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Hydrolytic reactions of 3′-*N*-phosphoramidate and 3′-*N*-thiophosphoramidate analogs of thymidylyl-3′,5′-thymidine

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The diastereomeric thiophosphoramidate analogs [(R_P)- and (S_P)-3′,5′-Tnp(s)T] **2** and the phosphoramidate analog [3′,5′-TnpT] **3** of thymidylyl-3′,5′-thymidine were prepared and their hydrolytic reactions over the pH-range 1–8 at 363.2 K were followed by RP HPLC. At pH < 6, an acid-catalyzed P–N3′ bond cleavage (first-order in [H^+]) takes place with both 3′,5′-Tnp(s)T and 3′,5′-TnpT, the former being about 12 fold more stable than the latter. At pH > 4, Tnp(s)T undergoes two competing pH-independent reactions, desulfurization (yielding TnpT) and depyrimidination (cleavage of the *N*-glycosidic bond) the rates of which are of the same order of magnitude. Also with 3′,5′-TnpT the pH-independent depyrimidination competes with P–N3′ cleavage at pH > 5.

Introduction

Phosphoramidate and phosphorothioate analogues of oligodeoxyribonucleotides belong to the most extensively studied potential antisense oligonucleotide agents.[1] Many of these modifications have been shown to be resistant toward nucleases, and to form duplexes with complementary oligonucleotide sequences.[2] For instance, oligodeoxynucleotide phosphoramidates with a 3′-*N*-bridging internucleosidic linkage have shown promising properties.[2] More recently, synthesis and characterisation of various oligonucleotide thiophosphoramidates have been reported. Gryaznov and Pongracz have described a solid support method for preparation of oligodeoxyribonucleotide thiophosphoramidates containing internucleosidic 3′NH–P(O)(S^-)–O5′ linkages.[3] Stawinski *et al.* have reported the synthesis of dinucleoside thiophosphoramidate analogues containing 3′O–P(O)(S^-)–NH5′ linkage using the *H*-phosphonate methodology.[4]

We have recently reported on a kinetic analysis of the base- and acid catalyzed hydrolysis of the diribonucleoside 3′,5′-(3′-*N*-phosphoramidate) UnpU **1**.[5] The present study is aimed at providing kinetic data on the hydrolysis of di(deoxyribonucleoside) thiophosphoramidates **2** and phosphoramidates **3**. Semi-quantitative data on the stability of deoxyribonucleoside phosphoramidates[6–9] and thiophosphoramidates[3] in aqueous acid have recently been published, along with the characterisation of the products of hydrolysis, but there are no detailed kinetic investigations available. Nevertheless, it has been shown that under acidic conditions dinucleoside 3′-*N*-phosphoramidates are hydrolysed *via* P–N bond-cleavage.[6–9] With the 3′-*N*-thiophosphoramidate analogs, however, desulfurization competes with the P–N bond cleavage.[3]

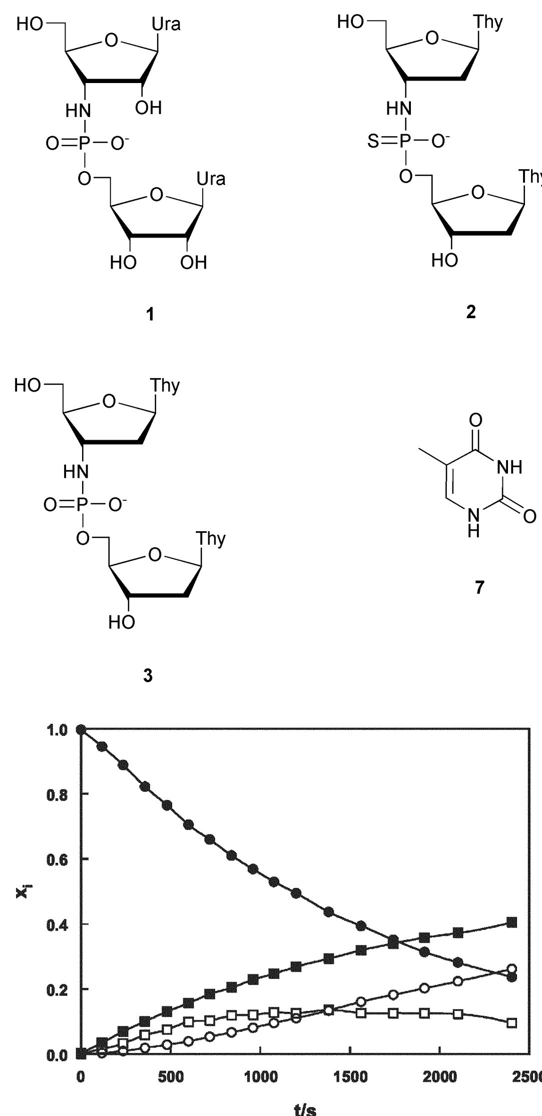

Fig. 1 Time-dependent product distribution for the hydrolysis of Tnp(s)T (**2**) in a formic acid buffer at pH 3 ([HA]/[A^-] = 0.05/0.01 mol L^{-1}; I = 0.1 mol L^{-1} with NaCl) and 363.2 K. Notation: (●) Tnp(s)T (**2**), (■) 3′-amino-3′-deoxythymidine (**4**), (□) thymidine 5′-thiophosphate (**12**) and (○) thymidine (**6**).

