Hydrolytic stability of nucleoside phosphotriesters derived from bis(hydroxymethyl)-1,3-dicarbonyl compounds and their congeners: towards a novel pro-drug strategy for antisense oligonucleotides

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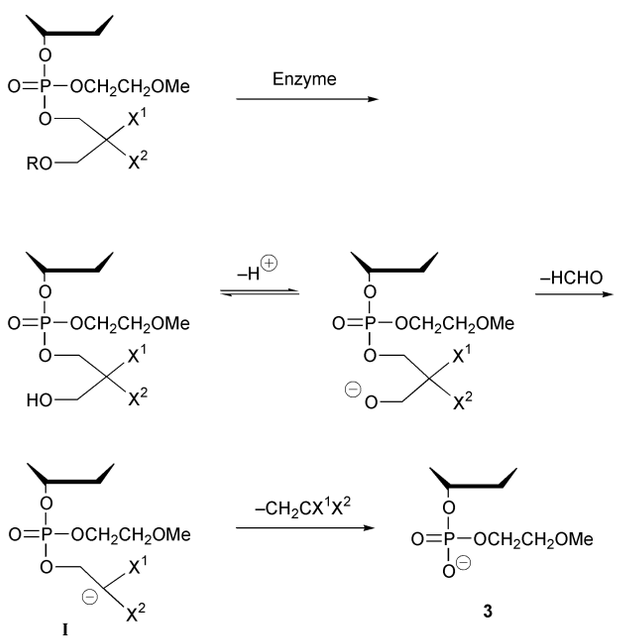
Four different nucleoside phosphodiester protecting groups derived from bis(hydroxymethyl)-1,3-dicarbonyl compounds and their congeners have been prepared and introduced to 5′-*O*-pivaloylthymidine 3′-(2-methoxyethyl)-phosphate and its monothioate analogs. Nucleoside phosphotriesters having either 2,2-bis(ethoxycarbonyl)-3-(4,4′-dimethoxytrityloxy)propyl (**1a**), 2-cyano-2-methoxycarbonyl-3-(4,4′-dimethoxytrityloxy)propyl (**1b**), 2,2-bis(cyano)-3-(4,4′-dimethoxytrityloxy)propyl (**1c**) or 2-acetyl-2-benzoyl-3-(4,4′-dimethoxytrityloxy)propyl (**1d**) protecting group have been prepared. Additionally were synthesized the *O*- and *S*-esterified phosphoromonothioate analogs of **1b**. After removal of the dimethoxytrityl group under acidic conditions, each of the detritylated protecting groups is readily cleaved from the phosphate/thiophosphate moiety by a reaction suggested to involve a base-catalyzed retro-aldol condensation and following elimination of the phosphodiester from the formed carbanion intermediate. The kinetics of the hydroxide ion-catalyzed cleavage have been studied by HPLC over a pH range 2–7. The half-lives of the cleavage at pH 7 and 25 °C vary from 0.3 s (for **1c**) to 5500 s (for **1a**). The results confirm that these protecting groups have promising chemical properties for use in the development of antisense oligonucleotide pro-drug strategies.

Introduction

Structurally modified oligonucleotides, the so-called antisense oligonucleotides, constitute a promising class of chemotherapeutic agents, which enable highly selective inhibition of gene expression. Phosphoromonothioates are the most widely studied oligonucleotide analogs introduced for these purposes.[1] One of the main hurdles for the use of phosphodiester analogs, such as phosphorothioates, as drugs is the poor cellular uptake of the charged oligomers. A possible way to overcome this problem is the masking of the phosphodiester functions with a protecting group that is removed by the action of intracellular enzymes and rapid subsequent chemical reactions. For example, acylthioethyl (SATE groups)[2] and acyloxymethyl[3] groups have been shown to be potential candidates for these purposes. In the present contribution we report on alternative phosphoester protecting groups, derived from bis(hydroxymethyl)-1,3-dicarbonyl compounds and their congeners.[4] The principle of removal of these groups is outlined in Scheme 1. Deprotection of the hydroxymethyl function by intracellular enzymes enables base-catalyzed retro-aldol condensation, accompanied by phosphate elimination giving the desired phosphodiester.

To study the structural effects on the kinetics of the non-enzymatic steps of the removal of this type of protecting group, phosphotriesters **1a**–**d** were prepared as model compounds. The dimethoxytrityl group was used in these compounds, instead of an enzymolabile protecting group, since the detritylated compounds **2a**–**d** are sufficiently stable under the conditions, where dimethoxytrityl is removed. Accordingly, treatment of **1a**–**d** with trifluoroacetic acid in a mixture of dichloromethane and methanol gave the detritylated compounds **2a**–**d** (Scheme 2), the hydrolysis of which could then be studied over a wide pH range (pH 2–7) by HPLC. The



Scheme 1

methoxyethyl group was used as a mimic for the 5′-*O*-linked nucleoside, since the methoxyethoxide ion is known to be in phosphoester hydrolysis about as good a leaving group as a nucleoside 5′-oxyanion.[5]

Besides the phosphotriesters **1a**–**d**, the phosphorothioate analogs **4a**,**b** were synthesized, and the hydrolytic stability of the detritylated thioates **5a**,**b** was compared with that of the phosphate analog **2b**.

