenyl [6] substituted nucleoside monophosphates, as well as several cyclic [7] and acyclic *O*,*N*-disubstituted nucleoside phosphoramidates [8]. Similarly, the cellular uptake of polyanionic antisense oligonucleotides may be expected to be enhanced by protection of the internucleosidic phosphodiester or phosphorothioate linkages with biodegradable groups. However, only two of the strategies developed for monomeric nucleotides, *viz*. *S*-protection with a 2-(acylthio)ethyl (see **1**) [9] or (acyloxy)methyl (see **2**) [10] group, have been tested at the oligonucleotide level. With both of these groups, the liberation of the antisense oligonucleotide within the cell is initiated by hydrolytic removal of the acyl group by intracellular carboxyesterases, after which the remaining part of the protecting group undergoes a rapid chemical cleavage, yielding episulfide (= thiirane) in the former and formaldehyde in the latter case. Complete release of a dodecathymidine phosphorothioate from its *S*-[2-(acylthio)ethyl] prodrugs in cell extracts has been recently verified [11].

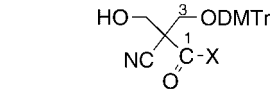
We have recently shown that protecting groups derived from 2,2-bis(hydroxymethyl)-1,3-dioxo compounds and their congeners exhibit also properties that make them promising candidates for prodrug applications [12]. Studies with simple nucleoside 3′-phosphotriesters and their phosphorothioate analogs **3** indicated that such protections were entirely stable as long as the OH function was kept protected, while the exposure of this function resulted in rapid release of the nucleoside 3′-diester. The protecting group is, in all likelihood, cleaved by departure of the hydroxymethyl group as formaldehyde, and this *retro*-aldol condensation is accompanied by elimination of the phosphodiester (or phosphorothioate) from the resulting carbanion (*Scheme 1*). The stability of the carbanion intermediate largely depends on the ability of substituents X^1 and X^2 to withdraw electrons from the carbanion center by resonance interactions. Accordingly, the rate of the cleavage following the exposure of the OH function may be adjusted within wide limits by the identity of these groups. For example, when both X^1 and X^2 are CN groups, the half-life for the cleavage is less than 1 s at pH 7 [12], while the half-life for the departure of a 2-mercaptoethyl group by episulfide formation is *ca.* 20 s under the same conditions [13]. One may expect that, on using an ester protection for the OH function of phosphotriesters **4**, the deprotection may be triggered by carboxyesterases, and, hence, such protections form a reasonable basis for a prodrug strategy of antisense oligonucleotides.

Scheme 1. *Cleavage of the Studied Phosphodiester-Protecting Groups from the Alkyl Esters of Thymidine 3′-Monophosphate and 3′-Phosphorothioate*

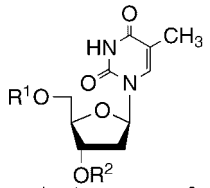


The present paper provides kinetic data for the chemical cleavage of the 2-cyano-2-(hydroxymethyl)-3-methoxy-3-oxopropyl group from the internucleosidic phosphodiester bond of thymidylyl-(3′→5′)-thymidine (TpT; **7a**) and its phosphorothioate analog Tp(s)T (**7b**). The removal of this group (previously named 2-cyano-3-hydroxy-2-methoxycarbonylpropyl) from the model phosphodiesters **4** has already been studied [12]. Accordingly, comparison of those results with the data of the present study allows one to conclude how well the more extensive kinetic results obtained previously with the simple model compounds **4** may be applied to predict the stability of the same protections when internucleosidic bonds are concerned. For this purpose, TpT and Tp(s)T **5a** and **5b**, respectively, having the internucleosidic linkage protected with a 2-cyano-2-{[(4,4′-dimethoxytrityl)oxy)]methyl}-3-methoxy-3-oxopropyl group were prepared, and their hydrolytic stability studied over a wide pH range after removal of the acid labile dimethoxytrityl protection (→**6a**,**b**; see *Scheme 3*). More importantly, the corresponding 3-(alkylamino)-3-oxopropyl analog **5c** was prepared to find out whether an aminocarbonyl substituent is able to induce a sufficiently fast cleavage of the protecting group. This piece of information is highly relevant, since peptides and other conjugate groups aimed at enhancing the cellular uptake of oligonucleotides could conveniently be attached through this kind of a prodrug protecting group to any position within the chain.

