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Hydrolytic Reactions of Thymidine 5′-*O*-Phenyl-*N*-Alkylphosphoramidates, Models of Nucleoside 5′-Monophosphate Prodrugs

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Abstract: To obtain detailed data on the kinetics of hydrolytic reactions of triester-like nucleoside 5′-*O*-aryl-*N*-alkylphosphoramidates, potential prodrugs of antiviral nucleoside monophosphates, the hydrolysis of diastereomeric (R_P/S_P) thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**), a phosphoramidate derived from the methyl ester of L-alanine, has been followed by reversed-phase HPLC over the range from $H_0 = 0$ to pH 8 at 90 °C. According to the time-dependent product distributions, the hydrolysis of **3** proceeds at pH < 4 by two parallel routes, namely by nucleophilic displacement of the alaninyl ester moiety by a water molecule and by hydrolysis of the carboxylic ester linkage that allows intramolecular attack of the carboxy group on the phosphorus atom, thereby resulting in the departure of either thymidine or phenol without marked accumulation of any intermediates. Both routes represent about half of the overall disappearance of **3**. The departure of phenol eventually leads to the formation of thymidine 5′-phosphate. At pH > 5, the predominant reaction is hydrolysis of the carboxylic ester linkage followed by intramolecular displacement of a phenoxide ion by the carboxylate ion and hydrolysis of the resulting cyclic mixed anhydride into an acyclic diester-like thymidine 5′-phosphoramidate. The latter product accumulated quantitatively without any indication of further decomposition. Hydroxide-ion-catalyzed P–OPh bond cleavage of the starting material **3** occurred as a side reaction. Comparative measurements with thymidine 5′-{*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**4**) revealed that, under acidic conditions, this diester-like compound is hydrolyzed by P–N bond cleavage three orders of magnitude more rapidly than the triester-like **3**. At pH > 5, the stability order is reversed, with **3** being hydrolyzed six times as rapidly as **4**. Mechanisms of the partial reactions are discussed.

Keywords: hydrolysis · kinetics · nucleotides · phosphoramidates · prodrugs

Introduction

The delivery of antiviral nucleoside analogues as appropriately protected monophosphate prodrugs has attracted increasing interest after approval of the bis(isopropoxycarbonyloxymethyl) esters of two acyclic nucleoside phosphonates, namely 9-[2-(phosphonomethoxy)ethyl]adenine and (*R*)-9-[2-(phosphonomethoxy)propyl]adenine, for clinical use.[1] Phosphoramidate pronucleotides constitute one class of such prodrug candidates.[2,3] In particular, aryl esters of nucleoside 5′-phosphoramidates derived from α-amino acid esters, such as **1**, have received attention.[4–8] This kind of prodrug has shown higher antiviral potency on cell lines than the parent nucleoside analogues.[4] It has been suggested that the increased potency results from enhanced cellular uptake of the prodrug and subsequent intracellular release of the monophosphate.[9] Accordingly, the rate-limiting conversion of the nucleoside analogue into its monophosphate by thymidine kinase is avoided. The α-amino acid moiety appears to be essential, since phosphoramidates derived from simple alkylamines or β-amino acids do not show antiviral activity.[10] The release of the monophosphate probably proceeds by intermediate formation of L-alaninyl phosphoramidate **2**.[11] Either release of phenol by chemical hydrolysis is followed by enzymatic cleavage of the alaninyl ester linkage, or these two processes take place in reverse order.[4,9,12] Unfortunately, the kinetics of the chemical hydrolysis of nucleoside *O*-aryl-*N*-alkylphosphoramidates have not previously been studied in such a detailed manner that mechanistic conclusions were possible. The present work is aimed at filling this gap. The hydrolysis of diastereomeric

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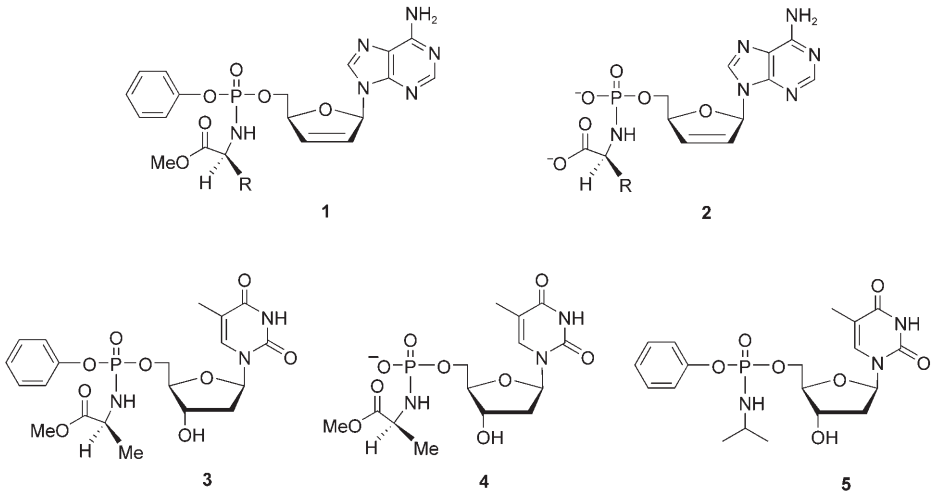
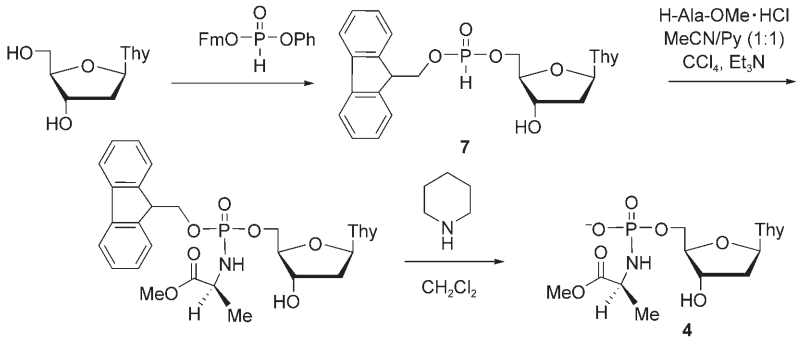


(R_P/S_P) thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**) has been studied over a wide pH range (from $H_0=0$ to pH 8) at 90 °C by analyzing the composition of aliquots withdrawn from the reaction mixture at appropriate time intervals by reversed-phase (RP) HPLC. Comparative measurements have been carried out with thymidine 5′-{*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**4**) and thymidine 5′-(*O*-phenyl-*N*-isopropylphosphoramidate) (**5**).

