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Synthesis, Chemical and Enzymatic Reactivity, and Toxicity of Dithymidylyl-3′,5′-phosphorofluoridate and -phosphorothiofluoridate

Konrad Misiura,[a,*] Daria Szymanowicz[a] and Halina Kuśnierczyk[b]

[a]*Department of Bioorganic Chemistry, Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, Sienkiewicza 112, 90-363 Łódź, Poland*

[b]*Department of Tumour Immunology, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, R. Weigla 12, 53-114 Wrocław, Poland*

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Abstract—Dithymidylyl-3′,5′-phosphorofluoridate and phosphorothiofluoridate were obtained by fluorinolysis of the P–Se bond in appropriate bisdimethoxytrityl selenomethyl esters. These compounds, which are hydrolytically unstable, are not inhibitors of snake venom, spleen phosphodiesterases and alkaline phosphatase. Neither compound is highly toxic. © 2001 Elsevier Science Ltd. All rights reserved.

Introduction

Oligonucleotide analogues possessing modified internucleotide phosphate moieties are extensively studied in aspects of their synthesis[1] and stereochemistry,[2] stability against nucleases[3] and their biological activity[4] (*antisense*, *antigene*, and *aptamer* concepts).

The introduction of a fluorine atom attached to phosphorus may influence the specific chemical and biological reactivity of such nucleoside phosphorofluoridates and phosphorothiofluoridates. Although the attachment of the fluorine to phosphorus involved in internucleotide linkage eliminates the formal negative charge, the analogue may still be involved in hydrophilic interactions with other biomolecules due to the ability of fluorine to form strong hydrogen bonds.[5] Horner and Gehring demonstrated[6] that phosphoric acid diesters containing a P(O)F moiety selectively react with HO nucleophiles even in the presence of NH_2 and SH groups. Due to such chemoselectivity, *O,O*-diisopropyl phosphorofluoridate could be used in the classical studies of the mechanism of serine proteases.[7] Simple *O,O*-dialkyl phosphorofluoridates are highly toxic due to their strong inhibition of acetylcholinesterases.[8] Despite the well-established chemistry of *O,O*-dialkyl phosphorofluoridates[9] and phosphorothiofluoridates, the corresponding dinucleoside analogues[10] were obtained only recently.

The oxathiaphospholane method for the synthesis of oligo(nucleoside phosphorothioate)s[11] (S-Oligo) and its dithiaphospholane modification leading to oligo(nucleoside phosphorodithioate)s[12] (S_2-Oligo) have been developed recently as the new strategy for the synthesis of P-modified oligonucleotides where one or both nonbridging oxygens at the internucleotide phosphate are replaced by one or two different atoms. It was interesting to examine if phospholane procedures can be applied for the synthesis oligo(nucleoside phosphorofluoridate)s (F-Oligo) and phosphorothiofluoridates (SF-Oligo). To assess the potential utility of F-Oligo or SF-Oligo for biological studies, the synthesis of model fully deprotected dinucleotides has been developed and their hydrolytic stability, chemical and enzymatic reactivity, and toxicity in mice were studied.

Results and Discussion

Our approach to the synthesis of F-Oligo and SF-Oligo was based on the concept of the solid-phase method to

*Corresponding author. Tel.: +48-42-681-9744; fax: +48-42-681-5483; e-mail: kmisiura@bio.cbmm.lodz.pl

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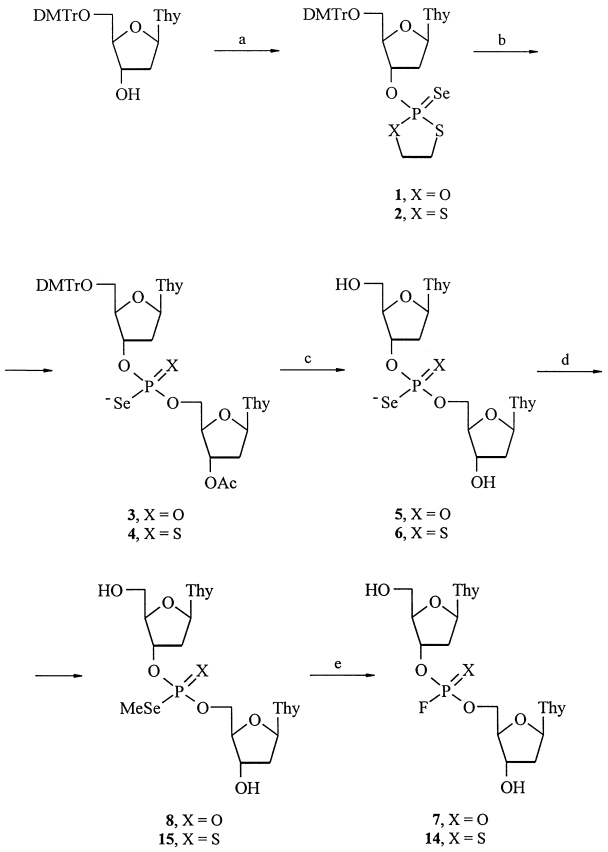
make oligonucleotide intermediates which, after release from the support and deprotection of amino groups in nucleobases, could be transformed into desired products. Such a concept was supported by early observations[13] of Stec et al. that dithymidylyl-3′,5′-phosphorofluoridate intermediate, formed in the reaction of appropriate phosphorothioate with 2,4-dinitrofluorobenzene in pyridine solution, reacted in situ with ethanol to give the corresponding *O*-ethylphosphotriester. Also, in our previous studies[10a] we proved that 3′-*O*-acetyl-5′-*O*-DMTr-dithymidylyl-3′,5′-phosphorofluoridate treated with ammonia solution was completely hydrolysed within 15 min. Thus one could expect that exposure of F-Oligo or SF-Oligo to this reagent commonly used in the solid-phase synthesis of oligonucleotides for their release from the solid support and nucleobase deprotection may lead to the substantial hydrolytic loss of fluorine. Our interest therefore focused on oligo(nucleoside phosphoroselenoate)s (Se-Oligo) and phosphorothioselenoates (SSe-Oligo) which could be directly transformed into appropriate fluorinated derivatives or could be methylated to form *Se*-methyl phosphoroselenoate and phosphorothioselenoate triesters. The selenomethyl moiety is known to be a good leaving group when a nucleophile attacks a phosphorus atom[14] and the fluorine anion possesses strong nucleophilic avidity towards P-IV compounds;[15] such substitution was expected to provide F-Oligo and SF-Oligo.