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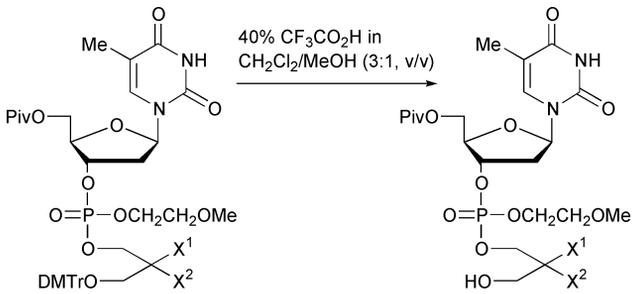
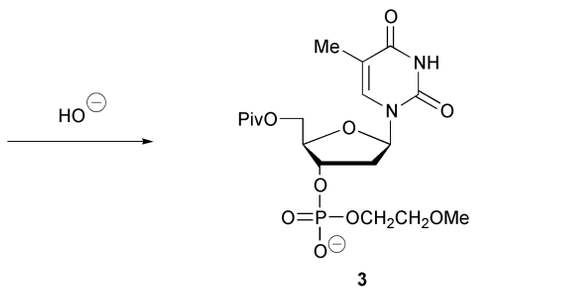


1a: $X^1 = X^2 = CO_2Et$
1b: X^1 = CN; $X^2 = CO_2Me$
1c: $X^1 = X^2$ = CN
1d: X^1 = C(O)Me; X^2 = C(O)Ph

2a: $X^1 = X^2 = CO_2Et$
2b: X^1 = CN; $X^2 = CO_2Me$
2c: $X^1 = X^2$ = CN
2d: X^1 = C(O)Me; X^2 = C(O)Ph



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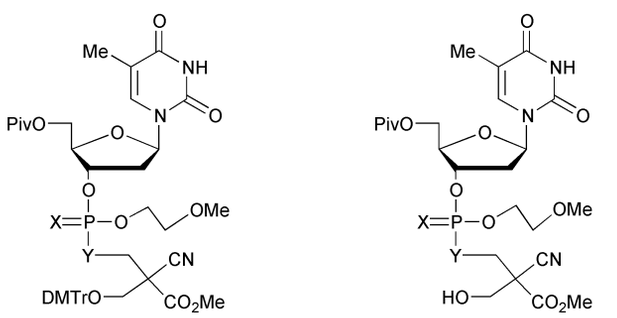
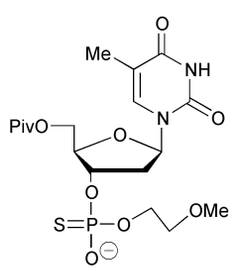


4a: X = O; Y = S
4b: X = S; Y = O

5a: X = O; Y = S
5b: X = S; Y = O



6

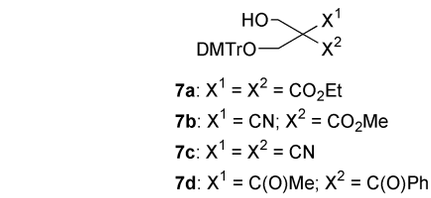
Piv = *tert*-butylcarbonyl; DMTr = 4,4′-dimethoxytrityl

Scheme 2

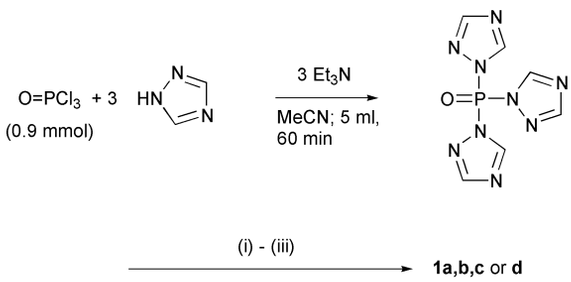
Results and discussion

Materials

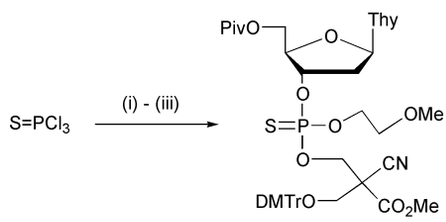
The phosphotriesters **1a**–**d** were prepared from the appropriate dimethoxytritylated bis(hydroxymethyl)-1,3-dicarbonyl derivatives[4] **7a**–**d**, 5′-*O*-pivaloylthymidine and 2-methoxyethanol by a phosphotriester method[6] using phosphoryl tris(triazolide) as the phosphorylating agent (Scheme 3). The phosphorothioate *O*,*O*,*O*-triester **4b** was prepared using thiophosphoryl trichloride as the thiophosphorylating reagent (Scheme 4). The phosphorothioate *O*,*O*,*S*-analog **4a** was synthesized by displacing the triflate group from methyl 2-cyano-2-(4,4′-dimethoxytrityloxymethyl)-3-(trifluoromethylsulfonyloxy)propanoate (**8**) with 5′-*O*-pivaloylthymidine 3′-*O*-(2-methoxyethyl)phosphorothioate (**6**), the sulfur atom of **6** serving as a nucleophile (Scheme 5). The phosphorothioate diester **6** was obtained from 5′-*O*-pivaloylthymidine by a hydrogen phosphonate method described earlier.[7]