Results

Preparation of thymidine 5′-phosphoramidates: Thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**) was obtained as a mixture of R_P and S_P diastereomers by oxidative amination of thymidine 5′-(*H*-phosphonate) phenyl ester (**6**) with L-alanine methyl ester (Scheme 1), a method previously used for the synthesis of nucleoside phosphoramidate diesters[13] and for the synthesis of oligoribonucleotide phosphoramidates on a solid support.[14] The diastereomers were separated by reversed-phase HPLC. No attempt was made to assign the absolute configuration of the slower and faster migrating diastereomers as R_P or S_P. Thymidine 5′-(*O*-phenyl-*N*-isopropylphosphoramidate) (**5**) was prepared similarly from **6** and diisopropylamine and was used in kinetic measurements as a mixture of the R_P and S_P diastereomers.

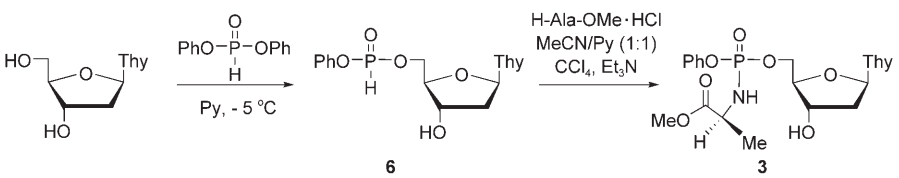
Thymidine 5′-{*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]-phosphoramidate} (**4**) was prepared by the methodology described by Zhao and co-workers.[13] Accordingly, thymidine was first converted into the fluoren-9-ylmethyl ester of thymidine 5′-(*H*-phosphonate) (**7**) by H-phosphonylation with fluoren-9-ylmethyl phenyl *H*-phosphonate and the product was subjected to oxidative amination with L-alanine methyl ester (Scheme 2). Finally, the fluoren-9-ylmethyl group was removed by treatment with piperidine in CH_2Cl_2.



Scheme 2. Preparation of thymidine 5′-{*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**4**). Fm: fluoren-9-ylmethyl.

Product distribution: The cleavage of the R_P and S_P diastereomers of thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**) was followed over a wide pH range (from $H_0=0$ to pH 8) at 90 °C by analyzing the composition of aliquots withdrawn from the reaction mixture at appropriate time intervals by RP HPLC. The products were characterized either by spiking with authentic samples or by mass spectrometric analysis (HPLC/ESI-MS).

The time-dependent product distributions obtained with the faster migrating diastereomer of **3** under acidic (pH 2) and slightly basic (pH 8) conditions are shown in Figures 1 and 2, respectively. At pH 2, three



Scheme 1. Preparation of thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**). Thy: thymine, Py: pyridine.

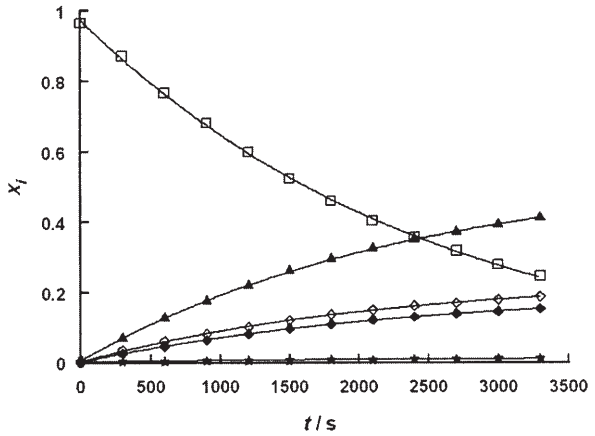


Figure 1. Time-dependent product distribution for the hydrolysis of the faster-eluting diastereomer of thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**) in 0.01 mol L^{-1} aqueous hydrogen chloride at 90 °C (I = 0.1 mol L^{-1} with NaCl). Compound **3**: □, thymidine 5′-phenylphosphate (**8**): ▲, thymidine (**9**): ◆, thymidine 5′-phosphate (**10**): ◇; thymine: ∗.

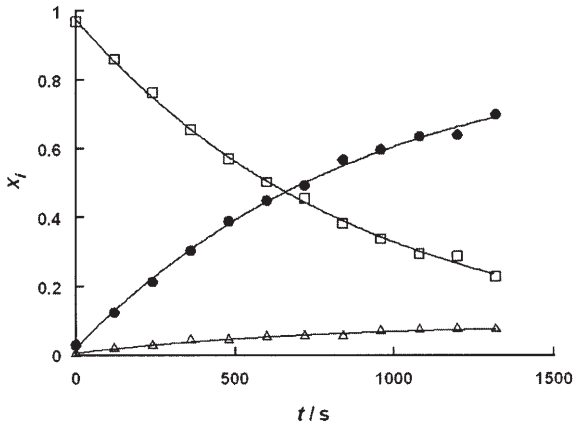
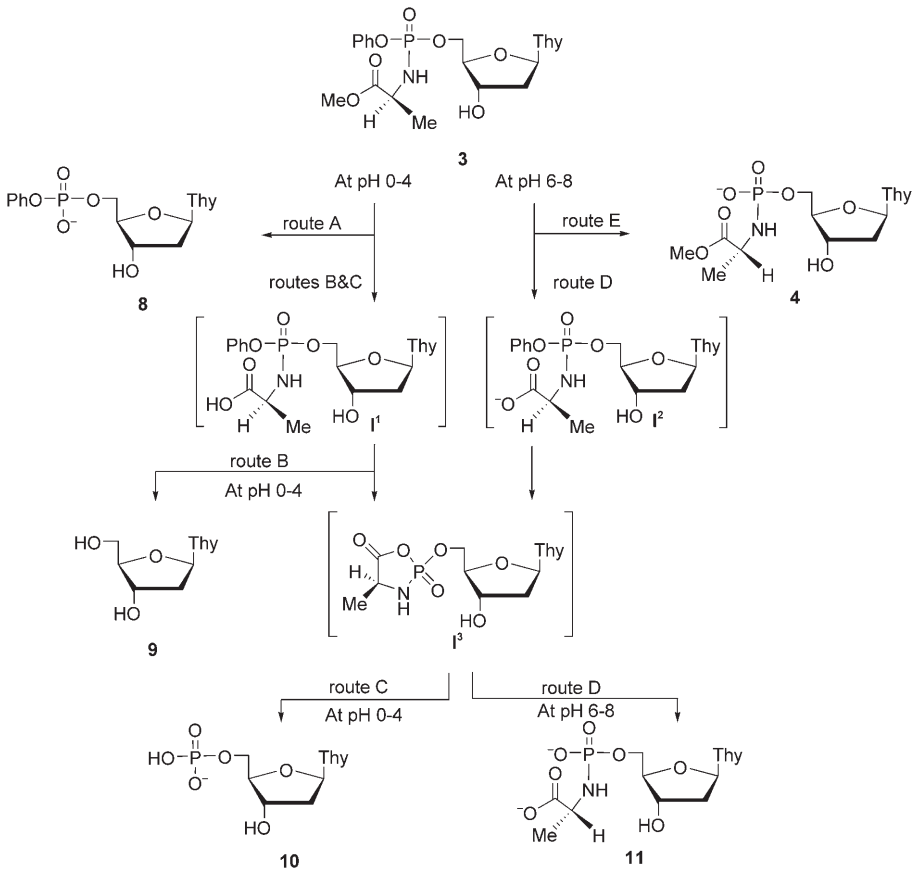