For the synthesis of Se-Oligo and SeS-Oligo, we developed a new method based on oxathia- and dithiaphospholane chemistry. To establish reaction conditions, we started with the synthesis 'in solution' (Scheme 1). 5′-*O*-DMTr-thymidine-3′-*O*-(2-seleno-1,3,2-oxathiaphospholane) (**1**) and -1,3,2-dithiaphospholane (**2**) were obtained in reaction of 5′-*O*-DMTr-thymidine with *N,N*-diisopropylamino-1,3,2-oxathiaphospholane and -1,3,2-dithiaphospholane in the presence of 1*H*-tetrazole, and subsequent oxidation of P-III intermediates with elemental selenium. Compounds **1** and **2** were condensed with 3′-*O*-acetylthymidine in the presence of 1,8-diazabicyclo[4.3.0]undec-7-ene (DBU) to give 3′-*O*-acetyl-5′-*O*-DMTr-thymidylyl-3′,5′-phosphoroselenoate (**3**) and -3′,5′-phosphorothioselenoate (**4**), which were, without isolation, deprotected by acetic acid followed by ammonia treatment. Crude products were purified by anion-exchange chromatography on DEAE-Sephadex. The overall yield of dithymidylyl-3′,5′-phosphoroselenoate (**5**) and -phosphorothioselenoate (**6**) were 71 and 58%, respectively. Compounds **5** and **6** were obtained as a mixture of diastereoisomers in nearly equimolar ratio. Subsequently, the applicability of this method under conditions of 'solid-phase' synthesis was examined. 5′-*O*-DMTr-thymidine (1 μmol) anchored to controlled pore glass support was detritylated and treated with a mixture of **1** (0.2 M) and DBU (0.5 M) in dry acetonitrile. After 10 min, the reaction column was washed with acetonitrile and, after detritylation, dithymidylyl-3′,5′-phosphoroselenoate (**5**) was released from solid support by ammonolysis. It was found that **5** was formed in 97% yield (HPLC assay). In a similar experiment starting from dithiaphospholane **2**, dithymidylyl-3′,5′-phosphorothioselenoate (**6**) was obtained in 96% yield.

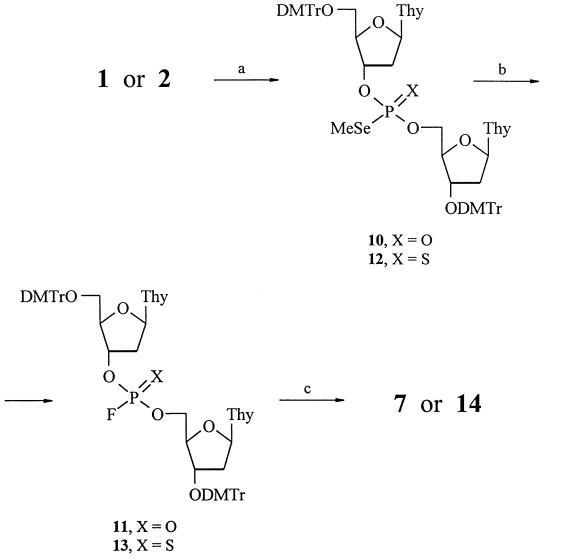
Dithymidylyl-3′,5′-phosphoroselenoate (**5**) was then used as a substrate for studies on the synthesis of appropriate phosphorofluoridates. Phosphoroselenoate **5** reacted with 25% aq AgF in acetonitrile solution providing dithymidylyl-3′,5′-phosphorofluoridate (**7**) as the mixture of diastereoisomers in ca. 1:1 ratio, (^{31}P NMR control, δ_P -9.45 ($J_{P\text{-}F} = 981$ Hz), δ_P -9.06 ($J_{P\text{-}F} = 973$ Hz)) in 100% yield in spite of the presence of water introduced into the reaction mixture by AgF solution. However, any attempts to isolate **7** from these reaction mixtures by silica gel chromatography using several eluting solutions failed, probably due to silica gel-assisted hydrolysis of the desired product. Also attempts to purify phosphorofluoridate **7** by its extraction from aqueous solutions into chloroform or ethyl acetate were unsuccessful since this compound is highly hydrophilic. Recently, AgF solution was employed for efficient conversion[16] of simple phosphorothioates and phosphoroselenoates into appropriate phosphorofluoridates, however these products were not isolated. Independently, Stawinski described transformation of **5** into **7** by means of triethylamine trihydrofluoride and iodine in a pyridine solution, but attempts to isolate this product have also failed.[17] This method was used for synthesis of tetra-thymidine phosphorofluoridate[18] but the product hydrolysed during its purification by means of RP-HPLC.