Results and discussion

Product distributions

The hydrolysis of 3′,5′-Tnp(s)T **2** and 3′,5′-TnpT **3** was followed over a wide pH range by determining by RP HPLC the composition of the aliquots withdrawn at appropriate intervals from the reaction solution (two examples given in Figs 1 and 2). The products were identified by spiking with authentic reference samples and by a mass spectrometric (HPLC-ESI-MS) analysis. With both 3′,5′-Tnp(s)T **2** and 3′,5′-TnpT **3**, the hydronium ion-catalysed cleavage of the P–N3′ bond (Routes A

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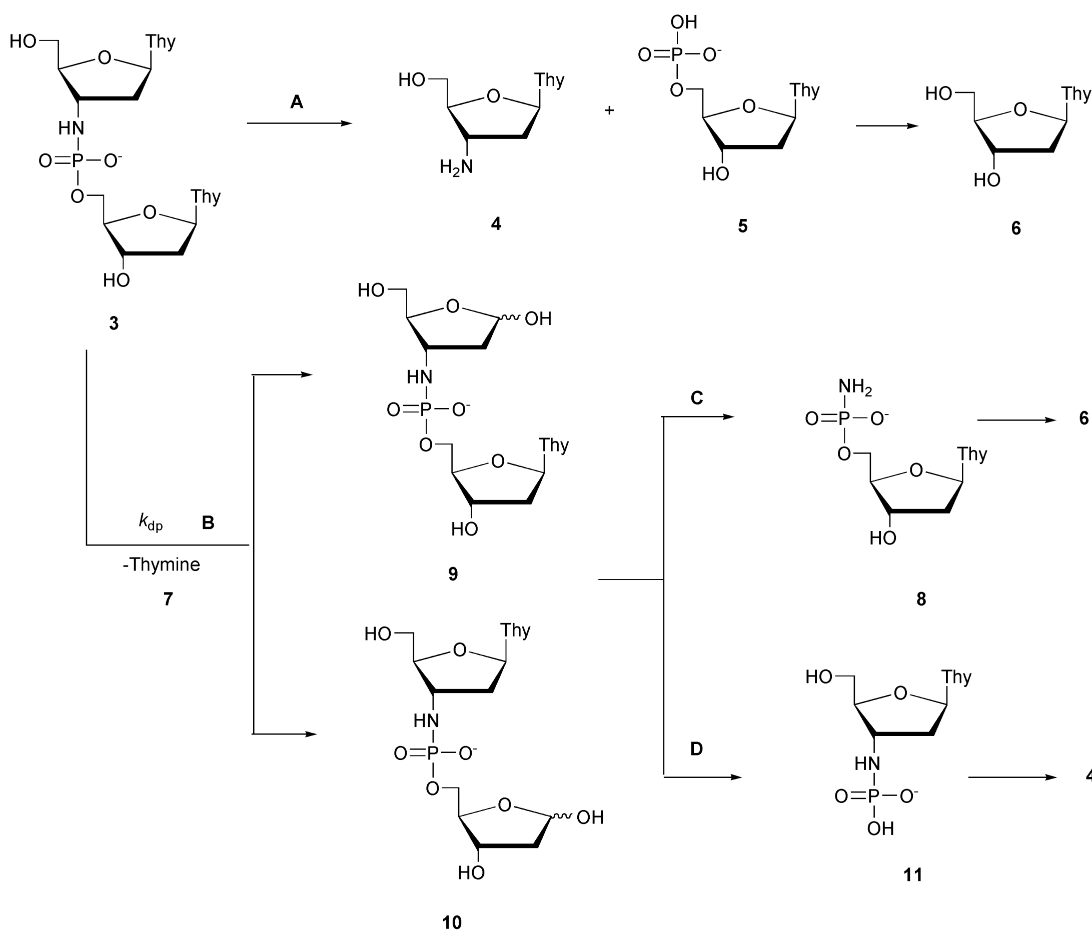


Scheme 1

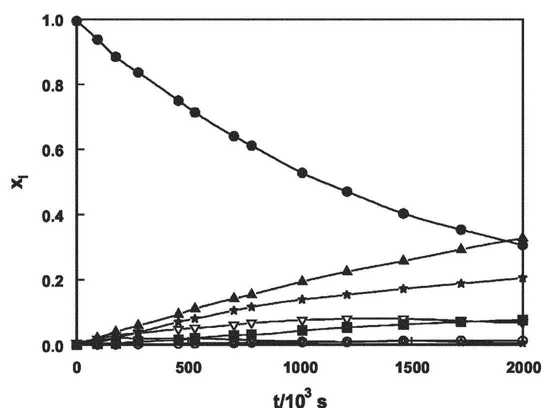


Fig. 2 Time-dependent product distribution for the hydrolysis of Tnp(s)T (**2**) in a glycine buffer at pH 8.0 ([HA]/[A$^-$] = 0.02/0.01 mol L^{-1}; I = 0.1 mol L^{-1} with NaCl) and 363.2 K. Notation: (●) Tnp(s)T (**2**), (■) 3′-amino-3′-deoxythymidine (**4**), (▽) TnpT (**3**), (★) thymidine 5′-amidothiomonophosphate (**15**), (☆) depyrimidinated product (**13/14**), (▲) thymine and (○) thymidine (**6**).

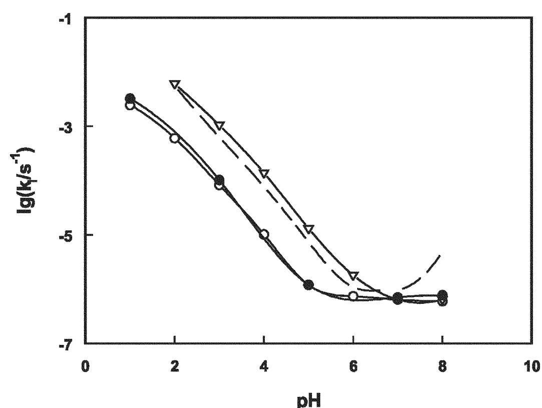


Fig. 3 pH-rate profile for the decomposition of R_P- and S_P-3′,5′-Tnp(s)T (**2**; ● and ○) and 3′,5′-TnpT (**3**; ▽) at 363.2 K. The ionic strength of the solutions adjusted to 0.1 mol L^{-1} with sodium chloride. The dotted line shows the corresponding curve for the 3′,5′-UnpU at 363.2 K.

and E in Schemes 1 and 2, respectively) was found to be the predominant reaction under acidic conditions (pH < 4), whereas under less acidic and neutral conditions the pH-independent cleavage of the *N*-glycosidic linkages (Routes B and F in Schemes 1 and 2, respectively) and desulfurisation of the thiophosphoramidate moiety of Tnp(s)T (Route G in Scheme 2) become the main pathways of degradation.