Figure 2. Time-dependent product distribution for the hydrolysis of the faster-eluting diastereomer of thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**) in a glycine buffer at pH 8 and 90 °C ([HA]/[A^-] = 0.02/0.01 mol L^{-1}; I = 0.1 mol L^{-1} with NaCl). Compound **3**: □, thymidine 5′-{*N*-[(1*S*)-2-oxo-2-hydroxy-1-methylethyl]-phosphoramidate} (**11**): ●, thymidine 5′-{*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**4**): △.

nucleosidic products, that is, thymidine 5′-phenylphosphate (**8**), thymidine (**9**), and thymidine 5′-phosphate (**10**), were formed in parallel. In addition, a small amount (<2%) of thymine was released, mainly by subsequent decomposition of the initial products. Only at pH 4 was the amount of thymine marked, up to 14%. Otherwise, the product distribution remained constant over the pH range 0–4. None of the products obtained at pH 0–4 accumulated at pH 8, but the hydrolysis instead yielded thymidine 5′-{*N*-[(1*S*)-2-oxo-2-hydroxy-1-methylethyl]phosphoramidate} (**11**) and thymidine 5′-{*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**4**).

For the reasons discussed in the following section, the nucleosidic products listed above are believed to be formed along the pathways indicated in Scheme 3. Formation of thymidine 5′-phenylphosphate (**8**) as the major product of the acid-catalyzed hydrolysis is expected. Previous studies with *N*,*O*-dialkyl-*O*-arylphosphoramidates[15] and *O*,*O*-dialkyl-*N*-arylphosphoramidates[16] have shown that the substrate monocation obtained in a rapid pre-equilibrium step undergoes rate-limiting breakdown by nucleophilic attack of water on the phosphorus atom with concomitant departure



Scheme 3. Pathways for the acid- and base-catalyzed hydrolyses of thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**). Structures in parentheses (**I^1**–**I^3**) indicate intermediates that were not detected.

of the amine ligand. Evidently, **3** can follow a similar pathway. By contrast, an explanation for the formation of thymidine (**9**) and its 5′-phosphate **10** in parallel with the formation of **8** is not so self-evident. Measurements in ^{18}O-enriched water indicated that no ^{18}O was incorporated into the thymidine. In other words, thymidine was released by P–O bond rupture, not by C–O fission. Thymidine 5′-(*O*-phenyl-*N*-isopropylphosphoramidate) (**5**) did not give any thymidine or thymidine 5′-phosphate at pH 1, but it was quantitatively hydrolyzed to thymidine 5′-phenylphosphate (**8**), twice as fast as **3**. These two observations leave intramolecular nucleophilic participation by the carboxy function as the most plausible alternative for the formation of thymidine and its 5′-phosphate. A prerequisite for the nucleophilic attack by the carboxylic acid group on the phosphorus atom is obviously hydrolysis of the carboxylic ester linkage. The second-order rate constants for the acid-catalyzed hydrolysis of saturated carboxylic esters are of the order of $10^{-2}\,L\,mol^{-1}\,s^{-1}$ at 90 °C[17] and, hence, formation of **9** and **10** through initial hydrolysis of the alaninyl ester linkage (**I**^{1}) appears possible. Nucleophilic attack of the carboxylic acid group on the phosphorus atom then results in displacement of thymidine (**9**) or phenol. Departure of phenol gives a cyclic phosphoramidate intermediate (**I**^{3}), which may decompose to thymidine 5′-phosphate (**10**) by consecutive cleavage of the P–N and P–O (or C(O)–O) bonds, in this order. Mass spectrometric analyses verified release of alanine (*m*/*z* value for $[M+H]^+$: 91.9) in addition to alanine methyl ester (*m*/*z* value for $[M+H]^+$: 104.1). Thymidine 5′-phosphate undergoes dephosphorylation to thymidine under slightly acidic conditions.[18] Evidently, this reaction is of minor importance, since the concentration ratio of **9** and **10** remains constant with time. In principle, intermediate **I**^{1} could also be hydrolyzed to form **8**. This intermediate does not, however, accumulate, which means that intramolecular attack of the carboxy group to give **9** and **I**^{3} is too fast to allow hydrolysis to **8** by intermolecular attack of water to compete. In striking contrast to triester-like thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**), its diester-like analogue, thymidine 5′-{*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**4**), reacts at pH values <4 only by P–N bond rupture, as observed previously with other diester-like nucleoside phosphoramidates.[19]

At pH values >5, hydroxide-ion-catalyzed P–OPh bond cleavage to thymidine 5′-{*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**4**) and hydrolysis of the alaninyl methyl ester linkage, thereby giving thymidine 5′-{*N*-[(1*S*)-2-oxo-2-hydroxy-1-methylethyl]phosphoramidate} (**11**), take place. While **4** is hydrolyzed at pH values <4 more rapidly than **3** by the P–N bond cleavage, the stability order of these two compounds is reversed with higher pH values and, hence, **4** accumulates as a stable product at pH values >6, although only as a minor product (8%). The major product, **11**, having the alaninyl ester linkage hydrolyzed, cannot be formed from **4**, since the concentration ratio of these two products remains constant with time. Moreover, independent studies into the hydrolysis of **4** indicated that the conversion of **4** into **11** is considerably slower than the disappearance of **3**. Accordingly, if **11** were formed via **4**, the latter should markedly accumulate as an intermediate. Since this is not the case, the formation of **11** is most likely initiated by hydroxide-ion-catalyzed hydrolysis of the carboxylic ester linkage. The rate of this reaction at pH 6–8 is expected to be comparable to the rate of the acid-catalyzed hydrolysis at pH 1–2.[17] Accordingly, sufficiently rapid formation of intermediate **I**^{2} appears possible, although no direct evidence for its existence could be obtained. Evidently, rapid intramolecular attack of the carboxylate ion on the phosphorus atom takes place, with concomitant departure of the phenoxide ion. The cyclic phosphoramidate intermediate obtained (**I**^{3}) then undergoes ring-opening by attack of water on the phosphorus atom and departure of the carboxylate ion. The acid-catalyzed cleavage of the P–N bond is too slow at pH >6 to compete with the hydrolysis of the mixed anhydride. It should be noted that a similar intramolecular participation has previously been reported for displacement of a phenoxide ion from *O*-phenyl-*N*-(carboxymethyl)phosphoramidate.[20]

pH rate profiles: The pH rate profiles for the breakdown of the two diastereomers of thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**) and the *O*-unsubstituted analogue, thymidine 5′-{*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**4**), are depicted in Figure 3. The hydrolytic stability of the diaseteromeric forms of **3** are very similar. The pH rate profiles for the individual partial reactions of the breakdown of phosphoramidate **3** are depicted in Figure 4. Numerical values for the partial rate constants are given in Table 1.