Scheme 1. (a) (i) *N,N*-diisopropylamino-1,3,2-oxathia(dithia)phospholane, 1H-tetrazole; (ii) Se_8; (b) 3′-*O*-acetylthymidine, DBU; (c) (i) CH_3COOH, (ii) NH_4OH; (d) MeI; (e) AgF for **8**, Et_3NHF for **15**.

The failure to isolate phosphorofluoridate **7** formed during direct PSe→PF transformation prompted us to examine the possibility of synthesis of PF-Oligo via selenomethyl intermediates. Phosphoroselenoate **5** was methylated with methyl iodide providing the selenomethyl triester **8** in 81% yield. Similarly as for simple dialkyl phosphoroselenoates, methylations occurred exclusively at the selenium atom, as proved by ^{31}P NMR analysis of the reaction mixture. In our previous studies,[10a] it was found that the fluoride anion can smoothly substitute the MeSe group and that 3′-*O*-acetyl-5′-*O*-DMTr-dithymidylyl-3′,5′-phosphorofluoridate, isolated by extraction, was obtained in a good yield. Indeed, reactions of *Se*-methyl phosphoroselenoate **8** with AgF in acetonitrile, pyridine, and DMSO solutions were also fast and efficient, providing, after 1 h, phosphorofluoridate **7** in 100% yield (^{31}P NMR assay) but any attempts to isolate this compound failed.

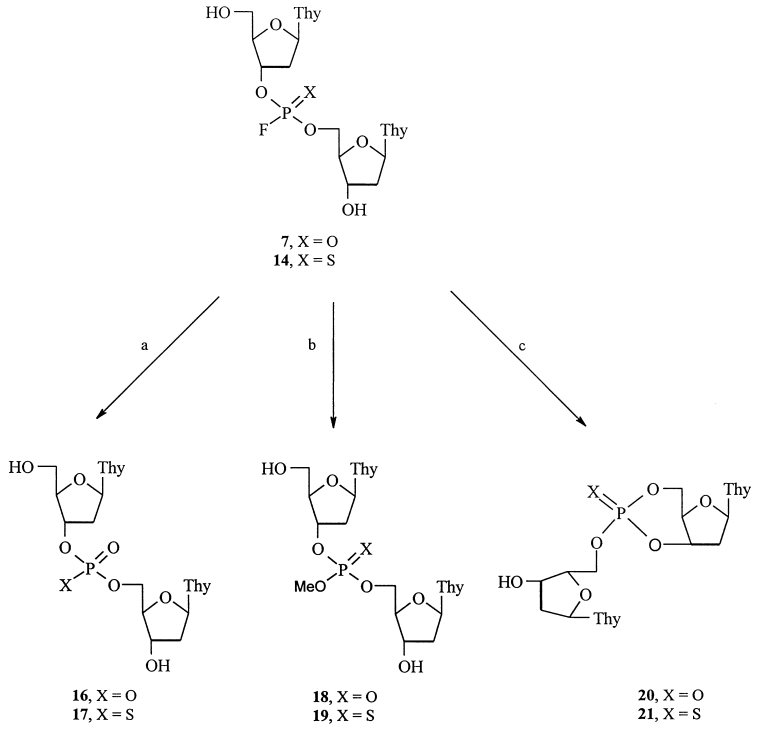
Problems with purification of dithymidylyl-3′,5′-phosphorofluoridate (**7**) mentioned above suggested that, for the isolation of F-Oligo, the presence of fluoride anions during the silica gel purification should be avoided. Thus, it was decided to modify the synthetic procedure by keeping the DMTr protection group of the 3′- and 5′-hydroxyls during PSeMe→PF transformation. The presence of two lipophilic groups should enable good solubility of the compound in organic solvents like chloroform and allow its purification by means of extraction. So oxathiaphospholane **1** reacted with 3′-*O*-DMTr-thymidine in a presence of DBU giving 3′,5′-*O,O*-bis-DMTr-dithymidylyl-3′,5′-phosphoroselenoate (**9**), which was, without, isolation methylated with methyl iodide providing selenomethyl triester **10** (mixture of diastereoisomers in ca. 1:1 ratio) with 68% yield (Scheme 2). Compound **10** reacted with 25% aq AgF in acetonitrile solution and, after 1 h, 3′,5′-*O,O*-bis-DMTr-dithymidylyl-3′,5′-phosphorofluoridate (**11**) was formed in 100% yield (^{31}P NMR assay). Crude **11** was dissolved in chloroform and washed with brine to remove an excess of silver fluoride. Pure phosphorofluoridate **11** (mixture of diastereoisomers ca. 1:1) was obtained with 85% yield. DMTr protecting groups were removed from **11** by exposure to 3% *p*-toluenesulfonic acid providing the desired dithymidylyl-3′,5′-phosphorofluoridate (**7**). Crude **7** was purified by fast column chromatography on silica gel. Pure phosphorofluoridate **7** (mixture of diastereoisomers ca. 1:1) was obtained with 53% yield.



Scheme 2. (a) (i) 3′-*O*-DMTr-thymidine, DBU; (ii) MeI; (b) AgF for **10**, Et_3NHF for **12**; (c) *p*-toluenesulfonic acid.