7a: $X^1 = X^2 = CO_2Et$
7b: X^1 = CN; $X^2 = CO_2Me$
7c: $X^1 = X^2$ = CN
7d: X^1 = C(O)Me; X^2 = C(O)Ph

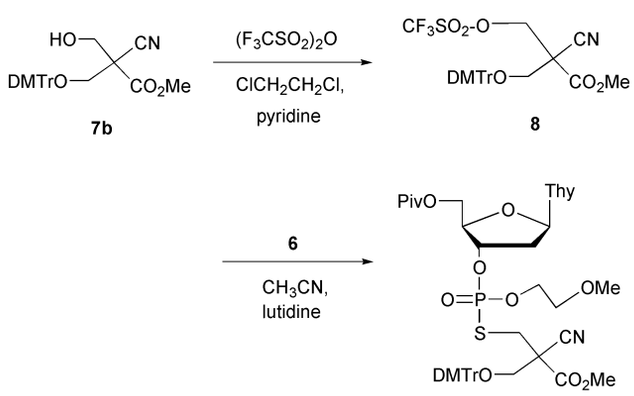


Scheme 3 (i) **7a**, **b**, **c** or **d**, 0.6 mmol, 45–80 min; (ii) 5′-*O*-pivaloylthymidine, 0.9 mmol, 5–8 h; (iii) 2-methoxyethanol, 5 ml, 16 h.



4b

Scheme 4 (i) 5′-*O*-pivaloylthymidine, pyridine, 3 h; (ii) **7b**, 3 h; (iii) 2-methoxyethanol, 14 h.



7b

8

4a

Scheme 5

The compounds were prepared and used as diastereomeric mixtures. The presence of the R_P- and S_P-diastereoisomers in the samples of **1a**–**d** could be verified both by ^{31}P NMR spectroscopy and by HPLC chromatography. Each of the samples of **2a**–**d** could also be rather easily fractionated by HPLC into two fractions. Accordingly, compounds **2b** and **2d**, having an asymmetric protecting group and thus consisting of 4 diastereoisomers, were analyzed as a mixture of two pairs of diastereomers. In each kinetic run the stereoisomers were found to diminish as rapidly as each other and strictly obeying the first-order kinetics. Thus, the stereochemistry at either the phosphorus atom or at the asymmetric carbon atom of the protecting group does not markedly affect the stability of the phosphate protection.

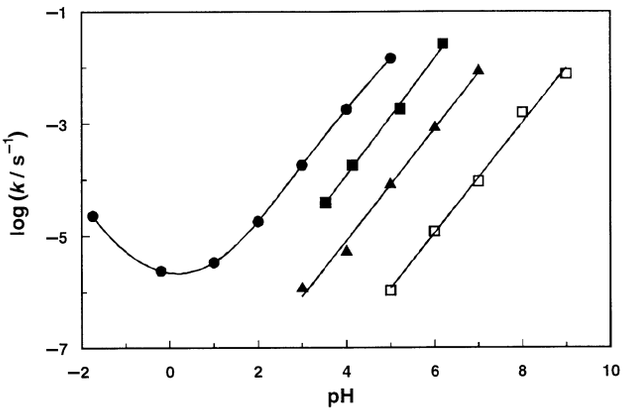


Fig. 1 pH–rate profiles for the hydrolysis of **2a** (open squares), **2b** (filled squares), **2c** (filled circles) and **2d** (filled triangles) at 298.2 K. The ionic strength of the solutions was adjusted to 0.1 mol dm^{-3} with sodium chloride.

Also with the phosphorothioates **4a** and **4b** the ^{31}P NMR spectra indicated the presence of four diastereomers in an almost equimolar ratio. The R_P- and S_P-diastereomers of **4a** were isolated by HPLC and analyzed separately. They showed identical ^1H NMR spectra.

Hydrolysis of the detritylated compounds

After removal of the 4,4′-dimethoxytrityl group from the hydroxymethyl groups of compounds **1a**–**d**, each of the detritylated compounds (**2a**–**d**) was readily hydrolyzed to the desired phosphodiester, 5′-*O*-pivaloylthymidine 3′-(2-methoxyethyl)phosphate (**3**; Scheme 2). This was the only reaction observed over the pH range from 1 to 9. Under more acidic conditions (pH > 1), however, acid-catalyzed cleavage of the 5′-*O*-pivaloyl group becomes the predominant reaction (shown for **2c** in Fig. 1).

With all the phosphate triesters studied (**2a**–**d**), the cleavage of the protecting group and formation of **3** are of first order in hydroxide ion concentration (Fig. 1). The rate of the reaction is highly susceptible to the nature of groups X^1 and X^2. Replacing one of the ester groups of **2a** with a cyano group (**2b**) increases the rate by a factor of 10^3, while replacement of both of the ester groups (**2c**) results in a 10^4-fold rate-acceleration. If both of the ester groups are replaced by keto groups (**2d**), a 100-fold rate-acceleration is observed (Fig. 1).