At pH values <4, the breakdown of **3**, utilizing routes A–C in Scheme 3, is first-order with respect to oxonium ion concentration. The reaction by initial P–N bond cleavage, giving thymidine 5′-phenylphosphate (**8**; route A), is approx-

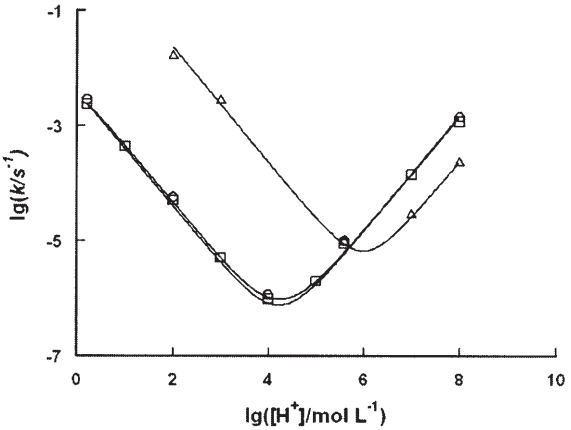


Figure 3. pH rate profiles for the decomposition of diastereomeric thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate}s (**3**; fast-eluting diastereomer □, slowly eluting diastereomer ○) and thymidine 5′-{*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**4**; △) at 90 °C. $I=0.1$ mol L^{-1} with NaCl.

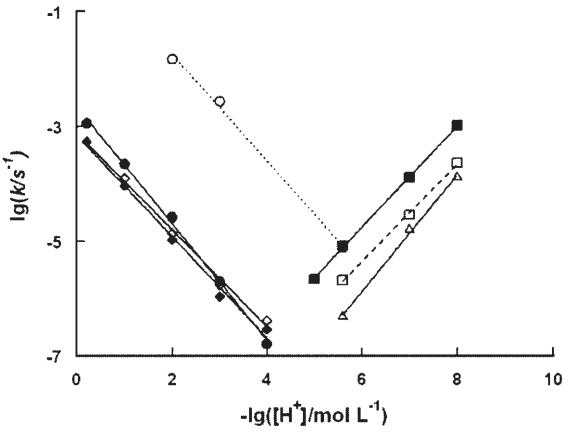


Figure 4. pH rate profiles for the hydrolysis of thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**) into thymidine 5′-phenylphosphate (**8**; k_A, ●), thymidine (**9**; k_B, ◆), thymidine 5′-phosphate (**10**; k_C, ◇), thymidine 5′-{*N*-[(1*S*)-2-oxo-2-hydroxy-1-methylethyl]phosphoramidate} (**11**; k_D, ■), and thymidine 5′-{*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**4**; k_E, △) at 90 °C (I= 0.1 mol L^{-1} with NaCl). The subscripts of the rate constants refer to the routes indicated in Scheme 3. The dotted (○) and dashed (□) lines show the corresponding curves for P–N bond cleavage and methyl ester hydrolysis of **4**, respectively.

Table 1. Rate constants, given as $k_i \times 10^5$ s^{-1}, for the partial reactions of the hydrolysis of the two diastereomeric forms of thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**) at 90 °C (I=0.1 mol L^{-1} with NaCl): k_A (formation of **8** by route A), k_B (formation of **9** by route B), k_C (formation of **10** by route C), k_D (formation of **11** by route D), and k_E (formation of **4** by route E). In addition, the corresponding data for the hydrolysis of thymidine 5′-{*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**4**) are given: k_F (formation of **10** from **4**) and k_G (formation of **11** from **4**).

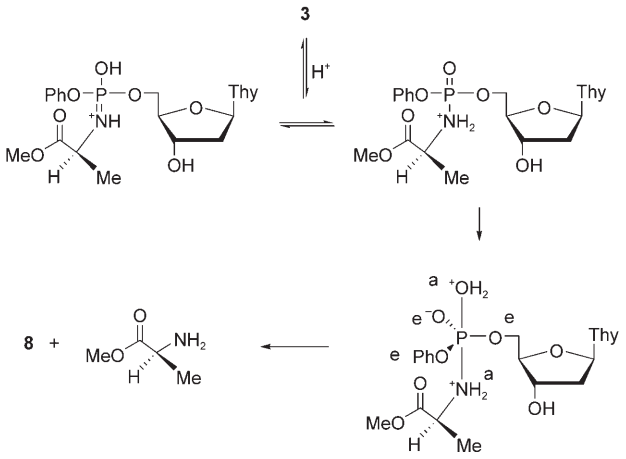
	3 (fast diastereomer)					**3** (slow diastereomer)					**4**	
pH	k_A	k_B	k_C	k_D	k_E	k_A	k_B	k_C	k_D	k_E	k_F	k_G
0.2	112	54.3	54.0			131	60.3	60.3				
1.0	21.7	9.1	12.4									
2.0	2.55	1.04	1.34			2.84	1.18	1.48			1490	
3.0	0.20	0.11	0.17								270	
4.0	0.02	0.02	0.03			0.03	0.03	0.04				
5.6				0.83	0.05				0.95	0.05	0.69	0.27
7.0				12.5	1.59							2.83
8.0				103	12.8				121	16.4		22.8

imately as fast as the carboxylic ester hydrolysis, which gives intermediate **I**[1] (routes B and C). The latter intermediate gives thymidine (**9**; route B) and intermediate **I**[3], and, hence, eventually thymidine 5′-phosphate (**10**; route C), in an equimolar ratio. The diester-like analogue, **4**, is hydrolyzed under acidic conditions 600 times more rapidly than **3**. As discussed above, this reaction proceeds entirely by cleavage of the P–N bond. In all likelihood, the reactive species is the zwitterionic minor tautomer of protonated (neutral) **4**, with the nitrogen atom protonated (cationic) and the phosphoryl oxygen atom anionic.[19] This ensures a good leaving group (neutral amine), together with a good remnant (metaphosphate-like structure). Hence, the breakdown of **4** is considerably faster than that of the triester-like *O*-phenyl derivative **3** and is too fast to allow competition by hydrolysis of the alaninyl ester linkage and subsequent intramolecular attack of the carboxy group.