The hydronium ion-catalysed hydrolysis of the P–N3′ bond of the phosphoramidate 3′,5′-TnpT **3** at pH 1–4 (Figs 3 and 4) yields a mixture of 3′-aminonucleoside **4** and thymidine 5′-phosphate **5** (route A). Under less acidic conditions the released phosphate monoester **5** is partly dephosphorylated during the kinetic run (yielding thymidine **6**). At pH > 5 the pH-independent cleavage of the *N*-glycosidic bond (release of thymine, route B) competes with P–N3′ cleavage and on going to less acidic and neutral conditions it becomes gradually the only reaction detected. During the hydrolysis of **3** at pH 8, thymine **7** and thymidine amidomonophosphate **8** (m/z = 320 was observed) were formed as the main chromophoric products, in equal amounts with each other, and besides these only a minor amount (4%) of 3′-amino-3′-deoxythymidine **4** was found to accumulate. The 5′-amidomonophosphate **8** is proposed to be formed by elimination of the carbohydrate moiety from the depyrimidination product **9**. The corresponding degradation of the other major depyrimidination product, **10**, would yield the 3′-*N*-phosphoramidate **11**, which can not be directly distinguished from **8** by the HPLC-MS analysis. However, **11** is

Scheme 2

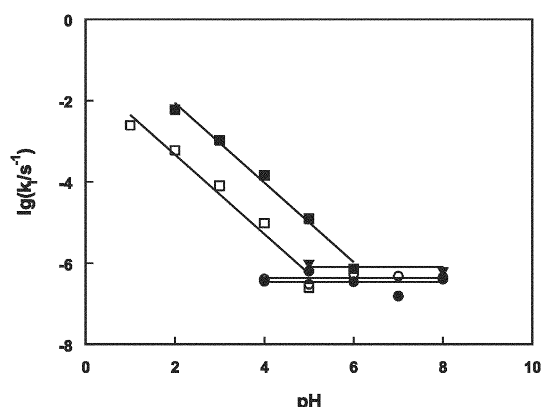


Fig. 4 pH-rate profiles for the partial reactions involved in the hydrolysis of Tnp(s)T (**2**) and TnpT (**3**) at 363.2 K. Notation: Route A (■) and Route B (▼) in Scheme 1 and (□) Route E, (●) Route G and (○) Route F in Scheme 2.

expected to be more labile than **8** and become readily dephosphorylated to yield 3′-amino-3′-deoxythymidine **4**.[10] While the accumulation of **4** remained low over the kinetic run, it seems that the thymine moiety is released more readily from the 3′-aminothymidine moiety than from the 5′-*O*-esterified thymidine moiety of **3**.

The intermediary accumulation of **9/10** during the hydrolysis of **3** at pH 8 remained too low (< 2%) to be quantified by HPLC with UV-detection. Nevertheless, an m/z value ($[M-H]^-$ = 436.3) corresponding to the molecular ion of **9/10** could be observed by the HPLC-ESI-MS analysis of the samples. By contrast, with the thiophosphoramidates **2**, the corresponding intermediate **13/14** accumulated at pH 8 to a much higher extent, close to 20% of the hydrolysis products (see discussion below).

The R_P- and S_P-diastereomers of the thiophosphoramidate **2** were separated from each other by reversed phase chromatography and their hydrolytic reactions were followed separately, although the absolute configurations of the isomers were not assigned. The two diastereomers showed similar reactivities with each other in all the reactions studied. The acid catalysed hydrolysis, which is the predominant reaction of Tnp(s)T **2** at pH < 4, yields a mixture of 3′-amino-3′-deoxythymidine **4** and thymidine 5′-thiomonophosphate **12** (route E in Scheme 2). The thiomonophosphate **12**, being more than 2 orders of

magnitude more labile than its phosphate analog **5**,[11] is further dephosphorylated to thymidine **6**. Fig. 1 shows, as an example, the time-dependent product distribution observed for hydrolysis of **2** at pH 3.

At pH 4–5, two pH-independent hydrolytic reactions of **2**, namely depyrimidination of either of the thymidine moieties (route F) and desulfurization of the thiophosphoramidate moiety (yielding 3′,5′-TnpT **3**; route G), compete with the acid-catalysed P–N3′ cleavage. Thymidine 5′-thiophosphate **12** was not detected to accumulate in this pH region, due to its relatively fast subsequent dephosphorylation[11] to thymidine. At pH > 5, the desulfurisation and depyrimidination become faster than the P–N3′ cleavage (Fig. 4). The product distribution of the hydrolysis under neutral conditions is rather complicated (Fig. 2), while in addition to TnpT **3** and thymine **7**, the mononucleosidic compounds **4**, **5** and **6**, thymidine 5′-amidothiomonophosphate **15** (up to 30% of the products at pH 8) and even the depyrimidinated compound **13/14** (up to 20% of the products at pH 7) accumulate. The appearance of the depyrimidinated product was clearly shown by HPLC-MS analysis, but only a single chromatographic peak corresponding to the appropriate molecular ion ($[M-H]^- = 452.2$) was observed. No direct evidence was obtained as to whether this represents a single compound or whether the possible isomeric compounds appear unseparated in the chromatogram (in fact, both **13** and **14** could appear also as *α*- and *β*-anomers, or possibly even partly as the corresponding pyranose isomers). Anyway, the isomeric **13** and **14** are expected to give different monoesters as hydrolysis products, thymidine 5′-amidothiophosphate **15** being formed from **13** and 3′-aminothymidine 3′-*N*-thiophosphoramidate **16** from **14** ($m/z = 336$ for $[M-H]^-$ of both **15** and **16**). Even for the latter step, only one chromatographic peak corresponding to the expected molecular ion was observed. However, this is to be expected, since the dephosphorylation involving cleavage of the P–N linkage is fast and there is some evidence[11] that the thiosubstitution (*cf.* **16**) still facilitates this reaction (a thiometaphosphate ion is a more stable intermediate than the metaphosphate anion). Accordingly, **16** is proposed to be too labile to accumulate during the performed kinetic run,[10] since it is expected to readily dethiophosphorylate to aminothymidine **4**. The latter was indeed observed to accumulate, but to a rather low extent (< 10% for the reaction at pH 8, see Fig. 2). It must be also noted that **4** can also be formed by other pathways, for example by degradation of TnpT **3** (Route G) and its appearance does not give exclusive evidence of the occurrence of hydrolysis through **14**. The mononucleosidic compounds may also be partly depyrimidinated during the kinetic run, which further complicates the analysis. Nevertheless, the analysis suggests that a major part of the depyrimidination of **2** takes place by release of thymine from the 3′-*N*-aminothymidine moiety. The accumulated compound showing the molecular ion $m/z = 336.0$ ($[M-H]^-$) is, tentatively, assigned as thymidine 5′-amidothiomonophosphate **15**, the formation of which may take place *via* intermediate **13**. Compound **15** is not markedly dephosphorylated or depyrimidinated during the course of the hydrolysis of **2** at pH 8.