Mechanistically the cleavage of the protection group in all likelihood includes the steps shown in Scheme 1. Firstly, the hydroxy group is deprotonated in a rapid pre-equilibrium step, then a molecule of formaldehyde is released by a retro-aldol reaction, and finally the phosphodiester **3** is eliminated from the carbanion intermediate **I**. Most probably, the rate limiting step of the reaction is the formation of the intermediate **I**. Accordingly, the overall reactivity is expected to correlate with the stability of the carbanion. Those X^1 and X^2 groups which result in charge delocalization by resonance and hence stabilize the carbanion, facilitate the departure of the protecting group. The lower stability of the bis-cyano derivative **2c** compared to the bis(ethoxycarbonyl) counterpart **2a** is consistent with the fact that the methylene group of malononitrile is considerably more acidic than that of diethyl malonate (pK_a values are 11.2 and 12.9 for malononitrile and diethyl malonate, respectively).[8] Somewhat unexpectedly, the keto functions in **2d** produce a smaller rate-accelerating effect than the cyano functions in **2c** (*cf.* the pK_a value for acetylacetone is as low as 8.95).

Fig. 2 shows the pH–rate profiles for the hydrolysis of the phosphorothioate derivatives **5a**,**b**, obtained from **4a**,**b** with a trifluoroacetic acid treatment analogous to that described above for the oxyphosphate analogs. As seen, the thiosubstitution of the phosphate linkage has only a minor effect on

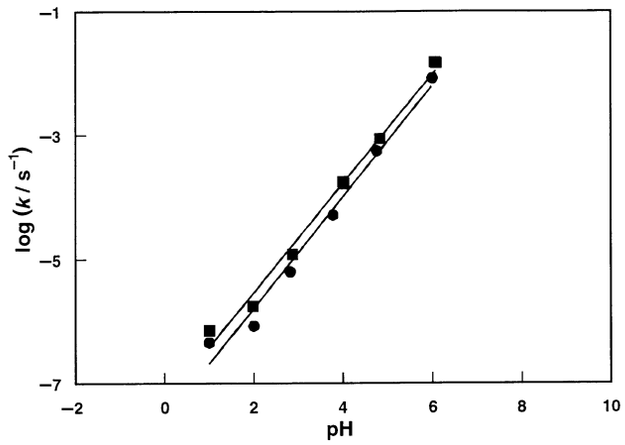


Fig. 2 pH–rate profiles for the hydrolysis of the diastereomers of **5a** (squares), and **5b** (circles) at 298.2 K. The ionic strength of the solutions was adjusted to 0.1 mol dm^{-3} with sodium chloride. No measurable difference was observed between the rates of hydrolysis of the diastereoisomers in either case.

the cleavage rate of the protecting group. The deprotection of the *O*-esterified analog **5b** is slightly slower than that of the *S*-esterified **5a**, but the difference is rather insignificant. Furthermore, even the rate difference between the deprotection of the oxyphosphate analog **2b** and the thioates **5a**,**b** is very small, **5a** being hydrolyzed about as readily as **2b**. As with **2b**, with both types of phosphorothioates the effect of the stereochemistry at the phosphorus atom on the rate of hydrolysis is negligible.

The phosphodiester protection strategy presented here exhibits promising properties and may well find applications in development of the pro-drug strategies for antisense oligonucleotides. With the cyano derivative **2b** and bis-cyano derivative **2c**, for example, the estimated half-lives for the cleavage of the protection group at pH 7 and 25 °C are only about 3 and 0.3 s, respectively. The cleavage of the protection group from the thiophosphate analogs is about as rapid as from the phosphate counterpart and no desulfurization was found to take place. Accordingly, the release of the phosphorothioate oligomer is entirely controlled by the deprotection of the hydroxymethyl function. On using an acyl protecting group instead of the acid-labile dimethoxytrityl, deprotection dependent on the intracellular carboxyesterase activity, as with the SATE[2] and acyloxymethyl[3] groups, may be expected. If a longer life-time is desired, variation of the structure of groups X^1 and X^2 allows tuning of the reactivity over wide limits.

Experimental

The NMR spectra were recorded on a Bruker AM 200 or JEOL Alpha 500 NMR spectrometer. Coupling constants are given in Hz. The ESI-MS analyses were performed on a Perkin-Elmer Sciex API 365 Triple Quadrupole LC/ESI/MS spectrometer. The solvents were dried by refluxing over calcium hydride. The solid starting materials were dried by coevaporations with an anhydrous solvent. 5′-*O*-Pivaloylthymidine was prepared by acylating thymidine with 1.2 equivalents of pivaloyl chloride in pyridine.[9]

Preparation of 5′-*O*-pivaloylthymidine 3′-[2-methoxyethyl, 2,2-disubstituted-3-(4,4′-dimethoxytrityloxy)propyl]phosphates 1a–d

