

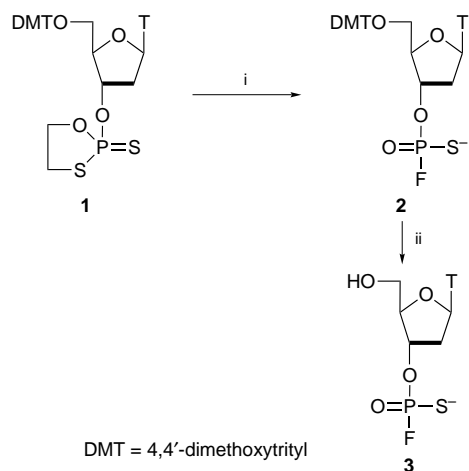
# Synthesis and chemical and enzymatic reactivity of thymidine 3'-O- and 5'-O-phosphorofluoridothioates

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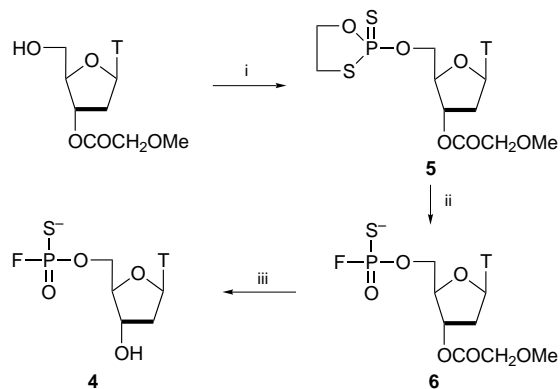
5'-O- or 3'-O-Protected thymidine 3'-O- or 5'-O-(2-thiono-1,3,2-oxathiaphospholanes) react with triethylammonium fluoride in a presence of DBU and furnish, after deprotection, thymidine 3'-O- and 5'-O-phosphorofluoridothioates; the latter undergoes stereoselective hydrolysis by snake venom phosphodiesterase.

Within the course of our studies on the development of oxathiaphospholane methodology for phosphorylation,<sup>1</sup> phosphorothioylation<sup>2</sup> and phosphorodithioylation<sup>3</sup> 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-dimethoxytrityl nucleoside 3'-O-phosphorofluoridothioates.<sup>4,5</sup> 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 triethylammonium fluoride in a presence of DBU is, unlike the 1,3,2-oxathiaphospholane ring-opening condensation with alcohols<sup>2</sup> or amines,<sup>6</sup> a non-stereospecific process. Starting from a mixture of partially separated diastereomers of **1** [ratio 73:27,  $\delta_P$  (CD<sub>3</sub>CN), 105.78 and 105.83] diastereomers of **2** were obtained in a ratio of 54:46 [ $\delta_P$  (CD<sub>3</sub>CN), –30.06 and –29.96]. The epimerisation at phosphorus was not unexpected in the light of

