Synthesis and chemical and enzymatic reactivity of thymidine 3′-*O*- and 5′-*O*-phosphorofluoridothioates

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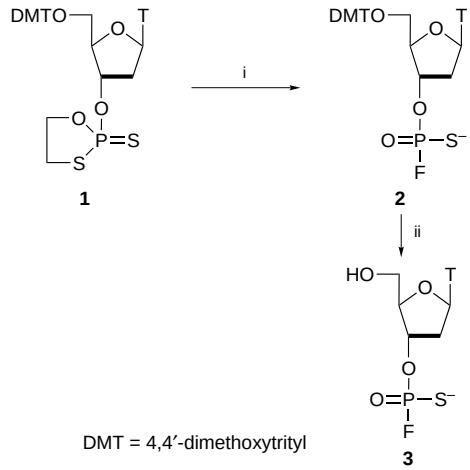
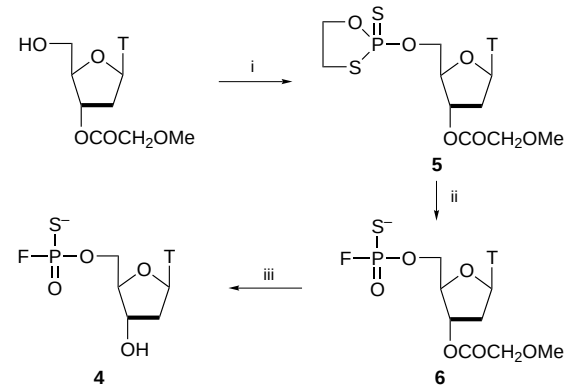
5′-*O*- or 3′-*O*-Protected thymidine 3′-*O*- or 5′-*O*-(2-thiono-1,3,2-oxathiaphospholanes) react with triethylammonium fluoride in a presence of DBU and furnish, after deprotection, thymidine 3′-*O*- and 5′-*O*-phosphorofluoridothioates; the latter undergoes stereoselective hydrolysis by *snake venom* phosphodiesterase.

Within the course of our studies on the development of oxathiaphospholane methodology for phosphorylation,[1] phosphorothioylation[2] and phosphorodithioylation[3] of biomolecules we have found that the endocyclic P–S bond of a 2-thio-substituted 1,3,2-oxathiaphospholane ring attached to nucleoside moiety can be easily broken in the presence of DBU by fluoride ions leading, after spontaneous episulfide elimination, to 3′-*O*-methoxyacetyl nucleoside 5′-*O*-phosphorofluoridothioates or 5′-*O*-dimethoxytrilyl nucleoside 3′-*O*-phosphorofluoridothioates.[4,5] Thus, 5′-*O*-dimethoxytritylthymidine 3′-*O*-(2-thiono-1,3,2-oxathiaphospholane) **1** (ratio of diastereomers *ca.* 1:1), after treatment with triethylammonium fluoride‡ in a presence of DBU gives 5′-*O*-dimethoxytritylthymidine 3′-*O*-phosphorofluoridothioate **2**§ in 78% yield (Scheme 1). The dimethoxytrityl protecting group was removed by the treatment of **2** with 80% AcOH. The final product, thymidine 3′-*O*-phosphorofluoridothioate **3**,¶ was purified by anion-exchange chromatography on Sephadex A-25 using triethylammonium hydrogen carbonate buffer (pH 7.5, 0.02–0.5 M) as eluent. Pure **3** has also been obtained from **1** in 68% yield in a one-pot synthesis. Reaction of **1** with triethylammmonium fluoride in a presence of DBU is, unlike the 1,3,2-oxathiaphospholane ring-opening condensation with alcohols[2] or amines,[6] a non-stereospecific process. Starting from a mixture of partially separated diastereomers of **1** [ratio 73:27, δ_P (CD_3CN), 105.78 and 105.83] diastereomers of **2** were obtained in a ratio of 54:46 [δ_F (CD_3CN), −30.06 and −29.96]. The epimerisation at phosphorus was not unexpected in the light of published earlier results on the stereochemistry of nucleophilic substitution at phosphorus by fluoride ion.[7,8] Also, thymidine 5′-*O*-phosphorofluoridothioate **4** has been synthesized according to the reaction sequence presented in the Scheme 2. Phosphitylation of 3′-*O*-methoxyacetylthymidine with *N*,*N*-diisopropylamino-1,3,2-oxathiaphospholane[2] in a presence of 1*H*-tetrazole, followed by sulfurization yielded oxathiaphospholane **5**. Reaction of **5** with triethylammonium fluoride–DBU furnished intermediate 3′-*O*-methoxyacetylthymidine 5′-*O*-phosphorofluoridothioate **6** which subsequently was deprotected with a concentrated solution of ammonia providing, after purification on Sephadex A-25, the final product **4**** in 75% yield. The phosphorofluoridothioate monoesters **3** and **4** were hydrolytically stable even under basic conditions (conc. ammonia, room temp., 1 h), as proven by ^{31}P NMR assay. Similarly, the resistance of **3** and **4** towards methanolysis, attempted in the presence of triethylamine or pyridine, has been observed. Attempts at internucleotide bond formation in reaction of **2** with 3′-*O*-acetylthymidine in the presence of strong bases such as Bu^{t}OK, DBU and 2-*tert*-butylimino-2-diethylamino-1,3-dimethyl-1,3,2-diazaphosphinane (BEMP) have failed. Reactions were performed in DMF and their progress was followed by ^{31}P NMR spectroscopy. Under these conditions, even after 18 h, formation of dithymidylyl (3′,5′)phosphorothioate ($T_{PS}T$) was not observed. Instead, both **3** and **4** underwent intramolecular cyclization in the presence of an excess of Bu^{t}OK (five-fold molar excess) leading to thymidine cyclic (3′,5′)phosphorothioate (cTMPS). Similarly, as previously observed[9] for Bu^{t}OK-catalyzed cyclization of nucleoside 5′-*O*-*p*-nitrophenyl phosphorothioates, in the reactions of phosphorofluoridothioates **3** and **4** [S_P]-cTMPS[10] was also formed preferentially (ratio of [S_P]-: [R_P]-cTMPS *ca.* 2:1). Yields of Sephadex-purified cTMPS obtained from **3** and **4** were 57 and 38%, respectively. The lack of formation of $T_{PS}T$ in Bu^{t}OK assisted condensation of **2** with 3′-*O*-acetylthymidine was rather unexpected in the light of the results of von Tigerstrom and Smith[11] on effective formation of T_PT and other medium-sized oligothymidylates in the reaction of protected thymidine 3′-*O*-phosphorofluoridate with 3′-*O*-acetylthymidine.