The appropriate bis(hydroxymethyl) compound,[4] either diethyl 2,2-bis(hydroxymethyl)malonate, methyl 2,2-bis(hydroxymethyl)-2-cyanoacetate, 2,2-bis(hydroxymethyl)malononitrile or 2,2-bis(hydroxymethyl)-1-phenylbutane-1,3-dione, was reacted with 1 equiv. of 4,4′-dimethoxytrityl chloride (Aldrich) in dry 1,4-dioxane containing 1 equiv. of pyridine.[4] The

dimethoxytritylated product (**7a**–**d**) was purified by silica column chromatography (dichloromethane–methanol) and used for preparation of **1a**–**d** by a phosphotriester method[6] (Scheme 3). The free hydroxy function of the protecting group reagent **7a**, **b**, **c** or **d** (typically 0.6 mmol) was phosphorylated with phosphoryl tris(triazolide) (0.9 mmol) in acetonitrile (5 ml), then 0.9 mmol of 5′-*O*-pivaloylthymidine was added to the mixture to replace the second triazole ligand, and after completion of this reaction (5–8 h, followed by TLC) an excess (5 ml) of 2-methoxyethanol was added. After a conventional sodium bicarbonate work-up, the product was purified by reversed phase chromatography on a Lobar RP-18 column (37–440 mm, 40–63 m) eluted with a mixture of acetonitrile and water (65 : 35%, v/v). The amounts of the isolated purified products corresponded to 6–41% of the theoretical yield, referred to the amount of **7a**–**d** used.

1a: δ_H (200 MHz, $CDCl_3$, ppm from TMS): 8.00 (s, N*H*), 7.17–7.38 (10 H, m, 6-H and DMTr protons), 6.81 (4 H, d, DMTr), 6.21 (dd, J_1 8.8, J_2 5.7, 1′-H), 4.86 (m, 3′-H), 4.70 (2 H, d, *J* 4.2, CH_2), 4.2 (9 H, m, 4′-H, 4 × CH_2), 3.78 (6 H, s, DMTr), 3.65 (2 H, s, CH_2), 3.52 (2 H, t, *J* 4, CH_2), 3.33 (3 H, s, OCH_3), 2.53 (m, 2′-Ha), 2.01 (m, 2′-Hb), 1.91 (3 H, s, CH_3 of Thy), 1.21 (6 H, t, 2 × CH_2CH_3), 1.20 (9 H, s; *Pivaloyl*). δ_P ($CDCl_3$; ppm from external H_3PO_4): 0.87 and 0.73. ESI$^+$-MS: *m*/*z* 969.0 for $[M + 1]^+$, 991.4 for $[M + Na]^+$.

1b: δ_H (200 MHz, $CDCl_3$): 8.56 (s, N*H*), 7.40–7.15 (10 H, 6-H and *DMTr*), 6.84 (4 H, d; *DMTr*), 6.27 (dd, J_1 8.2, J_2 5.6 Hz, 1′-H), 5.00 (m, 3′-H), 4.40 (3 H, m, 4′-H and CH_2), 4.35 (2 H, m, CH_2), 4.20 (2 H, m, CH_2), 3.85 (3 H, s, C(O)OCH_3), 3.80 (6 H, s, *DMTr*), 3.60 (2 H, m, CH_2), 3.49 (2 H, s, CH_2), 3.34 (3 H, s, OCH_3), 2.65 (m, 2′-Ha), 2.15 (m, 2′-Hb), 1.92 (3 H, s, CH_3 of Thy), 1.22 (9 H, s, *Pivaloyl*). δ_P ($CDCl_3$): 4.53 and 4.85. ESI$^+$-MS: *m*/*z* 908.5 for $[M + 1]^+$, 930.3 for $[M + Na]^+$.

1c: δ_H (200 MHz, $CDCl_3$): 8.76 (s, N*H*), 7.40–7.10 (10 H; 6-H and *DMTr*), 6.89 (4 H, d; *DMTr*), 6.27 (dd, J_1 8.9, J_2 5.4, 1′-H), 5.02 (m, 3′-H), 4.43 (4 H, m, 2 × CH_2), 4.27 (3 H, m, 4′-H, 2 × CH_2), 3.80 (6 H, s, *DMTr*), 3.60 (4 H, s, 2 × CH_2), 3.34 (3 H, s, OCH_3), 2.64 (m, 2′-Ha), 2.10 (m, 2′-Hb), 1.91 (3 H, s, CH_3 of Thy), 1.21 (9 H, s, *Pivaloyl*). δ_P ($CDCl_3$): −3.26 and −2.92. ESI$^+$-MS: *m*/*z* 875.6 for $[M + 1]^+$, 892.4 for $[M + NH_4^+]$.