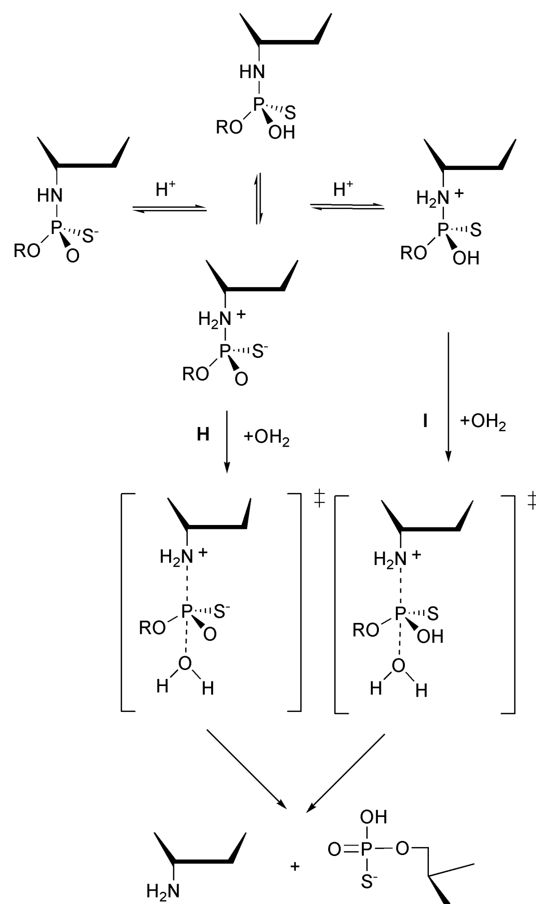
The pH-rate profiles

Fig. 3 shows the pH-rate profiles for the decomposition of the diastereomers of the thiophosphoramidate **2** in comparison with those of the phosphoramidates **1** and **3**. Fig. 4 shows the pH-rate profiles for the partial reactions involved in the hydrolysis of 3′,5′-Tnp(s)T (**2**) and TnpT (**3**). The rate of the acid-catalysed P–N3′ cleavage of 3′,5′-TnpT is close to that of 3′,5′-UnpU[5] **1**, TnpT being 1.7 times more reactive than UnpU. The thiosubstitution, however, significantly decreases the rate of this reaction, while 3′,5′-Tnp(s)T **2** is about 12 times more stable than 3′,5′-TnpT **3** in the pH-region 2–5. The reactivities of the two diastereomers of the thiophosphoramidate, S_P- and R_P-forms, are equal with each other.

On going to slightly acidic and neutral conditions (pH > 5), the pH-independent desulfurisation (route G in Scheme 2) and depyrimidination (route F) become the only reactions detected for Tnp(s)T. The rates of these two reactions are very close to each other (Fig. 4). The depyrimidination of the phosphoramidate **3** is slightly faster than that of the thiophosphoramidate **2**.

Reaction mechanisms

We have previously suggested[5] that the acid-catalysed P–N3′ bond rupture of UnpU **1** differs mechanistically from the typical phosphoester hydrolysis reactions of RNA fragments, since it does not involve participation of the neighbouring 2′-hydroxyl function as a nucleophile. The similarity of the pH-rate profiles of the 2′-deoxynucleoside phosphoramidate 3′,5′-TnpT and the ribonucleoside analog 3′,5′-UnpU gives further support to this interpretation. Furthermore, also with Tnp(s)T, the P–N3′ bond cleavage shows a linear first-order dependence on the hydronium ion concentration, and even the 12 fold lower reactivity of Tnp(s)T compared to that of TnpT (Figs 3 and 4) is consistent with the same mechanistic scheme. Under slightly acidic conditions (pH 2–6) the prevailing ionic form of the compounds is in all likelihood the monoanionic *N*,*O*-disubstituted phosphoramidate (**1**, **3**)[12] or thiophosphoramidate (**2**), but the reactive tautomer is the neutral molecule. The preferred site of protonation of the phosphoramidates is not exclusively clear,[13] but it seems in any case likely that the reactive tautomer of the phosphoramidate is the *N*-protonated zwitterionic species, from which the leaving group may depart as a neutral amine (Scheme 3). In all likelihood the P–N bond rupture proceeds, as earlier discussed in the case of UnpU,[5] by a mechanism involving participation of a water molecule in the transition state. Thus, the formation of the new P–O bond (by