At pH values >5, the breakdown of **3** becomes base catalyzed. The reaction by hydrolysis of the carboxylic ester linkage, followed by rapid intramolecular displacement of the phenoxy group, that is, formation of **11** by route D, predominates. Intermolecular displacement of the phenoxide ion without preceding hydrolysis of the carboxy ester (route E) is one order of magnitude slower. The hydrolysis of the carboxy ester linkage of triester-like phosphoramidite **3** under these conditions is 6 times as fast as the corresponding reaction of its diester-like analogue **4**.

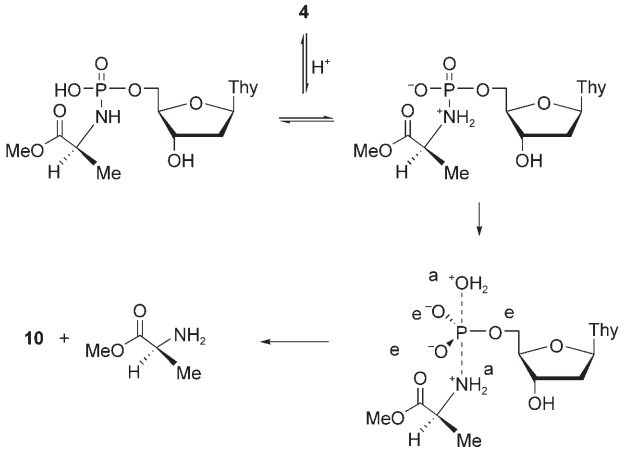
Discussion

Mechanisms of acid-catalyzed P–N bond cleavage (route A): As indicated above, thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**) is hydrolyzed under acidic conditions by two concurrent routes: nucleophilic displacement of the alaninyl ester moiety by a water molecule (route A in Scheme 3) and hydrolysis of the carboxylic ester linkage that allows intramolecular attack of the carboxy group on the phosphorus atom, thereby resulting in departure of either thymidine or phenol without marked accumulation of any intermediates (routes B and C). Both routes represent about half of the overall disappearance of **3**. Consistent with intermolecular displacement of the amine ligand with water, studies in ^{18}O-enriched water have shown that the methyl ester of alanine (*m*/*z* value for $[M+H]^+$: 104.1) is released without ^{18}O-atom incorporation, while one ^{18}O atom is incorporated into the thymidine 5′-phenylphosphate product (**8**). The reaction most likely proceeds as depicted in Scheme 4. As suggested previously[15] for *N*,*O*-dialkyl-*O*-arylphosphoramidates, rapid initial protonation of the starting material probably gives a mixture of *N*- and *O*-protonated monocations, but the reaction proceeds through the *N*-protonated species. Upon nucleophilic attack of water, the positively charged nitrogen atom takes an apical position and departs by an S_N2-like mechanism. It is worth noting that this is the only reaction detected with the *N*-isopropyl analogue of **3**, which contains no functional group on the *N*-alkyl moiety. The diester-like phosphoramidate **4**, which is hydrolyzed 600 times faster than **3**, evidently utilizes a rather similar mechanism. Protonation of monoanionic **4** is easier than protonation of neutral triester-like **3**. Again, the site of protonation remains obscure,[21] but the *N*-protonated tautomer in all likelihood lies on the reaction pathway. Upon departure of the amine ligand, a dianionic metaphosphate-like structure is developed. This increases



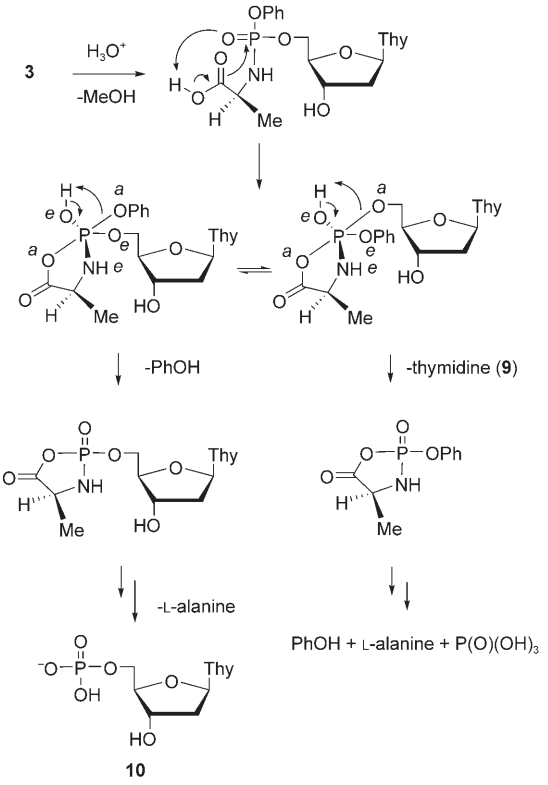
Scheme 4. Mechanism for the acid-catalyzed hydrolysis of the P–N bond of thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**). a: axial position, e: equatorial position.

the electron density at the phosphorus atom and, hence, the cleavage of the P–N bond is facilitated (Scheme 5). The fact that the solvolysis of phosphoramidic acid,[22] *O*-alkylphosphoramidates,[23] and *N*-alkylphosphoramidates[24] in aqueous alcohol favors the alcoholysis product over the hydrolysis product, however, suggests that the mechanism is not purely dissociative and that the entering nucleophile participates in the transition state.



Scheme 5. Mechanism for the acid-catalyzed hydrolysis of the P–N bond of thymidine 5′-{*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**4**).