2. Results and Discussion. – 2.1. *Preparation of the Phosphate or Phosphorothioate-Protected (3′→5′)-Dinucleotides.* The TpT phosphotriester **5a** was obtained as a mixture of [*P*(*R*)]- and [*P*(*S*)]-diastereoisomers by stepwise displacement of the triazole ligands from phosphoryl tris(1,2,4-triazolide) (=1,1′,1″-phosphinylidynetris[1,2,4-triazole]) [14] with the appropriate alcohols. Accordingly, the OH function of methyl 2-cyano-3-[(4,4′-dimethoxytrityl)oxy]-2-(hydroxymethyl)propanoate (**8a**) [15] was first phosphorylated with phosphoryl tris(1,2,4-triazolide) in MeCN in the presence of Et_3N. Then the second triazole moiety was replaced with 5′-*O*-[(*tert*-butyl)dimethylsilyl]thymidine (**9a**), and finally 3′-*O*-[(*tert*-butyl)dimethylsilyl]thymidine (**9b**) was added. The silyl groups were removed with Bu_4NF in THF in the presence of AcOH. The ^{31}P-NMR analysis of the crude product mixture showed the presence of four different P-resonances, their intensities being almost equal. This is consistent with the expected formation of four diastereoisomers upon phosphorylation; owing to the chirality of the protecting group, both [*P*(*R*)]- and [*P*(*S*)]-isomers appear as two diastereoisomers. The desilylated product mixture was fractionated by reversed-phase HPLC into two pairs of diastereoisomers. Within each fraction, the ^{31}P-chemical



8a X = MeO, DMTr = $(MeO)_2Tr$
b X = $PhCH_2CH_2NH$, DMTr = $(MeO)_2Tr$

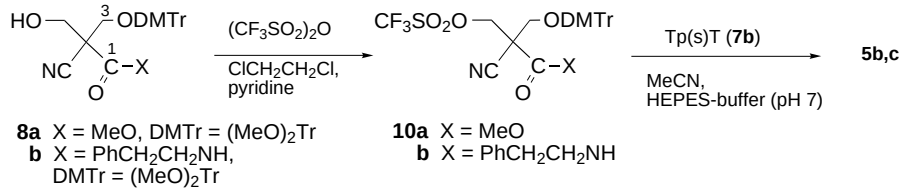


9a R^1 = ^{t}BuMe$_2$Si, R^2 = H
b R^1 = H, R^2 = ^{t}BuMe$_2$Si

shifts of the two isomers were almost identical (fraction *A:* δ(P) 0.18 and 0.12; fraction *B:* δ(P) 0.63 and 0.54). In all likelihood, the fractions represent different absolute configurations at the P-atom (*R*/*S*-stereoisomerism), whereas the two compounds in each fraction differ from each other with the respect to the absolute configuration at the branching C-atom of the protecting group.

The *S*-protected dinucleoside phosphorothioates **5b**,**c** were prepared from Tp(s)T (**7b**) by alkylating the S-atom with the triflates **10a**,**b** of alcohols **8a**,**b**, respectively (*Scheme 2*). Since the nucleophilicity of the S-atom is superior to that of the O-atom, only traces of the *O*-alkylated phosphorothioate triesters were formed.

Scheme 2. *Preparation of the* S-*Protected Phosphoromonothioate Derivatives* **5b**,**c** *of Thymidylyl-(3′→5′)-thymidine*