1d: δ_H (200 MHz, $CDCl_3$): 8.22 (s, N*H*), 7.65 (2 H, m, *Bz*), 7.55 (1 H, d, *Bz*), 7.46 (2 H, m, *Bz*), 7.4–7.1 (10 H, 6-H and 9 *DMTr* protons), 6.79 (4 H, d, *DMTr*), 6.19 (m, 1′-H), 5.00 (2 H, m, CH_2), 4.80 (m, 3′-H), 4.30 (5 H, m, 4′-H, 2 × CH_2), 4.00 (2 H, m, CH_2), 3.76 (6 H, s, *DMTr*), 3.55 (2 H, m, CH_2), 3.33 (3 H, s, OCH_3), 2.50 (m, 2′-Ha), 2.08 (m, 2′-Hb), 2.00 (3 H, s, C(O)CH_3), 1.91 (3 H, s, CH_3 of Thy), 1.22 (9 H, s, *Pivaloyl*). δ_P ($CDCl_3$): −2.23 and −1.75. ESI$^+$-MS: *m*/*z* 971.5 for $[M + 1]^+$, 993.5 for $[M + Na]^+$.

5′-*O*-Pivaloylthymidine 3′-(2-methoxyethyl, 2,2-disubstituted-3-hydroxypropyl)phosphates 2a–d

The 4,4′-dimethoxytrityl group of the appropriate fully protected triester **1a**–**d** was removed with trifluoroacetic acid in a mixture of dichloromethane and methanol [CH_2Cl_2 : MeOH : CF_3COOH = 1 : 3 : 3 (v : v : v)]. According to HPLC analysis, the detritylation was completed in 12 min at 22 °C. Neither competing reactions nor degradation of the detritylated compound were observed. The acidic solution was evaporated to dryness and the residue was dissolved in a mixture of water and acetonitrile (50 : 50, v/v; 40 µl). The detritylated compounds **2a**–**d** were characterized by HPLC–ESI-MS. The mass spectrometric and chromatographic data are given in Table 1.

5′-*O*-Pivaloylthymidine 3′-{*O*-(2-methoxyethyl) *S*-[2-cyano-2-methoxycarbonyl-3-(4,4′-dimethoxytrityloxy)propyl]phosphoromonothioate} (4a)

Methyl 2-cyano-2-(4,4′-dimethoxytrityloxy)methyl-3-hydroxypropanoate (**7b**) was sulfonylated with trifluoromethanesulfonic anhydride in 1,2-dichloroethane containing 2 equiv. of pyridine (Scheme 5).[10] The triflate derivative **8** (1.37 g, 2.3 mmol) was reacted with 5′-*O*-pivaloylthymidine 3′-*O*-(2-methoxyethyl)-phosphoromonothioate (**6**; 0.12 g, 0.21 mmol; for preparation, see below) in anhydrous acetonitrile (30 ml) for 3 days in the presence of 2,6-lutidine (0.18 g, 1.7 mmol). The reaction was quenched with 1 M aqueous triethylammonium acetate buffer (20 ml) and the mixture was evaporated to dryness. The residue was taken up in dichloromethane (30 ml) and washed with 0.1 M aqueous sodium bicarbonate (2 × 20 ml) and water (15 ml). The product was purified on a silica gel column, eluting with a mixture of dichloromethane and methanol. The product fractions were evaporated to dryness to give 0.10 g (52%) of **4a** as a mixture of diastereomers. The R_P- and S_P-diastereomers were separated on a Hypersil ODS5 column (4–250 mm, 5 m), eluting with a mixture of methanol and water (74 : 26%, v/v).

The diastereomers of 4a derived from the "slower migrating" isomer of 6. HPLC: t_R = 22.5 min on a Hypersil ODS 5 column (4–250 mm, 5 µm) eluted with 74% aqueous methanol. δ_H (500 MHz, DMSO-d_6): 11.4 (s, N*H*), 7.39 (d, *J* 1.2, 6-H), 7.33–7.17 (9 H; *DMTr*), 6.91 (4 H, d, *DMTr*), 6.14 (dd, $J_1 = J_2 = 7.2$, 1′-H), 5.03 (m, 3′-H), 4.21 (3 H, m, 4′-H and 2 × 5′-H), 4.13 (2 H, m, CH_2 of methoxyethyl), 3.78 (3 H, s, C(O)OCH_3), 3.73 (6 H, s, *DMTr*), 3.51 (2 H, m, CH_2), 3.39 (2 H, m, CH_2), 3.25 (2 H, m, CH_2), 3.21 (3 H, s, OCH_3 of methoxyethyl), 2.49 (m, 2′-Ha), 2.45 (m, 2′-Hb), 1.77 (d, *J* 1.1, CH_3 of Thy), 1.13 (9 H, s, *Pivaloyl*). δ_P (202.35 MHz, DMSO-d_6, ppm from external phosphoric acid): 29.83 and 29.81. ESI$^+$-MS: *m*/*z* 941 ([M + NH_4]$^+$), 946 ([M + NH_4]$^+$).

The diastereomers of 4a derived from the "faster migrating" isomer of 6. HPLC: t_R = 19.9 and 20.8 min by the method described for the other stereoisomers. ^{31}P NMR: 30.27 and 30.24. ^{1}H NMR and ESI-MS identical with the "slower migrating" isomers.

4a was detritylated to **5a** (Table 1) as described above for the conversion of **1a**–**d** to **2a**–**d**.