Mechanisms of acid-catalyzed P–O bond cleavage (routes B and C): As mentioned above, the phosphoramidate triester **3** also yields thymidine (**9**) and its 5′-phosphate **10** under acidic conditions. Both products are formed by P–O bond rupture, since ^{18}O is not incorporated into the released alcohols, that is, thymidine or phenol, but is incorporated into thymidine 5′-phosphate (**10**). Since no acid-catalyzed P–O bond cleavage takes place with thymidine 5′-(*O*-phenyl-*N*-isopropylphosphoramidate) (**5**), the carboxy function obtained by hydrolysis of the alaninyl ester linkage most likely serves as an intramolecular nucleophile (routes B and C), which triggers the rupture of P–O bonds. It should be noted that, while intramolecular attacks of oxygen nucleophiles on protonated (monocationic) phosphotriesters proceed by attack on the phosphorus atom and, hence, by P–O bond cleavage,[25] the corresponding intermolecular reactions take place at the carbon atom, that is, by C–O bond cleavage.[26] As discussed in the preceding section, ester hydrolysis appears to be sufficiently fast to allow intramolecular participation of the carboxy group. Attack of the carbonyl oxygen atom of the exposed carboxy group on the phosphorus atom concerted with transfer of the carboxylic acid proton to the phosphoryl oxygen atom gives a phosphorane intermediate with either the phenoxy or thymidine ligand in an apical position (Scheme 6). Cleavage of the apical P–O bond concerted with proton transfer from the phosphoryl oxygen atom to the departing oxygen atom results in release of either phenol or thymidine (**9**). Departure of phenol yields a cyclic triester-like phosphoramidate, which eventually releases thymidine 5′-phosphate (**10**) by consecutive departure of the carboxy and amino groups of the alanine moiety. It appears reasonable to assume that the reactions take place in this order, since the carboxylate ion is a conjugate base of a stronger acid than the amide ion. Hydrolysis of the mixed anhydride then gives a diester-like phosphoramidate, known



Scheme 6. Mechanism for the acid-catalyzed hydrolysis of thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**) into thymidine (**9**) and its 5′-phosphate **10**.

to be hydrolytically much less stable than its triester analogue.

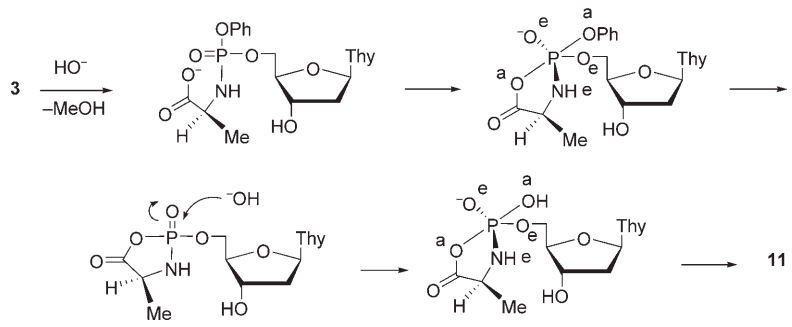
One might also speculate that the formation of thymidine (**9**) or its 5′-phosphate **10** is initiated by nucleophilic attack of the 3′-OH group, and not the carboxy group, on the phosphorus atom. This pathway is not, however, utilized under acidic conditions, because no sign of accumulation of the very stable thymidine 3′,5′-cyclic monophosphate[25c] or its phenyl ester[25d] could be detected.

Mechanism of base-catalyzed intramolecular P–O bond cleavage (route D): Hydrolysis of thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**) becomes hydroxide-ion-catalyzed at pH values higher than 5. The predominant product is thymidine 5′-{*N*-[(1*S*)-2-oxo-2-hydroxy-1-methylethyl]phosphoramidate} (**11**; route D in Scheme 3). This product is most probably formed by an initial attack of a hydroxide ion on the carbonyl carbon atom and concomitant elimination of a methoxide ion, followed by attack of the resulting carboxylate group on the phosphorus atom. A pentacoordinated intermediate (or transition state) is obtained and subsequently decomposes through departure of the phenoxide ion, which is a much better leaving group than thymidine 5′-oxyanion (Scheme 7). A highly unstable cyclic mixed anhydride intermediate is obtained. This intermediate then undergoes fast subsequent hydrolysis by attack of a hydroxide ion on the phosphorus atom. Since the endocylic oxygen atom of the mixed anhydride is a more electronegative atom than the endocyclic nitrogen atom, it occupies an apical within the five-membered ring upon attack of the hydroxide ion. Accordingly, only the P–O bond is cleaved.[19g,26] Consistent with the mechanism proposed, the final product (**11**) obtained in ^{18}O-enriched water contained two ^{18}O atoms. The accumulation of the first intermediate, with the carboxylic ester linkage hydrolyzed (**I²** in Scheme 3), remained too low to be reliably quantified by HPLC with UV detection, but an *m*/*z* value ($[M-H]^+$ 472.3) corresponding to the molecular ion containing one ^{18}O atom could be observed by HPLC/ESI-MS.

The results discussed above do not strictly rule out the possibility that the phenoxide ion is released from intermediate **I²** by nucleophilic attack of a hydroxide ion on the phosphorus atom. This alternative appears, however, less attractive. Previous comparative studies with *O*-phenyl-*N*-carboxymethylphosphoramidate and *O*-phenylphosphoramidate have shown that intramolecular catalysis by the neighboring carboxylate group accelerates the rupture of the P–OPh bond by a factor of approximately 10^4 at pH 8.[20] Evidently, the carboxylate function also produces a remarkable rate enhancement of P–OPh bond cleavage in **I²**.

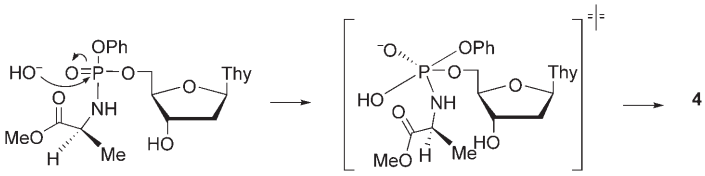
While the carboxylate group readily attacks on the neutral triester-like phosphoramidate center, no similar attack on the monoanionic diester-like phosphoramidate center of **11** takes place, but **11** accumulates as a stable product at pH 7–8. An intramolecular attack of the carboxylate group would give a pentacoordinated intermediate (transition state) in which the attacking oxygen atom and thymidine occupy the apical positions, while the nitrogen ligand remains equatorial, as it is a member of the same five-membered ring as the attacking oxygen atom. The dianionic intermediate, even if it exists, is too unstable to pseudo-rotate,[27] but thymidine should be released "in-line" with nucleophilic attack. The fact that **11** does not decompose by this pathway is not self-evident, as the rate constant for a similar intramolecular displacement of a phenoxide ion from *O*-phenyl-*N*-carboxymethylphosphoramidate is 1.4×10^{-4} s^{-1} at 75 °C.[20] One should, however, bear in mind that the 5′-oxyanion of thymidine is a poor leaving group compared to the phenoxide ion.