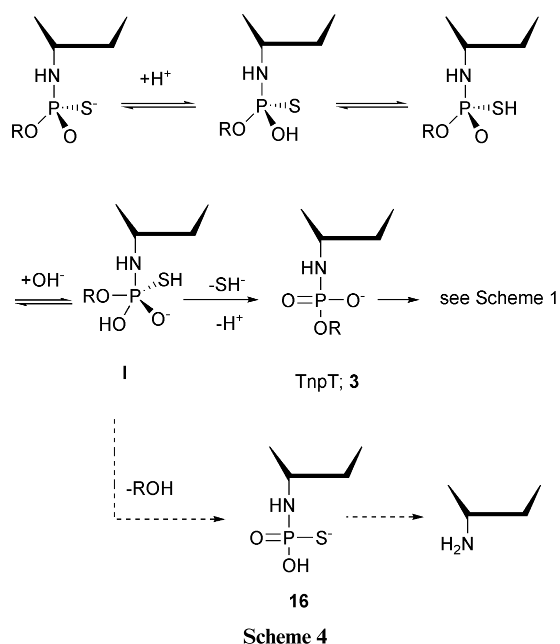
Scheme 3

attack of water) is already long advanced during the rupture of the P–N3′-linkage and no free alkyl metaphosphate (or thiometaphosphate) ion is formed. This type of mechanism has been earlier described for the solvolysis of phosphoramidic acid,[14] *O*-alkylphosphoramidates[15] and *N*-alkylphosphoramidates[16] in aqueous alcohol. The entropy of activation for the hydrolysis of TnpT at pH 2 was determined as −63 ± 4 J K^{-1} mol^{-1} (five experimental points at 298.2 < *T* < 363.2 K), which is equal to that determined[5] for UnpU (−65 J K^{-1} mol^{-1}). Being clearly negative, these values are inconsistent with a unimolecular mechanism, but on the other hand, they are actually not as negative as expected for a reaction with a purely bimolecular transition state. Rather, they represent borderline values of those expected for a unimolecular and bimolecular hydrolysis.

The replacement of a nonbridging phosphoryl oxygen with sulfur retards the hydronium ion-catalyzed hydrolysis of TnpT, the kinetic thio-effect (k_{PO}/k_{PS}) being 12, as noted above. This effect may well be due to the higher acidity of the thiophosphoramidate moiety compared to the phosphoramidate, *i.e.* the less ready protonation of the thiophosphoramidate diester to the reactive neutral (zwitterionic) form. For comparison, the pK_{a1} and pK_{a2} values of thiophosphoric acid are 0.5 and 1.8 units lower, respectively, than those of phosphoric acid.[17,18] Unfortunately, as far as we know no comparable pK_a values for thiophosphoramidates have been reported.

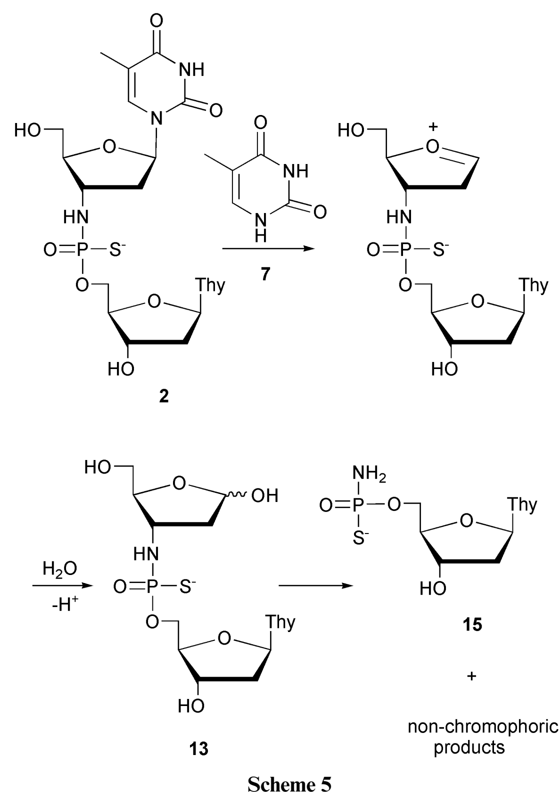
The pH-rate profile of the hydrolysis of Tnp(s)T shows slight curvature towards a constant value on going below pH 2 (Fig. 3). In principle, this kind of curvature could result from the thiophosphoramidate having a pK_a value in the region 1–2 and the monocationic species (at pH < pK_a) being unreactive in P–N3′ bond hydrolysis. However, the present data does not reveal whether the water catalysed hydrolysis of the neutral (zwitterionic) thiophosphoramidate (route H in Scheme 3) is accompanied by hydronium ion-catalysed (route I) hydrolysis at very low pH, *i.e.* on passing the pK_a of the thiophosphoramidate. Anyway, it may be noted for comparison that the rate profile of the hydrolysis of the P–N3′ bond of UnpU[5] does not show any deviation from the first-order dependence of the rate on $[H^+]$ in the pH range from −1 to 4.

The rate of the desulfurization of 3′,5′-Tnp(s)T is pH-independent at pH 4–8 (Fig. 4), which means that the reaction takes place either by attack of a water molecule on monoanionic thiophosphoramidate linkage or by attack of a hydroxide ion on a neutral thiophosphoramidate (Scheme 4). The sulfur ligand may depart as hydrogen sulfide ion after protolytic rearrangement, which leads to the observed intermediary accumulation of the phosphoramidate TnpT **3**. In principle, the breakdown of the pentacoordinated intermediate **I** could alternatively take place *via* departure of the 5′-esterified thymidine or even by departure of the 3′-aminothymidine. However, although the latter pathways cannot be completely excluded, they may not be proposed to be of significant importance. The accumulation of the appropriate monomeric products expected to be released by these pathways remains relatively low and the same products can be formed even by competing pathways. For example, a significant part of the formation of the aminothymidine (**4**) in all likelihood takes place by degradation of the TnpT formed *via* P–S bond cleavage of intermediate **I**. In any case, the desulfurization is proposed to be faster than the cleavage P–O5′ linkage, remembering that hydrogen sulfide ion is less basic than alkoxide and hence a better leaving group under the experimental conditions (pK_a's of H_2S and the 5′-OH are 6.5 and 13–15, respectively).[18,19]



Scheme 4

The rate of the hydrolysis of the *N*-glycosidic bond of TnpT and Tnp(s)T is pH-independent over the pH-region 4–8 (Fig. 4), analogously to the degradation of thymidine.[20] The uncatalysed depyrimidination of thymidine has been suggested to involve a unimolecular cleavage of the *N*-glycosidic linkage, resulting in formation of the cyclic glycosyl oxocarbenium ion derived from the sugar moiety (Scheme 5).[20] This is in contrast to the acid-catalysed cleavage, which most likely takes place by opening of the sugar ring after protonation of the O4′-oxygen.[21] The uncatalysed degradation of thymidine has been proposed[20] to involve a rate-limiting cleavage of the C–N bond followed by rapid protonation of the anionic base moiety and nucleophilic attack of a water molecule on the oxocarbenium ion. The formed depyrimidinated products of **2** and **3** (**13/14** and **9/10**, respectively) undergo readily a decomposition of the depyrimidinated sugar ring and release the appropriate monomeric nucleoside phosphor- or thiophosphoramidates. The rate constants obtained for the depyrimidination of TnpT and Tnp(s)T are comparable to the values previously reported[22] for the uncatalysed cleavage of the C1′–N bond of thymidine ($k = 1 \times 10^{-6}$ s^{-1} at pH 3–7 at 95 °C).