Scheme 1 *Reagents and conditions*: i, Et_3NHF, DBU, MeCN, 15 min; ii, 80% AcOH, 1 h



Scheme 2 *Reagents and conditions*: i, *N*,*N*-diisopropylamino-1,3,2-oxathiaphospholane, 1*H*-tetrazole, 2 h, then elemental sulfur, 18 h; ii, Et_3NHF, DBU, CH_2Cl_2 MeCN, 15 min; iii, conc. NH_4OH, EtOH, 2.5 h

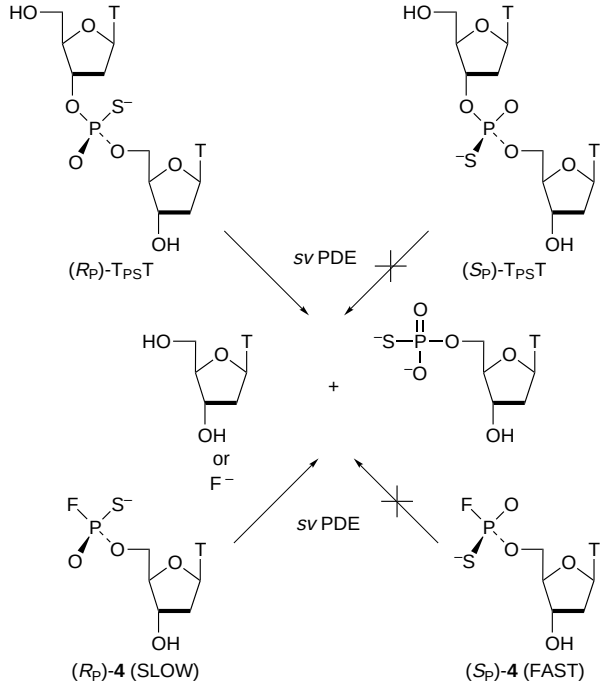


Fig. 1 Tentative assignment of absolute configuration of diastereomers of **4**

In the light of earlier results on an enzymatic cleavage of thymidine 5′-*O*- or 3′-*O*-phosphorofluoridate assisted by *snake venom* (*sv* PDE)[12,13] and *spleen*[12] phosphodiesterases (*spleen* PDE) it was tempting to study the thio analogues **4** and **3** as substrates for these enzymes. From the pioneering work of Eckstein[14] and Benkovic[15] demonstrating the stereoselectivity of *sv* PDE towards P-chiral diesters of phosphorothioic acid it was of interest to check if this enzyme can discriminate between the diastereomers of **3** or **4**.