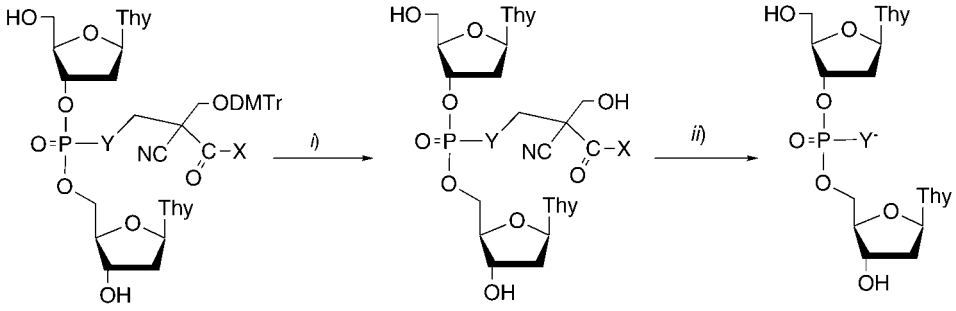


8a X = MeO, DMTr = $(MeO)_2Tr$
b X = $PhCH_2CH_2NH$, DMTr = $(MeO)_2Tr$

10a X = MeO
b X = $PhCH_2CH_2NH$

2.2. *Cleavage of the Protecting Groups.* The dimethoxytrityl group of the phosphotriesters **5a**–**c** was removed with CF_3COOH in a mixture of CH_2Cl_2 and MeOH (CF_3COOH/MeOH/CH_2Cl_2 3:3:1 (*v*/*v*)) (*Scheme 3*). According to TLC and HPLC analyses, the dimethoxytrityl group was completely removed, yielding **6a**–**c**, respectively, without any significant side reactions or further degradation of the starting material. After completion of the detritylation, the mixture was evaporated to dryness, and the residue was dissolved in a pre-equilibrated reaction buffer. The release of TpT (**7a**) or Tp(s)T (**7b**) from the detritylated compounds **6a**–**c** was followed by HPLC.

Scheme 3. *Cleavage of the Phosphotriester Protecting Groups of* **5a**–**c** *Yielding TpT* (**7a**) *and Tp(s)T* (**7b**)



5a Y = O, X = MeO, DMTr = $(MeO)_2Tr$
b Y = S, X = MeO, DMTr = $(MeO)_2Tr$
c Y = S, X = $PhCH_2CH_2NH$, DMTr = $(MeO)_2Tr$

6a Y = O, X = MeO
b Y = S, X = MeO
c Y = S, X = $PhCH_2CH_2NH$

7a Y = O
b Y = S

i) CF_3COOH/MeOH/CH_2Cl_2 3:3:1 (*v*/*v*); 12 min at 22°. *ii*) OH^-/H_2O.

For each compound **6a** – **c**, the cleavage of the protecting group was observed to be first-order in OH^- ion over the pH range studied (*Fig.*), and TpT (**7a**) or Tp(s)T (**7b**) was the only nucleosidic product formed. In other words, no sign of products corresponding to cleavage of the internucleosidic linkage or desulfurization of the thioate analogs was observed. Furthermore, no racemization at the stereogenic P-atom took place during the deprotection of the [*P*(*R*)]- and [*P*(*S*)]-isomers of **6b** and **6c** to the respective isomers of Tp(s)T (**7b**).