Mechanism of base-catalyzed intermolecular P–O bond cleavage (route E): At pH 6–8, release of the phenoxide ion from thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**) also takes place by direct intermolecular displacement by a hydroxide ion. On the basis of ^{18}O-incorporation studies, the diester-like phosphoramidate obtained (**4**) includes one ^{18}O atom. In all likelihood, this P–OPh bond fission proceeds by a bimolecular S_N2(P)-type mechanism (Scheme 8) by attack of a hydroxide ion on the phosphorus atom concerted with the departure of the phenoxide ion, as was also suggested for *N*-monosubstituted *O*-aryl-*O*-methyl- and *O*,*O*-diphenylphosphoramidates and their their unsubstituted analogues.[28] Consistent with the suggested mechanism, thymidine 5′-(*O*-phenyl-*N*-isopropylphosphoramidate) (**5**) yields only thymidine 5′-(*N*-isopropylphosphoramidate). The reaction is one order of magnitude slower than with **3**, probably because the isopropyl group does not exert a similar electron-withdrawing effect to that of the 2-methoxy-2-oxo-1-methylethyl group.



Scheme 7. Mechanism for the hydroxide-ion-catalyzed hydrolysis of thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**) into thymidine 5′-{*N*-[(1*S*)-2-oxo-2-hydroxy-1-methylethyl]phosphoramidate} (**11**).

In summary, thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**) has been shown to be hydrolyzed under acidic conditions (pH < 4) partly by displacement of the *N*-proton-



Scheme 8. Mechanism for the hydroxide-ion-catalyzed hydrolysis of thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**3**) into thymidine 5′-{*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]-phosphoramidate} (**4**) and a phenoxide ion.

ated amino ligand with water and partly by oxonium-ion-catalyzed methyl ester hydrolysis followed by intramolecular nucleophilic attack of the resulting carboxy group on the phosphorus atom. The latter reaction gives thymidine and its 5′-phosphate as nucleosidic products. The acid-catalyzed hydrolysis turns into a base-catalyzed hydrolysis above pH 5. The predominant product of the base-catalyzed hydrolysis is thymidine 5′-{*N*-[(1*S*)-2-hydroxy-2-oxo-1-methylethyl]phosphoramidate} (**11**), obtained by hydroxide-ion-catalyzed methyl ester hydrolysis and subsequent intermolecular attack of a hydroxide ion on the phosphorus atom. Intermolecular displacement of the phenoxide ion with a hydroxide ion occurs as a side reaction. In the pH range 0–4, the oxonium-ion-catalyzed P–N bond cleavage makes the diester-like thymidine 5′-{*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]-phosphoramidate} (**4**) 2–3 orders of magnitude more reactive than its triester analogue **3**. At pH values >6, the stability order is reversed, with triester **3** being decomposed six times as fast as the diester **4**.

Experimental Section

Thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (3): Thymidine (**9**, 0.24 g, 1.00 mmol) in an anhydrous pyridine (2.5 mL) was added dropwise to diphenyl phosphite (288 μL, 1.50 mmol) in anhydrous pyridine (2.5 mL) at −5 °C. The thymidine 5′-hydrogenphosphonate phenyl ester obtained was not isolated, but the solution was stirred for 20 min at room temperature, after which L-alanine methyl ester (0.21 g, 1.5 mmol), MeCN (5.0 mL), CCl_4 (6.2 mL), and Et_3N (0.5 mL) were added. After being stirred for 75 min, the solution was evaporated to dryness. The crude product was isolated by a conventional aqueous work up and purified on a silica gel column eluted with a mixture of CH_2Cl_2 and MeOH (90:10). The diastereomers were purified and separated by reversed-phase chromatography on a Lobar RP-18 column (37×440 mm, 40–63 μm) by elution with a mixture of water and MeCN (80:20, v/v).

*The faster-eluted diastereomer of **3***: ^{1}H NMR (500 MHz, [D]$CHCl_3$, 25 °C, TMS): δ = 9.35 (s, 1H; NH), 7.45–7.17 (m, 6H; H6, Ph), 6.33 (dd, J(H,H) = 6.5, 6.5 Hz, 1H; H1′), 4.52 (m, 1H; H3′), 4.40 (dd, J(H,H) = 7.0, 3.0 Hz, 1H; H5′), 4.35 (dd, J(H,H) = 7.0, 3.0 Hz, 1H; H5″), 4.01–4.11 (m, 3H; H4′, NH, CH), 3.71 (s, 3H; OCH_3), 2.34 (m, 1H; H2′), 2.02 (m, 1H; H2″), 1.90 (s, 3H; CH_3), 1.38 ppm (d, J(H,H) = 6.5 Hz, 3H; CH_3); ^{13}C NMR (125 MHz, [D]$CHCl_3$, 25 °C, TMS): δ = 174.1, 163.9, 150.5, 135.5, 129.8, 125.3, 120.0, 111.2, 84.8, 84.6, 70.6, 65.9, 52.7, 50.4, 39.8, 20.8, 12.5 ppm; ^{31}P NMR (202 MHz, D_2O, 25 °C, H_3PO_4): δ = 3.38 ppm; ESI-MS: m/z: 484.6 $[M-H]^+$.

*The more slowly eluted diastereomer of **3***: ^{1}H NMR (500 MHz, [D]$CHCl_3$, 25 °C, TMS): δ = 9.10 (s, 1H; NH), 7.37–7.18 (m, 6H; H6, Ph), 6.27 (dd, J(H,H) = 6.5, 6.5 Hz, 1H; H1′), 4.49 (m, 1H; H3′), 4.35 (m, 2H; H5′, H5″), 4.11–3.99 (m, 3H; H4′, NH, CH), 3.73 (s, 3H; OCH_3), 2.40 (m, 1H; H2′), 2.14 (m, 1H; H2″), 1.90 (s, 3H; CH_3), 1.39 ppm (d, J = 7.0 Hz, 3H; CH_3); ^{13}C NMR (125 MHz, [D]$CHCl_3$, 25 °C, TMS): δ = 174.1, 163.8, 150.4, 135.6, 129.9, 125.3, 120.1, 111.3, 85.0, 84.6, 71.0, 66.1, 52.7, 50.2, 39.8, 20.9, 12.5 ppm; ^{31}P NMR (202 MHz, D_2O, 25 °C, H_3PO_4): δ = 3.06 ppm; ESI-MS: m/z: 484.6 $[M+H]^+$.