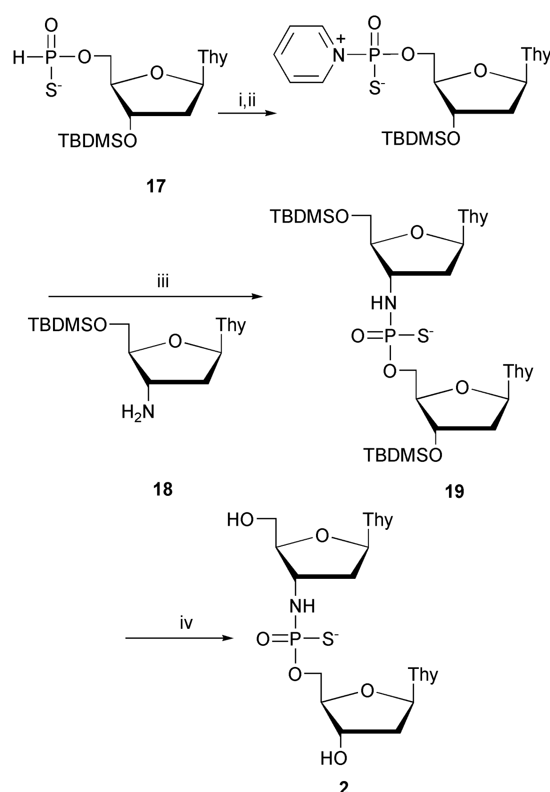
Scheme 5

Experimental

Materials

The NMR spectra were recorded on a Bruker AM 200 or a Jeol 400 spectrometer. The ^{1}H NMR chemical shifts (at 300 K) were referred to internal TMS, and the ^{31}P NMR shifts (202 MHz, 300 K) to external orthophosphoric acid. The mass spectra were acquired using a Perkin Elmer Sciex API 365 triple quadrupole LC/MS/MS spectrometer.

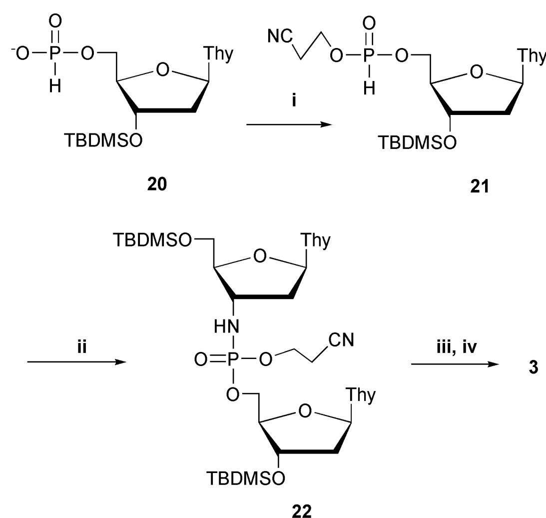
The organic solvents were dried by refluxing over calcium hydride and distilled. The solid starting materials were dried by coevaporation with the anhydrous solvent. Thymidine, thymine and thymidine 5′-phosphate were products of Sigma. 3′-Azido-3′-deoxythymidine was prepared as described previously[23] and converted to 3′-amino-3′-deoxythymidine by treatment with triphenylphosphine in anhydrous pyridine followed by addition of aqueous ammonia.[24] Dinucleoside thiophosphoramidate analogs of thymidylyl-3′,5′-thymidine (**2**) were prepared using *H*-phosphonate methodology, in which nucleoside *H*-phosphonothioate **17** was converted into a pyridine adduct of a nucleoside thiometaphosphate followed by reaction with 5′-protected 3′-amino-3′-deoxythymidine **18** (Scheme 6), as described previously by Stawinski *et al.*[4] Dinucleoside phosphoramidate analog of thymidylyl-3′,5′-thymidine (**3**) was obtained by oxidative amination of the appropriately protected thymidine 5′-(*H*-phosphonate) 2-cyanoethyl ester **21** in the presence of 3′-amino-3′-deoxythymidine (Scheme 7). The same reaction sequence has previously been used for the synthesis of oligoribonucleotide phosphoramidates on a solid support.[6,7]



Scheme 6 *Reagents and conditions*: (i) TMSCl, py, (ii) I_2, (iii) Et_3N, py, (iv) TBAF–THF.

The (R_P)- and (S_P)-thiophosphoramidate analogs of (3′-amino-3′-deoxythymidylyl)-3′,5′-thymidine (2)