The thio-analogue of **7** was synthesised by the same method starting from 5′-*O*-DMTr-thymidine-3′-*O*-(2-seleno-1,3,2-dithiaphospholane) (**2**) (Scheme 2). The only difference was that triethylammonium fluoride was used during the fluorination step of 3′,5′-*O,O*-bis-DMTr-dithymidylyl-3′,5′-*Se*-methylphosphorothioselenoate (**12**) because AgF caused rapid oxidation of thio compound **13** into oxo congener **11**. Dithymidylyl-3′,5′-phosphorothiofluoridate (**14**) (mixture of diastereoisomers in ca. 1:1 ratio) was obtained with 12% total yield. Compound **14** was synthesized earlier[19] by us using direct fluorination of dithymidylyl-3′,5′-*Se*-methyl phosphorothioselenoate (**15**) but it could not be obtained in a pure form.

Hydrolytic lability of phosphorofluoridate **7** and phosphorothiofluoridate **14** leading to dithymidylyl-3′,5′-phosphate (**16**) or dithymidylyl-3′,5′-phosphorothioate (**17**), respectively, was examined by means of ^{31}P NMR and HPLC (Scheme 3). Compounds **7** and **14** (both 2 mM) in 100 mM Tris–HCl, pH 7.5 were hydrolysed at room temperature after 1 h in 77 and 26%, respectively, with complete hydrolysis after 2 h for **7** and 12 h for **14**. Compound **7** (0.1 mM) in the same buffer was completely hydrolysed after 5 min at 37 °C whereas 1 h was required to hydrolyse **14**. Compounds **7** and **14** (both 2 mM) were hydrolysed in less then 5 min (^{31}P NMR assay) under alkaline conditions in 14% NH_4OH. A great acceleration of the rate of hydrolysis of *O,O*-diisopropyl phosphorofluoridate in alkaline conditions versus neutral one ($k(OH)/k(H_2O) = 8.2\times10^6$) had been observed earlier by Hudson.[20] The only nucleoside phosphorofluoridate diester reported[21] to be stable to the ammonia solution was 2′-deoxycytidine cyclic 3′,5′-phosphorofluoridate (single diastereoisomer). The most probable explanation for this unusual hydrolytic stability is a ring strain effect operating during formation of the P-V intermediate possessing a six-membered ring in a diaxial orientation. On the other hand, no difference in the rates of hydrolysis between *O,O*-diethyl phosphorofluoridate and cyclic 2-fluoro-1,3,2-dioxaphosphinane 2-oxide were found.[22] So one can speculate that the bicyclic ring system present in 2′-deoxycytidine cyclic 3′,5′-phosphorofluoridate should be responsible for this unusual stability. Compounds **7** and **14** also underwent fast methanolysis leading to the appropriate methyl esters **18** and **19** when dissolved in methanol in the presence of bases like triethylamine or pyridine. In the presence of DBU an intramolecular cyclisation of **7** and **14** occurred giving compounds **20** and **21**. ^{31}P NMR analysis of **20** and **21** revealed the presences of only two signals (δ −3.17, −4.62 (ratio 1:2), δ 66.30, 63.66 (ratio 3:1), respectively) suggesting that the cyclisation is 5′-regioselective and stereoselective.



Scheme 3. (a) Tris–HCl pH8 or NH_4OH; (b) MeOH, Et_3N; (c) DBU.

The high reactivity of phosphorofluoridate **7** and phosphorothiofluoridate **14** towards OH nucleophiles and the known inhibitory effect of *O,O*-dialkyl phosphorofluoridates of serine proteases[7] prompted us to examine the inhibition of certain phosphodiesterases by **7** or **14**. Snake venom and spleen phosphodiesterases were chosen for those studies since both kinetic and stereochemical analysis[23] proved formation of a covalent enzyme substrate complex during catalysed reactions, indicating the presence of hydroxy amino acids in the active centres of these enzymes. HPLC analysis of hydrolysis of dithymidylyl-3′,5′-phosphate (**16**) by both snake venom and spleen phosphodiesterases performed in the presence of phosphorofluoridate **7** or phosphorothiofluoridate **14** in concentrations up to 200 nM showed that no inhibitory effect was observed. Earlier, we also proved[24] that **7** and **14** are not substrates for these enzymes. Similarly, **7** and **14** were not inhibitors of alkaline phosphatase,[25] which is known to possess serine residue in its active centre. Most probably, phosphorofluoridate **7** and phosphorothiofluoridate **14** undergo hydrolysis faster than they react with a hydroxy group of amino acid residue present in active centres of these enzymes. Lack of inhibition of these enzymes can also suggest low reactivity of **7** and **14** towards cholinoesterases. Indeed, it was found that **7** and **14** were not toxic to mice at the concentration up to 10 mg/kg, which is a much higher level than observed[8] for phosphorofluoridate nerve agents (LD_{50} in a range of 0.01–1 mg/kg). The observed lack of toxicity of **7** and **14** can be also explained by fast hydrolysis of these compounds in biological fluids.

The high hydrolytic reactivity of phosphorofluoridate **7** as compared to other simple *O,O*-dialkyl phosphorofluoridates cannot be explained by an anchimeric assistance of 5′- or 3′-hydroxyl groups since no major differences in the rate of hydrolysis between **7** and its 3′-acetyl-5′-DMTr protected analogue were observed. Most probably this reactivity is caused by the hydrophilic nature of nucleoside residues. In our opinion, such hydrolytic instability precludes the use of F-Oligo and SF-Oligo in biochemical and biological studies.