Thymidine 5′-{*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (4): Thymidine was converted into thymidine 5′-(fluoren-9-ylmethyl-*H*-phosphonate) (**7**) as described previously by Zhao and co-workers[13] and subjected to oxidative amination in a mixture of pyridine and MeCN in the presence CCl_4, EtN_3, and alanine methyl ester, as described above for **3**. The product was purified by silica-gel chromatography with a mixture of CH_2Cl_2 and MeOH as the eluent (90:10). In a final step, the fluorenylmethyl group was removed by treatment with piperidine in CH_2Cl_2. The product was purified by silica-gel chromatography with a mixture of CH_2Cl_2 and MeOH as the eluent (80:20) and by reversed-phase chromatography on a Lobar RP-18 column (37×440 mm, 40–63 μm) by elution with a mixture of water and MeCN (92:8). Finally, the product was passed through an Na^+-form Dowex 50-W (100–200 mesh) cation-exchange column. ^{1}H NMR (500 MHz, D_2O, 25 °C, TMS): δ = 7.65 (s, 1H; H6), 6.23 (dd, J(H,H) = 7.0, 7.0 Hz, 1H; H1′), 4.45 (m, 1H; H3′), 4.05 (m, 1H; H4′), 3.93–3.84 (m, 2H; H5′, H5″), 3.73 (m, 1H; H$^{\alpha}$), 2.28–2.24 (s, 2H; H2′, H2″), 3.66 (s, 3H; OCH_3), 1.80 (d, J(H,H) = 1.0 Hz, 3H; CH_3), 1.22 ppm (d, J(H,H) = 7.0 Hz, 3H; CH_3); ^{31}P NMR (202 MHz, D_2O, 25 °C, TMS): δ = 6.28 ppm; ESI$^+$-MS: m/z: 408.5 $[M+H]^+$.

Thymidine 5′-(*O*-phenyl-*N*-isopropylphosphoramidate) (5): Compound **5** was obtained as a mixture of R_P and S_P diastereomers by a similar method to that described for **3**. The diastereomers were not separated and the compound was used as a diastereomeric mixture. **5**: ^{1}H NMR (500 MHz, CD_3CN, 25 °C, TMS): δ = 8.56 (s, 2×1H; NH), 7.48–7.18 (m, 2×6H; Ph and H6), 6.90 (d, J(H,H) = 8.9 Hz, 1H; NH), 6.86 (d, J(H,H) = 8.9 Hz, 1H; NH), 6.20, 6.21 (dd, J(H,H) = 6.8, 2.5 Hz, 2×1H; H1′), 4.37 (m, 2×1H; H3′), 4.25 (m, 2×2H; H5′, H5″), 4.04 (m, 2×1H; H4′), 3.8 (d, 2×1H; 3′-OH), 3.39 (m, 2H, 2×CH), 2.20 (m, 2×1H; H2′), 2.10 (m, 2×1H; H2″), 1.81 (d, J(H,H) = 1.2 Hz, 3H; CH_3), 1.80 (d, J(H,H) = 1.2 Hz, 3H; CH_3), 1.11, 1.09 ppm (d, J = 6.5 Hz, 2×6H; CH_3); ^{31}P NMR (202 MHz, [D_3]CH_3CN, 25 °C, H_3PO_4): δ = 4.68, 4.59 ppm; ESI-MS: m/z: 440.7 $[M+H]^+$.

Kinetic measurements: The reactions were carried out in sealed tubes immersed in a thermostated water bath (90.0 ± 0.1 °C). The oxonium-ion concentration of the reaction solutions was adjusted with hydrogen chloride, sodium hydroxide and formate, acetate, (*N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulfonic acid]) (HEPES), and glycine buffers. The oxonium-ion concentrations of the buffer solutions were calculated with the aid of the known pK_a values of the buffer acids under the experimental conditions.[29] Low buffer concentrations were used (30–60 mmol L^{-1}). The initial substrate concentration was approximately 0.1 mmol L^{-1}. The composition of samples withdrawn at appropriate intervals was analyzed on a Hypersil ODS 5 column (4×250 mm, 5 μm) by using mixtures of MeCN and an acetic acid/sodium acetate buffer (0.045/0.015 mol L^{-1}) containing 0.1 mol L^{-1} ammonium chloride as an eluent. Good separation of the product mixtures of **3** and **4** was obtained by using a 3 min isocratic elution with the buffer containing 0.5% MeCN, followed by a linear gradient of the eluent (over 18 min) up to 20.0% MeCN. After this, isocratic elution with the final eluent was continued. Signals were recorded on a UV detector at a wavelength of 267 nm. The observed retention times (t_R) for the products of **3** with RP HPLC (flow rate was 1 mL min^{-1}) were 6.8 (**10**), 8.6 (thymine), 14.8 (**11**), 15.0 (**9**), 20.2 (**4**), 22.9 (**8**), and 24.6 min (phenol). The observed retention times for the starting materials were 36.5 (faster-eluting diastereomer of **3**) and 37.2 min (more slowly eluting diastereomer of **3**). The reaction products were identified by the mass spectra (LCMS). A mixture of MeCN and aqueous ammonium acetate (5 mmol L^{-1}) or formic acid (0.1%) was used as the eluent. The contribution of the buffer catalysis to the observed rate constants was insignificant even at the higher buffer concentration employed (0.2 mol L^{-1}). The product of alkaline hydrolysis of **3**, assigned as the phosphoramidate diester **11**, was additionally isolated by RP HPLC (C18 column) and characterized by ^{1}H NMR and ^{31}P NMR spectroscopy.

*Diester **11***: ^{1}H NMR (500 MHz, D_2O, 25 °C, TMS): $\delta = 8.56$ (s, 1H; NH), 7.71 (d, J(H,H) = 1.2 Hz, 1H; H6), 6.27 (dd, J(H,H) = 6.5, 6.5 Hz, 1H; H1′), 4.46 (m, 1H; H3′), 4.08 (m, 1H; H4′), 3.92–3.86 (m, 2H; H5′, H5″), 3.47 (d, J(H,H) = 7.0 Hz, 1H; Hα), 2.31–2.23 (m, 2H; H2′, H2″), 1.86 (s, J(H,H) = 1.1 Hz, 3H; CH_3), 1.20 ppm (d, J(H,H) = 7.0 Hz, 3H; CH_3); ^{31}P NMR (202 MHz,D_2O, 25 °C, H_3PO_4): $\delta = 7.11$ ppm.

Calculation of the rate constants: The pseudo-first-order rate constants for the disappearance of **3** and **4** (k_{di}) were obtained by applying the integrated first-order rate equation to the time-dependent diminution of the concentration of the starting material. The first-order rate constants for the formation of compounds **8** (k_A), **9** (k_B), **10** (k_C), **11** (k_D), and **4** (k_E) from **3** and compounds **10** (k_F) and **11** (k_G) from **4** were obtained by dividing k_{di} into contributions of parallel first-order reactions on the basis of the product distribution at the early stages of the reaction.

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