5′-*O*-Pivaloylthymidine 3′-{*O*-(2-methoxyethyl) *O*-[2-cyano-2-methoxycarbonyl-3-(4,4′-dimethoxytrityloxy)propyl]phosphoromonothioate} (4b)

5′-*O*-Pivaloylthymidine (0.59 g, 1.8 mmol) was reacted with thiophosphoryl trichloride (0.31 g, 1.8 mmol, Aldrich) in anhydrous pyridine (2 ml) for 3 h at 22 °C (Scheme 4). Methyl 2-

Table 1 Mass spectrometric (HPLC–ESI$^+$-MS) and HPLC analysis of the 5′-*O*-pivaloylthymidine 3′-phosphotriesters **2a**–**d**, their hydrolysis product 5′-*O*-pivaloylthymidine 3′-(2-methoxyethyl)phosphate (**3**), and thiophosphate analogs **5a**,**b**

Compound	*m*/*z* $[M + H]^+$	*m*/*z* $[M + Na]^+$	t_R/min[a]
2a	667.4	689.2	16.4
2b	606.2	628.2	13.1
2c	573.2	595.4	14.0
2d	669.2	691.2	26.0
3	465.2		6.0
5a	622.2	644.4	7.7 and 8.4[b]
5b	622.3	644.4	5.1 and 5.4[c]

[a] On a Hypersil ODS 5 column (4–250 mm, 5 µm) eluted with a mixture of formic acid buffer ([HCOOH] : [HCOONa] = 0.05 : 0.01 M; [ammonium chloride] = 0.1 M) and acetonitrile, using a linear 30 min gradient from 20% to 80% acetonitrile (flow rate 1.0 ml min^{-1}). [b] For the R_P and S_P diastereomers (configurations not assigned) by isocratic elution with the above mentioned buffer containing 38% of acetonitrile. [c] For the R_P and S_P diastereomers by isocratic elution with 50% aqueous acetonitrile.

cyano-2-(4,4′-dimethoxytrityloxy)methyl-3-hydroxypropanoate (**7b**) was added (0.84 g, 1.8 mmol, dissolved in 1 ml pyridine) and the mixture was stirred for a further 3 h, after which 1 ml of 2-methoxyethanol was added and the mixture was stirred for 14 h. Then 100 ml of dichloromethane was added, and the organic phase was washed with a phosphate buffer (pH 6.5; 3 × 75 ml). After evaporation of the solvents, the product was purified by HPLC on a Hypersil ODS 5 column (10–250 mm), eluting with a mixture of acetonitrile and water (65 : 35%, v/v). 0.13 g of **4b** was isolated as a mixture of diastereomers. δ_H (500 MHz, $CDCl_3$): 8.28 (s, N*H*), 7.40–7.20 (10 H, 6-H and 9 *DMTr* protons), 6.85 (4 H, d, *DMTr*), 6.26 (m, 1′-H), 5.03 (m, 3′-H), 4.48–4.15 (7 H, m, 4′-H, 2 × 5′-H, 2 × CH_2), 3.85 (3 H, s, C(O)OCH_3), 3.80 (6 H, s, OCH_3 of DMTr), 3.50 (2 H, m, CH_2), 3.37 (2 H, m, CH_2), 3.34 (s, 3 H, OCH_3 of methoxyethyl), 2.60 (ddd; 2′-H_a), 2.12 (m, 2′-H_b), 1.92 (s, 3 H, CH_3 of Thy), 1.23 (9 H, *Pivaloyl*). δ_P (202 MHz, $CDCl_3$, ppm from external H_3PO_4): 70.07, 70.15, 70.69 and 70.93 for the four diastereomers, respectively. ESI$^+$-MS: *m*/*z* 924 ([M + 1]$^+$), 946 ([M + Na]$^+$), 962 ([M + K]$^+$).

4b was detritylated to **5b** (Table 1) as described above for the conversion of **1a**–**d** to **2a**–**d**.

5′-*O*-Pivaloylthymidine 3′-[*O*-(2-methoxyethyl)phosphoromonothioate] (6)