Experimental

Methylene chloride, acetonitrile, tetrahydrofuran, and trietylamine were dried by theirs distillation from calcium hydride. Dry DBU was obtained by its distillation from calcium hydride under reduced pressure. Commercial 25% solution of AgF was used. 1 M TEAF in dry THF was obtained by neutralisation of triethylamine trihydrofluoride with two equivalents of dry Et_3N. Snake venom and spleen phosphodiesterases (svPDE and sPDE) were from Sigma and alkaline phosphatase (AP) from Boehringer Mannheim.

Unless stated otherwise, the progress of each reaction was monitored on TLC. HPLC analyses were performed on LDC Analytical using Econoshere C-18 (Alltech) reverse-phase column (flow 1 ml/min) and gradient 0→60% B (A, 0.1 M CH_3COONH_4, B, 0.02 M CH_3COONH_4 in 80% acetonitrile) in 15 min. ^1H and ^{31}P NMR spectra were recorded on a Brucker AC-200. Mass spectrometry analyses were performed using a Finnigan MAT 95 apparatus.

5′-*O*-DMTr-thymidine-3′-*O*-(2-seleno-1,3,2-oxathiaphospholane) (1). To a stirred solution of 5′-*O*-DMTr-thymidine (2.72 g, 5.0 mmol) and 1*H*-tetrazole (0.42 g, 6 mmol) in anhydrous methylene chloride (20 mL) was added 2-*N,N*-diisopropylamino-1,3,2-oxathiaphospholane[11b] (1.24 g, 6 mmol). After 1 h, selenium (0.60 g, 7.5 mmol) was added to the reaction mixture. P-III Intermediate (δ_P 172.9, 171.8) was oxidized within 12 h (^{31}P NMR assay). The reaction mixture was filtered and the filtrate was washed with water (2×25 mL). The organic layer was dried and filtered and the solvent was evaporated. Crude product was purified by silica-gel column chromatography eluted with methylene chloride containing 0–5% of ethanol. Product **1** was obtained as solid foam (2.80 g, 77% yield): TLC (methylene chloride/ ethanol 19:1) R_f 0.46; ^{1}H NMR ($CDCl_3$) δ 1.41, 1.42 (pair of d, J = 1.1 Hz, J = 1.0 Hz, 3H), 2.57 (m, 2H), 3.48 (m, 4H), 3.79 (s, 6H), 4.41 (m, 1H), 4.50 (m, 2H), 5.60 (m, 1H), 6.45 (dd, J = 8.8 Hz, J = 5.7 Hz, 1H), 6.84 (d, J = 9.0 Hz, 4H), 7.27 (m, 9 H), 7.60, 7.61 (pair of q, J = 1.1 Hz, J = 1.0 Hz, 1H), 8,45 (s, 1H); ^{31}P NMR ($CDCl_3$) δ: 99.0 ($J_{P\text{-}Se}$ = 953 Hz), 99.2 ($J_{P\text{-}Se}$ = 952 Hz); MS (−FAB) m/z 729.0 (M−H), (+FAB) m/z 731.3 (M+H); elemental analysis for $C_{33}H_{35}O_8N_2PSSe$: calcd/found C 54.32/54.42, H 4.83/4.91, N 3.84/3.80, P 4.25/4.58, S 4.39/4.33.

5′-*O*-DMTr-thymidine-3′-*O*-(2-seleno-1,3,2-dithiaphospholane) (2). To a stirred solution of 5′-*O*-DMTr-thymidine (2.72 g, 5 mmol) and 1*H*-tetrazole (0.38 g, 5.5 mmol) in anhydrous methylene chloride (20 mL) was added 2-*N,N*-diisopropylamino-1,3,2-oxathiaphospholane[26] (1.23 g, 5.5 mmol). After 1 h selenium (0.55 g, 6.9 mmol) was added to reaction mixture. The P-III Intermediate (δ_P 152.4) was oxidized within 12 h (^{31}P NMR assay). The reaction mixture was filtered and the filtrate was washed with water (2×25 mL). The organic layer was dried, filtered and the solvent was evaporated. Crude product was purified by silica-gel column chromatography eluted with methylene chloride containing 0–5% of ethanol. Product **2** was obtained as solid foam (2.76 g, 74% yield): TLC (methylene chloride/ ethanol 19:1) R_f 0.46; ^{1}H NMR: (CD_3Cl) δ: 1.44 (d, J = 1.1 Hz, 3H), 2.61 (m, 2H), 3.59 (m, 6H), 3.79 (s, 6H), 4.32 (m, 1H), 5.60 (dd, J = 15 Hz, J = 5.9 Hz, 1H), 6.46 (dd, J = 8.8 Hz, J = 5.5 Hz, 1H), 6.84 (d, J = 8.9, 4H), 7.28 (m, 9H), 7.60 (q, J = 1.1 Hz), 8.61 (s, 1H); ^{31}P NMR ($CDCl_3$) δ 106.0 ($J_{P\text{-}Se}$ = 884 Hz); MS (-FAB) m/z 745.2 (M-H), (+FAB) m/z 747.5 (M+H); elemental analysis for $C_{33}H_{35}O_7N_2PS_2Se$: calc./found C 53.15/53.48, H 4.73/4.83, N 3.76/3.73, P 4.15/4.22, S 8.60/8.44.