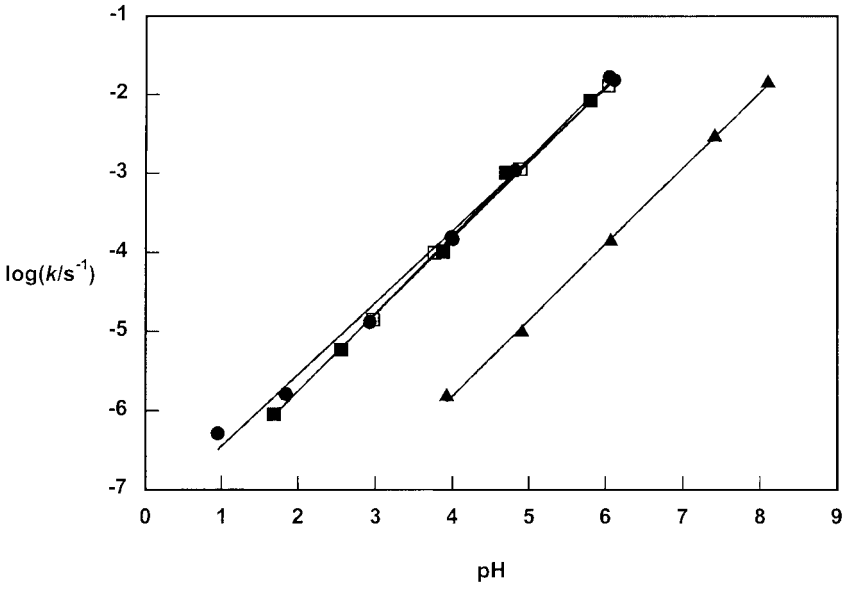


Figure. *pH-Rate profiles for hydrolysis of the diastereoisomers of* **6a** (□ and ■), *of [*P(R)*]*-**6b** (○), *of [*P(S)*]*-**6b** (●), *and of [*P(R)*]*-**6c** (▲). The ionic strength of the solutions was adjusted to 0.1M with NaCl.

The cleavage is as fast with the phosphate **5a** as with phosphorothioate **5b** (*Fig.*). Similarly, the effects of the configuration on the reaction rate, *i.e.*, the effect of the configuration at the P-atom and at the stereogenic C-atom of the protecting group, are negligible. The kinetics data shown in the *Figure* also indicate that the hydrolytic stability of TpT triesters **6a**,**b** is very similar to that of the simpler thymidine 3′-phosphotriesters **4** studied previously [12]. The most important piece of information is probably that the half-life for the cleavage of the 2-cyano-2-(hydroxymethyl)-3-methoxy-3-oxopropyl group from the internucleosidic phosphorothioate linkage of **6b** is 6 s at pH 7 and 25°. Since it is known [12] that the 2,2-dicyano-3-hydroxypropyl group is cleaved one order of magnitude more readily, the protecting strategy of oligonucleotides can be based on groups that are cleaved instantaneously after the exposure of the OH function by intracellular esterases.

It is worth noting that even the 2-cyano-2-(hydroxymethyl)-3-methoxy-3-oxopropyl group is cleaved from a Tp(s)T triester more readily than the 2-mercaptoethyl group, obtained by enzymatic deacylation of the extensively studied *S*-[2-(acylthio)ethyl] protection; the half-life for the release of both [*P*(*R*)]- and [*P*(*S*)]-Tp(s)T from their *S*-

(2-mercaptoethyl)phosphorothioates is *ca.* 30 s at pH 7 and 25° [13]. Furthermore, the release of toxic episulfide is avoided. The other seriously studied prodrug candidate, *S*-[(acyloxy)methyl] protection, yields upon enzymatic deacylation *S*-(hydroxymethyl)-protected Tp(s)T, which is decomposed to Tp(s)T even faster than **6b** [13]. The drawback of this protection, however, is that desulfurization appears to compete with the release of the phosphorothioate diester under physiological conditions [10].

As expected, replacing the alkoxycarbonyl group of **6b** with an (alkylamino)carbonyl group (see **6c**) retarded the cleavage of the protecting group. An aminocarbonyl group is known to stabilize the negative charge in the α-position less efficiently than an alkoxycarbonyl group, and, for this reason, the departure of formaldehyde by a *retro*-aldol condensation mechanism is slower. The retardation of the rate of hydrolysis of **6c** compared to **6b** is 100-fold, *i.e.*, the half-life for the release of Tp(s)T from **6c** is *ca.* 10 min at pH 7 and 25°. Although the cleavage is not instantaneous, this protecting group still offers a useful handle through which conjugate groups aimed at facilitating the cellular uptake may be temporarily attached to the oligonucleotide chain.

Experimental Part

General. MeCN, dioxane, and pyridine were dried by refluxing over CaH_2 and distilled before use. CC = column chromatography. NMR Spectra: *Bruker AM-200* or *Jeol Alpha-500* NMR spectrometer; δ(H) in ppm referenced to internal $SiMe_4$ and δ(P) to external orthophosphoric acid (85% in D_2O); coupling constants *J* in Hz. ESI-MS: *Perkin-Elmer Sciex-API-365* triple-quadrupole LC/ESI/MS spectrometer; in *m*/*z*.