Nucleoside *H*-phosphonothioate[25] **17** (0.25 g, 0.57 mmol) and trimethylsilyl chloride (0.19 g, 1.72 mmol) were dissolved in 10 mL of anhydrous pyridine. After stirring for 5 min at RT, iodine (0.22 g, 2.87 mmol) was added. After an additional 7 min of stirring, 5′-*O*-(*tert*-butyldimethylsilyl)-3′-amino-3′-deoxythymidine **18** (0.20 g, 0.57 mmol) and triethylamine (0.40 ml) in pyridine (2 ml) were added (Scheme 6). The crude product was isolated by a conventional aqueous work up, and purified on a silica gel column eluted with a mixture of dichloromethane and methanol (80 : 20%, v/v). The *tert*-butyldimethylsilyl protected thiophosphoramidate (**19**) was dissolved in 1 mol L^{-1} solution of tetrabutylammonium fluoride (0.265 g, 1.02 mmol) in tetrahydrofuran (1 mL), and the solution was stirred for 16 h at room temperature. The mixture was evaporated to dryness and the diastereomers were purified and separated from each other by reversed phase chromatography on a Lobar RP-18 column (37 × 440 mm, 40–63 µm) eluting with a mixture of water and acetonitrile (90 : 10%, v/v). Finally, the product was passed through a Na^{+}-form Dowex 50-W (100–200 mesh) cation exchange column. **2** (the faster eluted diastereomer): ^{31}P NMR: δ_P (202 MHz, D_2O) = 53.63. ^{1}H NMR: δ_H (400 MHz, D_2O) = 7.71 (s, 1H, H6), 7.70 (s, 1H, H6), 6.15 (t, 1H, H1′, *J* = 6.62), 5,80 (dd, 1H, H1′, *J* = 7.05, *J* = 2.14), 4.43 (m, 1H, H3′), 4.00 (m, 2H, H4′), 3.93 (m, 2H, H5′, H5″), 3.83–3.79 (m, 1H, H5′), 3.71 (m, 1H, H5″), 3.70 (m, 1H, H4′), 3.63–3.54 (m, 1H, H3′), 2.37–2.13 (m, 4H, H2′, H2″), 1.73 (s, 3H, CH_3), 1.70 (s, 3H,CH_3). ESI$^-$-MS: *m*/*z* 560.3 [M − H]$^-$. **2** (The slower eluted diastereomer): ^{31}P NMR: δ_P (202 MHz, D_2O) = 53.59. ^{1}H NMR: δ_H (400 MHz, D_2O) = 7.62 (s, 2H, 2 × H6), 6.16 (t, 1H, H1′, *J* = 6.62), 5.90 (dd, 1H, H1′, *J* = 6.83, *J* = 3.63), 4.42 (m, 1H, H3′), 4.00 (m, 1H, H4′), 4.00–3.95 (m, 1H, H5′), 3.91–3.86 (m, 1H, H5″), 3.81 (m, 1H, H5″), 3.72 (s, 2H, H3′, H4′), 3.76–3.68 (m, 1H, H5″), 2.33–2.18 (m, 4H, H2′, H2″), 1.80 (s, 3H, CH_3), 1.72 (s, 3H, CH_3). ESI$^-$-MS: *m*/*z* 560.3 [M − H]$^-$.



Scheme 7 *Reagents and conditions*: (i) $CNCH_2CH_2OH$, DPCP, CH_3CN–pyridine, (ii) **4**, CCl_4, Et_3N, (iii) NH_3–MeOH, (iv) TBAF–THF.

(3′-Amino-3′-deoxythymidylyl)-(3′→5′)-thymidine (3)

Compound **3** was prepared, analogously with the previously described method,[6,7] by reacting the triethylammonium salt of 5′-*O*-(*tert*-butyldimethylsilyl)thymidine 5′-hydrogenphosphonate **20** (0.52 g, 1.24 mmol) with cyanoethanol (76 µL, 1.11 mmol) and DPCP (304 µL, 1.49 mmol) in a mixture of anhydrous pyridine (4.0 mL) and acetonitrile (6.0 mL) (Scheme 7). The formed cyanoethyl ester **21** was not isolated. After 2 h of stirring at room temperature, 3′-amino-3′-deoxythymidine **4** (0.47 g, 1.34 mmol), carbon tetrachloride (5.0 mL) and triethylamine (0.22 mL) were added and the mixture was left to stand overnight. The mixture was then poured into 90 mL of dichloromethane and washed with saturated aqueous NaCl (3 × 50 mL). The organic layer was dried with Na_2SO_4 and concentrated. The residue was purified on a silica gel column using a mixture of dichloromethane and methanol as eluent (92 : 8%, v/v) to give the fully protected product **22**. The cyanoethyl group was removed by treatment with saturated

methanolic ammonia (3 mL) and the nucleotidic product was purified using silica gel chromatography using a mixture of dichloromethane and methanol as eluent, the methanol content of which was increased stepwise from 0 to 30%. The *tert*-butyldimethylsilyl protecting groups were removed with 1 mol L^{-1} tetrabutylammonium fluoride in tetrahydrofuran (1 mL). The mixture was evaporated to dryness and the product was purified by reversed phase chromatography on a Lobar RP-18 column (37 × 440 mm, 40–63 μm) eluting with a mixture of water and acetonitrile (92 : 2%, v/v). Finally, the product was passed through a Na^+-form Dowex 50-W (100–200 mesh) cation exchange column. ^{31}P NMR δ_P (202 MHz, D_2O) = 6.81. ^{1}H NMR δ_H (400 MHz, D_2O) = 7.70 (s, 1H, H6), 7.68 (s, 1H, H6), 6.15 (t, 1H, H1′, *J* = 6.4), 5.80 (dd, 1H, H1′, *J* = 7.0, *J* = 2.6), 4.43 (m, 1H, H3′), 3.96–3.90 (m, 2H, H4′, H5′), 3.83–3.78 (m, 2H, H5″, H5′), 3.72–3.67 (m, 2H, H4′, H5″), 3.46 (m, 1H, H3′), 2.34–2.28 (m, 1H, H2′), 2.26–2.15 (m, 3H, H2′, 2 × H2″), 1.70 (s, 2 × 3H). ESI$^-$-MS: *m/z* 544.9 $[M - H]^-$.

Kinetic measurements

The reactions were carried out in sealed tubes immersed in a thermostated water bath (363.2 K), the temperature of which was adjusted within ± 0.1 K. The hydronium ion concentration of the reaction solutions was adjusted with hydrogen chloride and formate, *N*-morpholinoethanesulfonic acid (MES), triethanolamine, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) and glycine buffers. The pH values of the buffer solutions were calculated from the literature data of the pK_a values of the buffer acids under the experimental conditions.[26] Low buffer concentration was used (30–60 mM). The initial substrate concentration in the kinetic runs was *ca.* 0.1 mM. The composition of the samples withdrawn at appropriate intervals was analyzed by HPLC on a Hypersil ODS 5 column (4 × 250 mm, 5 μm) using as eluent an acetic acid/sodium acetate buffer (0.045/0.015 mol L^{-1}) containing 0.1 mol L^{-1} ammonium chloride. A good separation of the product mixture was obtained, when a 5 min isocratic elution was followed by a linear gradient (32 min) up to 5% MeCN. The observed retention times (t_R/min) for the hydrolytic products of **2** (flow rate 1 mL min^{-1}) were as follows: 10.4 (**5**), 11.3 (**7**), 12.0 (**4**), 20.7 (**15**), 25.1 (**6**) 26.5 (**13/14**) 15.0 (**12**). The observed retention times for diastereomers of Tnp(s)T **2** were 37.8 and 48.0 min. The products were identified by spiking with authentic reference samples and the characterizations were further ascertained by LC/ESI-MS analysis. In the HPLC/MS analysis, a mixture of acetonitrile and 5 mmol L^{-1} aqueous ammonium acetate was used as an eluent.