Dithymidylyl-3′,5′-phosphoroselenoate (5). To a stirred solution of 5′-*O*-DMTr-thymidine-3′-*O*-(2-seleno-1,3,2-oxathiaphospholane) (**1**) (1.82 g, 2.5 mmol) and 3′-*O*-acetylthymidine (0.86 g, 3.0 mmol) in anhydrous acetonitrile (25 mL) was added DBU (0.45 mL, 3.0 mmol). The reaction was completed after 15 min providing 3′-*O*-acetyl-5′-*O*-DMTr-dithymidylyl-3′,5′-phosphoroselenoate (**3**) (δ_P 50.6 ($J_{P\text{-}Se}$ = 813 Hz), 50.1 ($J_{P\text{-}Se}$ = 814 Hz)) with 100% yield. The mixture was concentrated and the residue was treated with 80% acetic acid (25 mL) for 6 h. Then the solution was concentrated. The residue was dissolved in acetonitrile (25 mL) and concentrated ammonium hydroxide (25 mL). After 16 h, the reaction mixture was concentrated and the residue dissolved in water (100 mL). Obtained suspension was filtered, and the filtrate was evaporated. Crude product was purified on DEAE-Sephadex column eluting with 0.02–0.8 M TEAB, pH 7.0. Product **5** was obtained as solid foam (1.26 g, 71% yield): HPLC t_R 8.42, 8.57; ^{1}H NMR (D_2O) δ: 1.18 (t, J = 7.3 Hz, 9H), 1.79 (d, J = 1.0 Hz, 3H), 1.84, 1.85 (pair of d, J = 1.0 Hz, 3H), 2.27 (m, 4H), 3.10 (q, J = 7.3 Hz, 6H), 3.75 (m, 2H), 4.10 (m, 4H), 4.49 (m, 1H), 4.92 (m, 1H), 6.13 (t, J = 6.8 Hz, 1H), 6.23 (t, J = 6.8 Hz, 1H), 7.58 (q, J = 1.0 Hz), 7.65 (q, J = 1.0 Hz, 1H); ^{31}P NMR (D_2O) δ 49.8 ($J_{P\text{-}Se}$ = 777 Hz), 49.1 ($J_{P\text{-}Se}$ = 775 Hz); MS (−FAB) m/z 609.1 (M−H).

Dithymidylyl-3′,5′-phosphorothioselenoate (6). To a stirred solution of 5′-*O*- DMTr-thymidine-3′-*O*-(2-seleno-1,3,2-dithiaphospholane) (**1**) (1.84 g, 2.5 mmol) and 3′-*O*-acetylthymidine (0.86 g, 3.0 mmol) in anhydrous acetonitrile (25 mL) was added DBU (0.45 mL, 3.0 mmol). The reaction was completed after 15 min providing 3′-*O*-acetyl-5′-*O*-DMTr-dithymidylyl-3′,5′-phosphorothioselenoate (**4**) (δ_P 103.1 ($J_{P\text{-}Se}$ = 782 Hz), 103.4 ($J_{P\text{-}Se}$ = 783 Hz)) with 96% yield. The mixture was concentrated and the residue was treated with 80% acetic acid (25 mL) for 6 h. Then the solution was concentrated. Obtained residue was dissolved in acetonitrile (25 mL) and concentrated ammonium hydroxide (25 mL). After 16 h, the reaction mixture was concentrated and dissolved in water (100 mL). Obtained suspension was filtered and the filtrate was evaporated. Crude product was purified on DEAE-Sephadex column eluting with 0.025–1.0 M TEAB, pH 7.0. Product **6** was obtained as solid foam (1.05 g, 58% yield): HPLC t_R 8.47, 8.59; ^{1}H NMR (D_2O) δ 1.29 (t, J = 7.3 Hz, 9H), 1.89 (s, 3H), 1.98 (s, 3H), 2.38 (m, 4H), 3.22 (q, J = 7.3 Hz, 6H), 3.88 (m, 2H), 4.22 (m, 4H), 4.62 (m, 1H), 5.20 (m, 1H), 6.25 (t, J = 6.8 Hz, 1H), 6.34 (t, J = 6.9 Hz, 1H), 7.7 (s, 1H), 7.8 (s, 1H); ^{31}P NMR (D_2O) δ 102.2 ($J_{P\text{-}Se}$ = 753 Hz), 102.5 ($J_{P\text{-}Se}$ = 754 Hz); MS (−FAB) m/z 625.6 (M−H), (+FAB) m/z 627.8 (M+H).

Solid-phase synthesis of dithymidylyl-3′,5′-phosphoroselenoate (5) and dithymidylyl-3′,5′-phosphorothioselenoate (6). 5′-*O*-DMTr-thymidine (1 mmol) bound to the standard LCA CPG solid support was detritylated with 3% solution of dichloroacetic acid in methylene chloride (3 mL) within 3 min. Then the column was dried (water pump vacuum) and washed with dry acetonitrile (10 mL), dried again and filled with a mixture of **1** (29 mg, 0.04 mmol) and dry DBU (36 mL, 0.2 mmol) in dry acetonitrile (150 mL) and left for 10 min. Column was washed with dry acetonitrile (10 mL), 3% dichloroacetic acid in methylene chloride (3 mL) and acetonitrile (10 mL) again. The support was dried and the product was released by washing the column with 30% ammonia (1 mL) within 1 h. The solution was concentrated on a Speed-Vac. The residue was dissolved in water (1 mL) and filtered. The solution of **5** was analysed by RP-HPLC.

Starting from **2**, and using the same procedure, product **6** was obtained.