O^P-*(2-Cyano-2-[[(4,4′-dimethoxytrityl)oxy]methyl]-3-methoxy-3-oxopropyl)thymidylyl-(3′→5′)-thymidine* (**5a**). Methyl 2-cyano-3-hydroxy-2-(hydroxymethyl)propanoate [15] was treated with 4,4′-dimethoxytrityl chloride in anh. 1,4-dioxane in the presence of 1 equiv. of pyridine. The product *methyl 2-cyano-3-[(4,4′-dimethoxytrityl)oxy]-2-(hydroxymethyl)propanoate* (**8a**) was purified by CC (silica gel, benzene containing 2→12% AcOEt and 0.1% pyridine).

To a soln. of 1*H*-1,2,4-triazole (0.13 g, 1.9 mmol; recrystallized from toluene) in anh. MeCN (6 ml), $POCl_3$ (0.16 ml, 0.62 mmol) and Et_3N (0.27 ml, 1.9 mmol) were added. After stirring for 1 h, the mixture was filtered onto **8a** (0.23 g, 0.50 mmol). The mixture was stirred for 80 min at 22° and then poured onto 5′-*O*-[(*tert*-butyl)dimethysilyl]thymidine (**9a**; 0.27 g, 0.75 mmol). After 11 h, 3′-*O*-[(*tert*-butyl)dimethylsilyl]thymidine (**9b**; 0.33 g, 0.93 mmol) in MeCN (1.2 ml) was added. After stirring for 4 d at 22°, the mixture was evaporated, the residue dissolved in CH_2Cl_2 (40 ml), the soln. washed with a phosphate buffer (pH 7; 3 × 15 ml), dried (Na_2SO_4), and evaporated, and the product purified by HPLC (*LiChroSpher RP-18* column (10 × 250 mm, 5 μm), MeCN/H_2O): 80 mg of an equimolar mixture of four 5′,3″-bis(*O*)-silylated **5a** diastereoisomers. ^{31}P-NMR ($CDCl_3$): 0.57; 0.55; 0.40; 0.39. ESI-MS (pos.): 1240.9 ($[M+Na]^+$).

The (*tert*-butyl)dimethylsilyl groups were removed by treatment with Bu_4NF in THF under acidic conditions [16]: To a soln. of Bu_4NF (0.21 g, 0.66 mmol) in THF (4.25 ml), AcOH (0.75 ml, 13 mmol) was added, followed by the bis-silylated **5a** (40 mg, 0.033 mmol). After 24 h treatment at 22° (TLC monitoring), the mixture was neutralized with 1M aq. Et_3N and the soln. (pH 7.5) extracted with Et_2O (3 × 10 ml). The pooled org. phase was evaporated and the residue separated by reversed-phase HPLC (*Merck LiChroSpher RP-18* column (10 × 250 mm, 5 μm) MeCN/H_2O 45:55): 10 mg of fast migrating *Fraction A* and 8 mg of slow migrating *Fraction B*, each containing two isomers of **5a** (by MS and NMR).

Fraction A: ^{1}H-NMR ($CDCl_3$, 500 MHz): 9.26 (br. *s*, NH); 7.46 (*s*, 1 H, H–C(6)); 7.37 (*s*, 1 H, H–C(6)); 7.20–7.36 (9 H, $(MeO)_2Tr$); 6.84 (*d*, 4 H, $(MeO)_2Tr$); 6.19 (*dd*, *J* = 6, 6, 1 H, H–C(1′)); 6.14 (*dd*, *J* = 6.8, 6.8, 1 H, H–C(1′)); 5.16 (*m*, 1 H, H–C(3′) of the 3′-linked T); 4.50 (*m*, 1 H, H–C(3′)); 4.42 (*m*, 2 H, CH_2); 4.27 (*m*, 2 H, CH_2); 4.18 (*m*, 1 H, H–C(4′)); 4.06 (*m*, 1 H, H–C(4′)); 3.83 (5 H, CH_2, COOMe); 3.79 (*s*, 6 H, $(MeO)_2$Tr); 3.50 (*m*, 2 H, CH_2); 2.47 (*m*, 2 H, H–C(2′)); 2.36 (*m*, 1 H, H–C(2′)); 2.24 (*m*, 1 H, H–C(2′)); 1.87 (6 H, 2 Me). ^{31}P-NMR ($CDCl_3$): 0.18 (*s*); 0.15 (*s*). ESI-MS (neg.): 988.7. ESI-MS (pos.): 1012.5 ($[M+Na]^+$). TOF-ES-MS (pos.): 1012.2980 ($[M+Na]^+$, $C_{47}H_{52}N_5O_{17}NaP^+$; calc. 1012.2994).