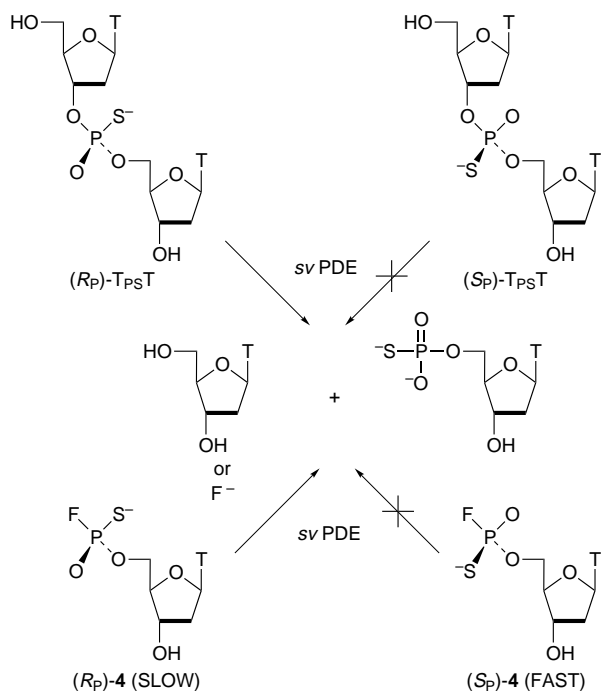


**Scheme 1** Reagents and conditions: i, Et<sub>3</sub>NHF, DBU, MeCN, 15 min; ii, 80% AcOH, 1 h

published earlier results on the stereochemistry of nucleophilic substitution at phosphorus by fluoride ion.<sup>7,8</sup> 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<sup>2</sup> 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 <sup>31</sup>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<sup>t</sup>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 <sup>31</sup>P NMR spectroscopy. Under these conditions, even after 18 h, formation of dithymidylyl (3',5')phosphorothioate (T<sub>PS</sub>T) was not observed. Instead, both **3** and **4** underwent intramolecular cyclization in the presence of an excess of Bu<sup>t</sup>OK (five-fold molar excess) leading to thymidine cyclic (3',5')phosphorothioate (cTMPS). Similarly, as previously observed<sup>9</sup> for Bu<sup>t</sup>OK-catalyzed cyclization of nucleoside 5'-O-*p*-nitrophenyl phosphorothioates, in the reactions of phosphorofluoridothioates **3** and **4** [*S*<sub>P</sub>]-cTMPS<sup>10</sup> was also formed preferentially (ratio of [*S*<sub>P</sub>]-: [*R*<sub>P</sub>]-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<sub>PS</sub>T in Bu<sup>t</sup>OK assisted condensation of **2** with 3'-O-acetylthymidine was rather unexpected in the light of the results of von Tigerstrom and Smith<sup>11</sup> on effective formation of T<sub>P</sub>T and other medium-sized oligothymidylylates in the reaction of protected thymidine 3'-O-phosphorofluoridothioate with 3'-O-acetylthymidine.



**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<sub>3</sub>NHF, DBU, CH<sub>2</sub>Cl<sub>2</sub> MeCN, 15 min; iii, conc. NH<sub>4</sub>OH, 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)<sup>12,13</sup> and *spleen*<sup>12</sup> 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<sup>14</sup> and Benkovic<sup>15</sup> 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<sup>††</sup> 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**<sup>††</sup> 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<sub>PS</sub>T). 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<sup>§§</sup> towards either phosphodiesterase. Results on the use of **4** for inhibition of thymidylate synthase will be published separately.<sup>16</sup>

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<sub>PS</sub>T and **4** undergoing *sv* PDE- assisted hydrolysis allows the tentative assignment of absolute configuration of the slow-eluted **4** as R<sub>P</sub> (Fig. 1). *Spleen* and *sv* PDE-assisted hydrolysis of P-F bonds in compounds **3** and **4** is in agreement with earlier findings<sup>12,13</sup> 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.*,<sup>17</sup> who characterized thymidine 3'-*O*-phosphorofluoridate as the product of *spleen* PDE-assisted degradation of thymidin-3'-yl 2'-deoxyadenosin-5'-yl phosphorofluor-

idate. 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<sup>18</sup> 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.

Studies presented here were financially supported by the State Committee of Scientific Research (KBN), grant no 4 PO5F 023 10.

## Notes and References

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<sup>‡</sup> Triethylammonium fluoride (1 M solution in THF) was obtained by mixing triethylamine trifluoride (1 equiv.) with triethylamine (2 equiv.).

<sup>§</sup> Compound **2** consists of a 1 : 1 mixture of diastereomers,  $\delta_p(\text{CD}_3\text{CN}, 81 \text{ MHz})$  54.52 ( $^1J_{\text{P-F}}$  1043 Hz), 54.58 ( $^1J_{\text{P-F}}$  1046 Hz);  $m/z$  (-FAB) 641.4 ( $M^+ - 1$ ).

<sup>¶</sup> Compound **3** consists of a mixture of diastereomers (ratio 48 : 52),  $\delta_p(\text{D}_2\text{O}, 81 \text{ MHz})$  53.72, ( $^1J_{\text{P-F}}$  1053 Hz), 53.74 ( $^1J_{\text{P-F}}$  1055 Hz);  $\delta_f(\text{D}_2\text{O}, 188 \text{ Mz})$  -31.4 ( $^1J_{\text{P-F}}$  1043 Hz), -31.2 ( $^1J_{\text{P-F}}$  1046 Mz);  $m/z$  (-FAB) 339.1 ( $M^+ - 1$ ).

<sup>||</sup> Compound **5** was obtained as a mixture of diastereomers,  $\delta_p(\text{CDCl}_3, 81 \text{ MHz})$  106.64, 106.82 (ratio 1 : 1);  $m/z$  (+FAB) 453.2 ( $M^+ + 1$ ).

<sup>\*\*</sup>Compound **4** consists of mixture of diastereomers (ratio 59 : 41),  $\delta_p(\text{D}_2\text{O}, 81 \text{ MHz})$  54.17, 54.20 ( $^1J_{\text{P-F}}$  1053 Hz);  $\delta_f(\text{D}_2\text{O}, 188 \text{ Mz})$  -35.6, -35.7 ( $^1J_{\text{P-F}}$  1053 Hz);  $m/z$  (-FAB) 339.2 ( $M^+ - 1$ ).

<sup>††</sup> The reaction mixture consists of 0.1 mM **4** or **3**, 100 mM Tris-HCl pH 8.0, 20 mM MgCl<sub>2</sub>, and *sv* PDE (0.01 U ml<sup>-1</sup>), 37 °C; incubation time: 0.5 h for **4** and 16 h for **3**.

<sup>‡‡</sup> 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<sup>-1</sup>), 37 °C; incubation time: 1 h for **3** and 16 h for **4**.

<sup>§§</sup> Enzymatic digestions were performed under conditions mentioned above. T<sub>PS</sub>T and **3** (or **4**) were used at equimolar concentrations (0.1 mM).

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Received in Glasgow, UK, 12th September 1997; 7/06636H