Dithymidylyl-3′,5′-*Se*-methyl phosphoroselenoate (8). To a stirred solution of dithymidyl-3′,5′-phosphoroselenoate (**5**) (0.71 g, 1.0 mmol) dissolved in a mixture of solvents CH_3CN/H_2O/0.5 M TEAB (pH 7.0) in a ratio 2.5:1.5:1 (10 mL) was added methyl iodide (0.12 mL, 2.0 mmol). After 2 h, reaction mixture was concentrated and the residue was dissolved in acetonitrile and concentrated again. The residue was twice dissolved in ethanol and concentrated. The crude product was purified by silica-gel column chromatography eluted with chloroform containing 0–15% ethanol. Product **8** was obtained as solid foam (0.52 g, 81% yield): TLC (chloroform/ethanol 4:1) R_f 0.36; ^{1}H NMR (DMSO-d_6) δ 1.77 (m, 6H), 2.18 (d, J = 13.9 Hz, 3H), 2.38 (m, 4H), 3.32 (m, 4H), 3.62 (m, 2H), 4.25 (m, 2H), 5.23 (t, J = 4.1 Hz, 1H), 5.45, 5.47 (pair of d, J = 1.3 Hz, 1H), 6.21 (m, 2H), 7.48, 7.50 (pair of d, J = 1.2 Hz, 1H), 7.67, 7.69 (pair of d, J = 1.2 Hz, 1H), 11.35 (m, 2H); ^{31}P NMR (d_6-DMSO) δ: 21.8, 21.9 ($J_{P\text{-}Se}$ = 496 Hz); MS (−FAB) m/z 623.9 (M−H), (+FAB) m/z 625.8 (M+H).

3′,5′-*O*,*O*-Bis-DMTr-dithymidylyl-3′,5′-Se-methyl phosphoroselenoate (10). To a stirred solution of 5′-*O*-DMTr-thymidine-3′-*O*-(2-seleno-1,3,2-oxathiaphospholane) (**1**) (1.46 g, 2.0 mmol) and 3′-*O*-DMTr-thymidine[27] (0.86 g, 1.6 mmol) in anhydrous acetonitrile (12 mL) was added DBU (0.24 mL, 1.6 mmol). The reaction was completed after 15 min (^{31}P NMR (CH_3CN) δ 50.38 (J = 809.7 Hz)) and the obtained mixture was treated with methyl iodide (0.12 mL, 2.0 mmol). Progress of the methylation was observed by ^{31}P NMR, which showed disappearance of the substrate after 2 h. The reaction mixture was concentrated and the crude product was purified by silica-gel column chromatography eluted with chloroform containing 0–5% ethanol. Product **10** was obtained as solid foam (1.12 g, 68% yield): TLC (chloroform/ethanol 19:1) R_f 0.45; ^{1}H NMR ($CDCl_3$) δ: 1.58 (m, 6H), 1.82 (m, 4H), 2.00 (d, J = 14.3 Hz, 3H), 3.45 (m, 2H), 3.69 (m, 1H), 3.77 (s, 6H), 3.78 (s, 6H), 3.94 (m, 2H), 4.25 (m, 2H), 6.10 (m, 1H), 6.36 (m, 2H), 6.83 (d, J = 8.7 Hz, 8H), 7.30 (m, 18H), 7.42 (m, 2H), 8.16 (s, 1H), 8.22 (s, 1H); ^{31}P NMR ($CDCl_3$) δ 22.1 ($J_{P\text{-}Se}$ = 503 Hz), 22.0 ($J_{P\text{-}Se}$ = 506 Hz); MS (−FAB) m/z 1227.9 (M−H).

3′,5′-*O*,*O*-Bis-DMTr-dithymidylyl-3′,5′-phosphorofluoridate (11). To a stirred solution of 3′,5′-*O*,*O*-Bis-DMTr-dithymidylyl-3′,5′-*Se*-methyl phosphoroselenoate (**10**) (0.162 g, 0.13 mmol) in acetonitrile (6 mL) was added 25% aqueous solution of silver fluoride (79 μl, 0.2 mmol). The ^{31}P NMR spectrum showed the reaction was completed after 1 h. Chloroform (10 mL) was added to the reaction mixture and resulting mixture was extraction with brine twice. The organic fraction was dried, filtered and the solvent was evaporated. Product **11** was obtained as solid foam (0.13 g, 85% yield): ^{31}P NMR (CD_3Cl) δ −9.2 ($J_{P\text{-}Se}$ = 979 Hz), −9.6 ($J_{P\text{-}Se}$ = 985 Hz); MS (−FAB) m/z 1152.8 (M−H).

Dithymidylyl-3′,5′-phosphorofluoridate (7). To a stirred solution of 3′,5′-*O*,*O*-Bis-DMTr-dithymidylyl-3′,5′-phosphorofluoridate (**11**) (0.426 g, 0.37 mmol) in chloroform (9 mL) was added 3% *p*-toluenesulfonic acid (3.7 mL, 0.57 mmol). After 5 min, 10% solution of pyridine in chloroform (0.48 mL, 0.6 mmol) was added to the reaction mixture to neutralise the acid. The crude product was purified by silica-gel column chromatography eluted with chloroform containing 0–15% ethanol. Product **7** was obtained as solid foam (0.11 g, 53% yield): TLC (chloroform/ethanol 4:1) R_f 0.36; ^{1}H NMR (DMSO-d_6) δ: 1.78 (s, 6H), 2.15 (m, 4H), 3.60 (m, 2H), 4.10 (m, 2H), 4.24 (m, 1H), 4.36 (m, 2H), 5.16 (m, 1H), 5.26 (m, 1H), 5.50 (d, J = 4.4 Hz, 1H), 6.21 (m, 2H), 7.46 (s, 1H), 7.66 (s, 1H), 11.33 (s, 1H), 11.37 (s, 1H); ^{31}P NMR (d_6-DMSO) δ: −9.2 ($J_{P\text{-}F}$ = 977 Hz), −9.5 ($J_{P\text{-}F}$ = 981 Hz); ^{19}F NMR (d_6-DMSO) δ: −77.6 ($J_{P\text{-}F}$ = 976 Hz), −77.9 ($J_{P\text{-}F}$ = 981 Hz); MS (−FAB) m/z 547.3 (M−H), (+FAB), m/z 549.5 (M+H).