Fraction B: ^{1}H-NMR: identical with that of *Fraction A*. ^{31}P-NMR ($CDCl_3$): 0.63 (*s*); 0.54 (*s*). ESI-MS (neg.): 988.7. ESI-MS (pos.): 1012.5 ($[M+Na]^+$).

O^P-*[2-Cyano-2-(hydroxymethyl)-3-methoxy-3-oxopropyl]thymidylyl-(3′ → 5′)-thymidine* (**6a**). A sample of **5a**, either from *Fraction A* or *B*, was treated with $CF_3COOH/MeOH/CH_2Cl_2$ 3:3:1 for 12 min at 22°. The mixture was evaporated at r.t. In a typical procedure, the residue (ESI-MS (pos.): 710.2 ($[M+Na]^+$) was used immediately in a kinetic run without purification. Accordingly, the residue was dissolved in a small volume (80 µl) of H_2O/MeCN 40:60 (*v*/*v*), and this soln. was added into the pre-equilibrated reaction buffer.

*[P(R)]- and [P(S)]-*S^P*-(2-Cyano-2-[[(4,4′-dimethoxytrityl)oxy]methyl]-3-methoxy-3-oxopropyl)-*P*-thiothymidylyl-(3′ → 5′)-thymidine* (**5b**). *P*-Thiothymidylyl-(3′ → 5′)-thymidine ammonium salt (**7b** · NH_4^+; 0.13 mmol of either [*P*(*R*)]- or [*P*(*S*)]-diastereoisomer) [13] was dissolved in MeCN (3 ml), and 0.25M HEPES (=4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) buffer (pH 7.05; 0.5 ml) was added. Then, 2-cyano-2-{[(4,4′-dimethoxytrityl)oxy]methyl}-3-methoxy-3-oxopropyl trifluoromethanesulfonate (**10a**; 0.32 g, 0.54 mmol) [12] was added, and the mixture was stirred at 37°. After 24 h with the [*P*(*S*)]-isomer or 43 h with the [*P*(*R*)]-isomer, the reaction was quenched by adding 0.5% aq. $NaHSO_3$ soln. (0.1 ml). The mixture was evaporated and the residue purified by CC (silica gel, $MeOH/CH_2Cl_2$ 1:99 → 16:84). The products were further purified by HPLC (*LiChroSpher-RP-18* column (10 × 250 mm, 5 µm; 45% $MeCN/H_2O$): 100 mg (77%) of [*P*(*R*)]-**5b** or 80 mg (59%) of [*P*(*S*)]-**5b**, resp.

Data of [*P*(*R*)-**5b**: ^{1}H-NMR ((D_6)DMSO, 500 MHz): 11.32 (*s*, 1 NH, Thy); 11.28 (*s*, 1 NH, Thy); 7.66 (*s*, 1 H, H−C(6)); 7.42 (*s*, 1 H, H−C(6)); 7.32 (*m*, 4 H, $(MeO)_2$*Tr*); 7.18 (*t*, 5 H, $(MeO)_2$*Tr*); 6.90 (*d*, 4 H, $(MeO)_2$*Tr*); 6.17 (*m*, 2 H, 2 H−C(1′)); 5.42 (*d*, 1 H, OH−C(3′)); 5.20 (*m*, 1 H, OH−C(5′)); 5.04 (*m*, 1 H, H−C(3′) of the 3′-linked T); 4.20 (*m*, 3 H, H−C(3′), H−C(5′), H′−C(5″) of the 5′-linked T); 4.07 (*m*, 1 H, H−C(4′) of the 3′-linked T); 3.92 (*m*, 1 H, H−C(4′) of the 5′-linked T); 3.78 (*d*, 3 H, COOMe); 3.73 (*s*, (*Me*O)$_2$Tr); 3.59 (*m*, 2 H, H−C(5′), H′−C(5″) of the 3′-linked T); 3.27–3.52 (4 H, 2 CH_2); 2.26–2.36 (*m*, 2 H, 2 H−C(2′)); 2.08–2.15 (*m*, 2 H, 2 H−C(2′)); 1.74 (6 H, 2 Me). ^{31}P-NMR (DMSO): 30.48 (*s*). ESI-MS (pos.): 1023 ($[M+NH_4]^+$), 1028 ($[M+Na]^+$), 1044 ($[M+K]^+$).