Calculation of the rate constants

The pseudo-first-order rate constants (k_{dec}) for the decomposition of **2** and **3** were obtained by applying the integrated first-order rate equation to the time-dependent diminution of the HPLC peak area of the starting material.

The first-order rate constants (k_{ds}) for the desulfurization of 3′,5′-Tnp(s)T (pH 5–7, route G in Scheme 2), were obtained by least-squares fitting to eqn. (1), where k_{dec} is the first-order rate constant for the disappearance of Tnp(s)T and k_1 the first-order rate constant for the disappearance of **3**. $[Tnp(s)T]_0$ stands for the initial concentration of the starting material and $[TnpT]_t$ for the concentration of **3** at moment *t*.

$$\frac{[TnpT]_t}{[Tnp(s)T]_0} = \frac{k_{ds}}{k_1 - k_{dec}}\left[\exp(-k_{dec}t) - \exp(-k_1 t)\right] \quad (1)$$

The first-order rate constants (k_{dp}) for the depyrimidination of 3′,5′-Tnp(s)T (pH 6–8, route F in Scheme 2), were obtained by least-squares fitting to eqn. (2), where k_{dec} is the first-order rate constant for the disappearance of Tnp(s)T and k_2 the first-order rate constant for the disappearance of **13/14**. $[Tnp(s)T]_0$ stands for the initial concentration of the starting material and $[\mathbf{13} + \mathbf{14}]_t$ for the observed concentration of **13** and **14** at moment *t*.

$$\frac{[\mathbf{13}+\mathbf{14}]_t}{[Tnp(s)T]_0} = \frac{k_{dp}}{k_2 - k_{dec}}\left[\exp(-k_{dec}t) - \exp(-k_2 t)\right] \quad (2)$$

At pH 4–5, where **13/14** does not accumulate, the first-order rate constants (k_{dp}) for the depyrimidination of 3′,5′-Tnp(s)T (route F in Scheme 2), were calculated by eqn. (3), by bisecting k_{dec} to the rate constants of parallel first-order reactions on the basis of the product distribution at the early stages of the reaction *i.e.*, under conditions where the formation of thymine from the hydrolysis products of **2** may be neglected. $[Thymine]_t$ and $[Tnp(s)T]_t$ stand for the concentration of thymine and **2**, respectively, at moment *t*, and $[Tnp(s)T]_0$ denotes the initial concentration of the starting material.

$$k_{dp} = \frac{[Thymine]_t}{[Tnp(s)T]_0 - [Tnp(s)T]_t} k_{dec} \quad (3)$$

Under conditions, where TnpT does not accumulate (pH 4) or the decomposition of TnpT is slow compared to its formation (pH 8), the first-order rate constants (k_{ds}) for the desulfurization of 3′,5′-Tnp(s)T (route G in Scheme 2) were calculated by eqn. (4) by bisecting k_{dec} to the rate constants of parallel first-order reactions on the basis of the product distribution at the early stages of the reaction *i.e.*, under conditions where the subsequent reactions TnpT may be neglected. $[Tnp(s)T]_0$ denotes the initial concentration of the starting material, $[TnpT]_t$ and $[Tnp(s)T]_t$ stand for the concentrations of **3** and **2**, respectively, at moment *t* and k_{dec} is the first-order rate constant for the disappearance of **2**.

$$k_{ds} = \frac{[TnpT]_t}{[Tnp(s)T]_0 - [Tnp(s)T]_t} k_{dec} \quad (4)$$

Eqn. (5) was applied to obtain the rate constants, k_{cl}, for the competing acid-catalyzed hydrolysis and pH-independent depyrimidination (k_{dp}) of Tnp(s)T.

$$k_{cl} = k_{dec} - k_{ds} - k_{dp} \quad (5)$$

At pH 5–6, the first-order rate constants k_{dp} for the depyrimidination of 3′,5′-TnpT (**3**) (route B in Scheme 1) were calculated by eqn. (6), analogously to 3′,5′-Tnp(s)T (**2**).

$$k_{dp} = \frac{[Thymine]_t}{[TnpT]_0 - [TnpT]_t} k_{dec} \quad (6)$$

Eqn. (7) was applied to obtain the rate constants, k_{cl}, for the competing acid-catalyzed hydrolysis of TnpT.

$$k_{cl} = k_{dec} - k_{dp} \quad (7)$$

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10 For comparison, the ribonucleoside analog 3′-deoxyuridine 3′-*N*-phosphoramidate (3′-Unp) was shown[5] to be hydrolytically unstable, being readily dephosphorylated to yield 3′-aminouridine. The dephosphorylation involving the P–N bond cleavage was estimated[5] to be as much as 5 orders of magnitude as fast as the corresponding dephosphorylation involving a P–O bond cleavage. The rate constant for the dephosphorylation of 3′-Unp could be determined only for alkaline conditions, the first order rate constant being 8.1×10^{-5} s^{-1} at pH 10.4 and 363.2 K (I = 0.1 mol L^{-1}).[5] The dephosphorylation of 3′-Unp proceeds by a dissociative mechanism without participation of the 2′-OH group. Accordingly, we propose by analogy that thymidine 3′-*N*-phosphoramidate **11** would be readily dephosphorylated to 3′-aminothymidine **4**, if formed during the hydrolysis of **3** at pH 8. However, it must be noted that **10** may possibly also degrade by some other routes to yield small amounts of free thymine and all the possible reactions cannot be distingushed on the basis of the present data.

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