5′-*O*-Phosphorofluoridothioate **4** was incubated with *sv* PDE†† and the progress of the enzymatic digestion was analyzed by RP-HPLC. It was found that *sv* PDE, if added to a diastereomeric mixture of **4**, causes the stereoselective hydrolysis of the P–F bond of slow-eluted **4** leaving the fast-eluted diastereomer intact. Also, in the case of diastereomeric mixture of **3**†† only slow-eluted **3** underwent hydrolysis in a presence of *sv* PDE, albeit the reaction proceeded much slower than that observed for slow-eluted **4**. Interestingly, under analogous conditions, the rate of hydrolysis of slow-**4** by *sv* PDE was similar to that obtained during digestion of dithymidylyl (3′,5′)phosphate (T_PT). In the presence of *spleen* PDE both diastereomers of **3** were hydrolyzed while both diastereomers of **4** were resistant to this enzyme.** It was also found that **3** and **4** have no inhibitory activity§§ towards either phosphodiesterase. Results on the use of **4** for inhibition of thymidylate synthase will be published separately.[16]

In conclusion, we have found that the 1,3,2-oxathiaphospholane ring can be opened in the presence of DBU by fluoride anion leading to the appropriate phosphorofluoridothioates. The nucleoside 5′-*O*- or 3′-*O*-phosphorofluoridothioates obtained can be used in studies of the mode of action of nucleolytic enzymes. Comparative topological analysis of diastereomers of $T_{PS}T$ and **4** undergoing *sv* PDE- assisted hydrolysis allows the tentative assignment of absolute configuration of the slow-eluted **4** as R_P (Fig. 1). *Spleen* and *sv* PDE-assisted hydrolysis of P–F bonds in compounds **3** and **4** is in agreement with earlier findings[12,13] that these enzymes split nucleoside 3′-*O*- or 5′-*O*-phosphorofluoridate, respectively, giving rise to the appropriate nucleoside phosphates. From this perspective our data on enzymatic hydrolyses of **3** and **4** disagree with the results of Dabkowski *et al.*,[17] who characterized thymidine 3′-*O*-phosphorofluoridate as the product of *spleen* PDE-assisted degradation of thymidin-3′-yl 2′-deoxyadenosin-5′-yl phosphorofluoridate. Adenosine 5′-*O*-phosphorofluoridate was found as the product of *sv* PDE-assisted hydrolysis of the same substrate. Besides the hydrolytic instability of the P–F bond of dinucleoside (3′,5′)phosphorofluoridates in buffered aqueous media[18] yielding appropriate phosphates, even if thymidine 3′-*O*-phosphorofluoridate or adenosine 5′-*O*-phosphorofluoridate were the respective products of PDE-catalyzed hydrolyses, they would necessarily undergo further enzymatic degradation to phosphomonoesters.

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Notes and References

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‡ Triethylammonium fluoride (1 M solution in THF) was obtained by mixing triethylamine tris(hydrofluoride) (1 equiv.) with triethylamine (2 equiv.).

§ Compound **2** consists of a 1 : 1 mixture of diastereomers, δ_P(CD_3CN, 81 MHz) 54.52 ($^1J_{P–F}$ 1043 Hz), 54.58 ($^1J_{P–F}$ 1046 Hz); *m/z* (−FAB) 641.4 ($M^{+\cdot}$ −1).

¶ Compound **3** consists of a mixture of diastereomers (ratio 48 : 52), δ_P(D_2O, 81 MHz) 53.72, ($^1J_{P–F}$ 1053 Hz), 53.74 ($^1J_{P–F}$ 1055 Hz); δ_F(D_2O, 188 Mz) −31.4 ($^1J_{P–F}$ 1043 Hz), −31.2 ($^1J_{P–F}$ 1046 Mz); *m/z* (−FAB) 339.1 ($M^{+\cdot}$ −1).

‖ Compound **5** was obtained as a mixture of diastereomers, δ_P($CDCl_3$, 81 MHz) 106.64, 106.82 (ratio 1 : 1); *m/z* (+FAB) 453.2 ($M^{+\cdot}$ +1).

Compound **4 consists of mixture of diastereomers (ratio 59 : 41), δ_P(D_2O, 81 Mz) 54.17, 54.20 ($^1J_{P–F}$ 1053 Hz); δ_F(D_2O, 188 Mz) −35.6, −35.7 ($^1J_{P–F}$ 1053 Hz); *m/z* (−FAB) 339.2 ($M^{+\cdot}$ −1).

†† The reaction mixture consists of 0.1 mM **4** or **3**, 100 mM Tris–HCl pH 8.0, 20 mM $MgCl_2$, and *sv* PDE (0.01 U ml^{-1}); 37 °C; incubation time: 0.5 h for **4** and 16 h for **3**.

‡‡ The reaction mixtures consist of 0.1 mM **3** (or **4**), 50 mM acetate buffer pH 5.0, and *spleen* PDE (0.15 U ml^{-1}), 37 °C; incubation time: 1 h for **3** and 16 h for **4**.

§§ Enzymatic digestions were performed under conditions mentioned above. T_PT and **3** (or **4**) were used at equimolar concentrations (0.1 mM).

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