*Data of [P(S)]-***5b**: ^{1}H-NMR: identical with that of [*P*(*R*)]-**5b**. ^{31}P-NMR (DMSO): 30.35 (*s*). ESI-MS (pos.): 1023 ($[M+NH_4]^+$), 1028 ($[M+Na]^+$), 1044 ($[M+K]^+$).

*[P(R)]- and [P(S)]-*S^P*-[2-Cyano-2-(hydroxymethyl)-3-methoxy-3-oxopropyl]-*P*-thiothymidylyl-(3′ → 5′)-thymidine* (**6b**). As described for **6a**, diastereoisomers [*P*(*R*)]- and [*P*(*S*)]-**5b** were detritylated to the respective isomers of **6b**.

*2-Cyano-3-hydroxy-2-(hydroxymethyl)-*N*-(2-phenylethyl)propanamide.* A mixture of ethyl cyanoacetate (4.0 g; 35.4 mmol) and (2-phenylethyl)amine (4.29 g; 39.3 mmol) was stirred for 16 h at 22° in the presence of Et_3N (200 µl). The solid cyanoacetamide was filtered off and bis(hydroxymethyl)ated by the method described previously [15]: The amide (3.54 g, 18.8 mmol) was dissolved in 1,4-dioxane (20 ml) and 37% aq. formaldehyde soln. (3.05 g) was added. Then 1.0M Et_3N in THF (0.9 ml) was added, and the mixture was stirred for 1 h at 22°. After addition of H_2O (50 ml), the mixture was extracted with CH_2Cl_2 (2 × 50 ml) and the org. phase dried (Na_2SO_4), and evaporated. ^{1}H-NMR ($CDCl_3$, 200 MHz): 8.0–7.1 (5 arom. H); 3.75 (*m*, 1 CH_2); 3.5–3.3 (2 CH_2); 2.75 (*m*, 1 CH_2). ESI-MS (pos.): 249.2 ($[M+H]^+$), 271.2 ($[M+Na]^+$), 287.2 ($[M+K]^+$).

*2-Cyano-3-[(4,4′-dimethoxytrityl)oxy]-2-(hydroxymethyl)-*N*-(2-phenylethyl)propanamide* (**8b**). The 2-Cyano-3-hydroxy-2-(hydroxymethyl)-*N*-(2-phenylethyl)propanamide was reacted with 1 equiv. of 4,4′-dimethoxytrityl chloride in anh. MeCN in the presence of pyridine [15]. After aq. workup, the product was purified by CC (silica gel, benzene/AcOEt containing 0.1% pyridine). ^{1}H-NMR ($CDCl_3$, 200 MHz): 7.5–7.0 (18 H, $(MeO)_2$*Tr*, *Ph*); 6.80 (*d*, 2 H, $(MeO)_2$*Tr*); 3.85 (*m*, 1 CH_2); 3.67 (*s*, (*Me*O)$_2$Tr); 3.7–3.4 (2 CH_2); 2.80 (*m*, 1 CH_2). ESI-MS (pos.): 573.7 ($[M+Na]^+$), 589.8 ($[M+K]^+$).