According to a hydrogen phosphonate method described earlier,[7] 2.0 g (5.5 mmol) of 5′-*O*-pivaloylthymidine and 2.3 g (28 mmol) of phosphorous acid (hydrogen phosphonic acid) were dissolved in 50 ml of anhydrous pyridine and 2.5 ml (20 mmol) of pivaloyl chloride were added. After 46 h stirring at 22 °C, 10 ml of 2-methoxyethanol were added and the stirring was continued for 20 h. The hydrogen phosphonate diester was oxidized to the phosphorothioate *in situ* by adding elemental sulfur (1.3 g) into the reaction mixture. After 4 h stirring, the crude product was isolated by a conventional aqueous work up. The product was purified on a silica column eluted with a mixture of dichloromethane and methanol (80 : 20%, v/v). The product fractions were evaporated to dryness to give 1.1 g of **6** as an equimolar mixture of two diastereomers. HPLC: t_R = 28.5 and 36.6 min on a Hypersil ODS 5 column (4–250 mm, 5 μm) eluted with a formic acid–sodium formate buffer (0.5 : 0.1 M) containing 0.1 M ammonium chloride and 18.5% (v/v) acetonitrile (flow rate 1.0 ml min^{-1}). *The slower migrating isomer*: δ_H (500 MHz, DMSO-d_6): 7.38 (d, *J* 1.2, 6-H), 6.12 (dd, J_1 8.2, J_2 5.8, 1′-H), 4.78 (m, 3′-H), 4.21 (3 H, m, 4′-H and 2 × 5′-H), 3.78 (2 H, m, CH_2 of methoxyethyl), 3.42 (2 H, t, *J* 5.0, CH_2 of methoxyethyl), 3.23 (3 H, s, OCH_3 of methoxyethyl), 2.30 (ddd, J_1 7, J_2 3.0, J_3 1.2, 2′-H_a), 2.16 (ddd, 2′-H_b), 1.77 (3 H, d, *J* 1.2, CH_3 of Thy), 1.15 (9 H, s, *Pivaloyl*). δ_P (202.35 MHz, DMSO-d_6, ppm from external phosphoric acid): 58.6. ESI-MS (negative): *m*/*z* 478.9. *The faster migrating isomer*: ^{1}H NMR identical with the other isomer. δ_P (202.35 MHz, DMSO-d_6): 58.5. ESI-MS (negative): *m*/*z* 478.9.

Kinetic measurements

The hydrolytic reactions were followed by an RP HPLC method (UV detection at 267 nm). The reactions were carried out in sealed tubes immersed in a thermostatted water bath (298.2 K). The hydronium ion concentrations of the reaction solutions were adjusted with hydrogen chloride, sodium hydroxide and formate, acetate, 2-morpholinoethanesulfonic acid (MES), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) (HEPES), and glycine buffers. To initiate a kinetic run, the 4,4′-dimethoxytrityl group of the appropriate phosphate or phosphorothioate triester (**1a**–**d**, **2a**–**d** or **4a**,**b**) was first removed as described above. The detritylated compound dissolved in 40 μl of a mixture of water and acetonitrile (50 : 50; v/v) was added to the reaction buffer solution (4 ml). The initial substrate concentration was *ca*. 10^{-4} M. The buffer concentrations were adjusted low (<0.06 M) to minimize the effects of buffer catalysis on the reactions. The composition of the samples withdrawn at appropriate time intervals was analyzed by RP HPLC. The separations were carried out on a Hypersil ODS 5 column (4–250 mm; 5 μm) using a mixture of formic acid–sodium formate buffer (0.05/0.01 M) containing 0.1 M tetramethylammonium chloride and acetonitrile as an eluent. The mass spectra of the hydrolysis products were recorded by LC-MS using a mixture of acetonitrile and 5 mM aqueous ammonium acetate as an eluent.

Calculation of the rate constants

The pseudo-first-order rate constants (k_{obs}) for the reactions were calculated by applying the integrated first-order rate equation to the diminution of the peak area of the phosphotriester.

References

1 (*a*) C. A. Stein and Y.-C. Cheng, *Science*, 1993, **261**, 1004; (*b*) S. T. Crooke and C. F. Bennet, *Annu. Rev. Pharmacol. Toxicol.*, 1996, **36**, 107.
2 (*a*) I. Barber, B. Rayner and J.-L. Imbach, *Bioorg. Med. Chem. Lett.*, 1995, **5**, 563; (*b*) G. Tosquellas, K. Alvarez, C. Dell'Aquila, F. Morvan, J.-J. Vasseur, J.-L. Imbach and B. Rayner, *Nucleic Acids Res.*, 1998, **26**, 2069; (*c*) J.-L. Bologna, F. Morvan and J. L. Imbach, *Eur. J. Org. Chem.*, 1999, 2353.
3 (*a*) R. P. Iyer, D. Yu and S. Agrawal, *Bioorg. Chem.*, 1995, **23**, 1; (*b*) R. P. Iyer, D. Yu, T. Devlin, N.-H. Ho and S. Agrawal, *Bioorg. Med. Chem. Lett.*, 1997, **7**, 871.
4 A. Guzaev and H. Lönnberg, *Synthesis*, 1997, 1281.
5 M. Kosonen, E. Yousefi-Salakdeh, R. Strömberg and H. Lönnberg, *J. Chem. Soc., Perkin Trans. 2*, 1998, 1589.
6 A. Krazewski and J. Stawinski, *Tetrahedron Lett.*, 1980, **21**, 2935.
7 J. Stawinski and M. Thelin, *Nucleosides, Nucleotides*, 1990, **9**, 129.
8 E.-P. Serjeant and B. Dempsey, *Ionization Constants of Organic Acids in Aqueous Solution. IUPAC Chemical Data Series, No. 23*, Pergamon, Oxford, 1979.
9 H. P. M. Fromageot, B. E. Griffin, C. B. Reese and J. E. Sulston, *Tetrahedron*, 1967, **23**, 2315.
10 A. M. Ozols, A. V. Azhayev, N. B. Dyatkina and A. A. Krayevsky, *Synthesis*, 1980, 557.