*[P(R)]-*S^P*-(2-Cyano-2-[[(4,4′-dimethoxytrityl)oxy]methyl]-3-oxo-3-[(2-phenylethyl)amino]propyl)-*P*-thiothymidylyl-(3′ → 5′)-thymidine* (**5c**). In the presence of 2 equiv. of pyridine, **8b** was sulfonylated with 1.3 equiv. of trifluoromethanesulfonic anhydride in 1,2-dichloroethane [17]. The resulting 2-{[(4,4′-dimethoxytrityl)oxy]methyl}-3-oxo-3-[(2-phenylethyl)amino]propyl trifluoromethanesulfonate (**10b**; 437 mg, 0.64 mmol) was added to a soln. of [*P*(*R*)]-*P*-thiothymidylyl-(3′ → 5′)-thymidine (86 mg, 0.15 mmol) [13] in a mixture of MeCN (55 ml) and 0.25M aq. HEPES buffer (pH 7; 8 ml). The mixture was stirred for 27 h at 37°, during which time **10b** entirely dissolved. The reaction was quenched with 10% aq. $NaHSO_3$ soln. (100 µl). The mixture was evaporated and the residue purified by CC (silica gel, $MeOH/CH_2Cl_2$ 10:90). The product was further purified by HPLC (*Hyperprep-HS-C-18* column (10 × 250 mm, 8µm), $MeCN/H_2O$ 1:1). ^{1}H-NMR ((D_6)DMSO, 500 MHz): 11.35 (*s*, 1 NH, Thy); 11.30 (*s*, 1 NH, Thy); 8.52 (*q*, 1 H, NH); 7.67 (*s*, 1 H, H−C(6)); 7.44 (*s*, 1 H, H−C(6)); 7.36–7.14 (14 H, Ph $(MeO)_2$*Tr*); 6.88 (*d*, 4 H, $(MeO)_2$*Tr*); 6.19 (*m*, 2 H, 2 H−C(1′)); 5.45 (*d*, 1 H, OH−C(3′)); 5.23 (*m*, 1 H, OH−C(5′)); 5.06 (*m*, 1 H, H−C(3′) of the 3′-linked T); 4.14–4.28 (3 H, H−C(3′), H−C(5′), H′−C(5′) of the 5′-linked T); 4.09 (*m*, 1 H, H−C(4′) of the 3′-linked T); 3.94 (*m*, 1 H, H−C(4′ of the 5′-linked T); 3.72 (*s*, 6 H, (*Me*O)$_2$Tr); 3.59 (*m*, 2 H, H−C(5′), H′−C(5′) of the 3′-linked T); 3.30–3.39

(*m*, 3 CH_2); 2.72 (*t*, 2 H, $PhCH_2$); 2.25–2.35 (*m*, 2 H, 2 H–C(2′)); 2.04–2.15 (*m*, 2 H, 2 H–C(2′)); 1.73 (6 H, 2 Me of Thy). ^{31}P-NMR ((D_6)DMSO): 29.10.

*[P(R)]-S^P-{2-Cyano-2-(hydroxymethyl)-3-oxo-3-[(2-phenylethyl)amino]propyl}-*P*-thiothymidylyl-(3′→5′)-thymidine* (**6c**). As described for **6a**, the mixture containing the two stereoisomers of [*P*(*R*)]-**5c** was detritylated.

Kinetics Measurements. The hydrolytic removal of the detritylated protecting group from the phosphodiester linkage of TpT or phosphorothioate linkage of Tp(s)T was followed by an HPLC method described previously [12]. Accordingly, the dimethoxytrityl group was first removed as described above, and the residue obtained by evaporation of the solvents was dissolved in pre-equilibrated reaction buffer. The pH of the reaction solns. was adjusted with HCl or with a formate, acetate, HEPES, MES, or glycine buffer. The composition of the aliquots withdrawn at appropriate intervals was analyzed by HPLC (*Hypersil-ODS-5* column (4 × 250 nm, 5 μm), formic acid/sodium formate buffer (pH 3.3) containing 0.1M NH_4Cl/MeCN). The hydrolysis products were identified chromatographically by spiking with authentic samples, and by HPLC-ESI-MS analysis.

The first-order rate constants were calculated by applying the integrated first-order rate equation to the time-dependent diminution of the HPLC peak area of the starting compound.

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