3′,5′-*O*,*O*-Bis-DMTr-dithymidylyl -3′,5′-*Se*-methyl phosphorothioate (12). To a stirred solution of 5′-*O*-DMTr-thymidine-3′-*O*-(2-seleno-1,3,2-dithiaphospholane) (**2**) (2.3 g, 3.08 mmol) and 3′-*O*-DMTr-thymidine (1.34 g, 2.46 mmol) in anhydrous acetonitrile (21 mL) was added DBU (0.44 mL, 3.0 mmol). The ^{31}P NMR spectrum showed the completion of the reaction in 1 h. The mixture was treated with methyl iodide (0.23 mL, 3.7 mmol). Progress of the methylation was observed by ^{31}P NMR, which showed disappearence of substrate after 1 h. The reaction mixture was concentrated and crude product was purified by silica-gel column chromatography eluted with chloroform containing 0–5% ethanol. The product was obtained as solid foam (2.89 g, 75% yield): TLC (chloroform/ethanol 19:1) R_f 0.46, ^{31}P NMR (CD_3Cl) δ 88.8 ($J_{P\text{-}Se}$ = 492 Hz), 88.2 ($J_{P\text{-}Se}$ = 492 Hz); MS (−FAB) m/z (M−H), (+FAB) m/z (M+H).

3′,5′-*O*,*O*-Bis-DMTr-dithymidylyl-3′,5′-phosphorothiofluoridate (13). To a stirred solution of 3′,5′-*O*,*O*-bis-DMTr-dithymidylyl-3′,5′-*Se*-methyl phosphoroselenothioate (0.5 g, 0.4 mmol) in acetonitrile (8 mL) was added 1 M TEAF in dry THF (0.87 mL, 0.87 mmol) and DBU (0.07 mL, 0.45 mmol). ^{31}P NMR spectrum showed the reaction was completed after 0.5 h. The reaction mixture was concentrated and crude product was purified by silica-gel column chromatography eluted with methylene chloride containing 0–5% methanol. The product was obtained as solid foam (0.21 g, 56% yield): TLC (methylene chloride/methanol 19:1) R_f 0.38; ^{31}P NMR (CH_3Cl) δ 61.8 ($J_{P\text{-}F}$ = 1086 Hz), 61.1 ($J_{P\text{-}F}$ = 1096 Hz); ^{19}F NMR, purity 92%, δ: −41.2 ($J_{P\text{-}F}$ = 1085 Hz), −42.1 ($J_{P\text{-}F}$ = 1096 Hz), MS (−FAB) m/z 1152.8 (M−H).

Dithymidylyl-3′,5′-phosphorothiofluoridate (14). To a stirred solution of 3′,5′-*O*,*O*-bis-DMTr-dithymidylyl-3′,5′-phosphorothiofluoridate (0.136 g, 0.11 mmol) in chloroform (2.8 mL) was added 3% *p*-toluenesulfonic acid (1.18 mL, 0.19 mmol). The reaction was completed after 5 min (TLC assay). A 10% solution of pyridine in chloroform (0.15 mL, 0.18 mmol) was added to the reaction mixture to neutralise the acid. The crude product was purified by silica-gel column chromatography eluted with chloroform containing 0–15% ethanol. Product **14** was obtained as solid foam (0.025 g, 38%

yield): TLC (chloroform/ethanol 4:1) R_f 0.58; ^{1}H NMR (DMSO-d_6) δ: 1.78 (s, 6H), 2.14 (m, 4H), 3.61 (m, 2H), .3.97 (m, 1H), 4.11 (m, 1H), 4.26 (m, 1H), 4.46 (m, 2H), 5.02 (m, 1H), 5.28 (m, 1H), 5.50 (d, J = 4.0 Hz, 1H), 6.21 (m, 2H), 7.43 (s, 1H), 7.67 (s, 1H), 11.34 (s, 2H); ^{31}P NMR (CD_3Cl/C_2H_5OH 3/1) δ 61.3 ($J_{P\text{-}F}$ = 1081 Hz), 60.7 ($J_{P\text{-}F}$ = 1093 Hz); ^{19}F NMR δ −42.8 ($J_{P\text{-}F}$ = 1081 Hz), −43.1 ($J_{P\text{-}F}$ = 1093 Hz); MS (−FAB) m/z 563.1 (M−H), (+FAB) m/z 565.1 (M+H).

Dithymidylyl-3′,5′-*O*-methyl phosphate (18). To a solution of dithymidylyl-3′,5′-phosphorofluoridate (**7**) (0.020 g, 0.036 mmol) in methanol (0.5 mL) was added triethylamine (0.025 mL, 0.182 mmol). ^{31}P NMR spectrum showed the reaction was completed after 1 h. The crude product was purified by preparative TLC (developing system: chloroform/ethanol, 4:1 (v/v)). Product **18** was obtained with 65% yield (0.013 g): TLC (chloroform/ethanol 4:1) R_f 0.45, ^{1}H NMR (CD_3OD) δ 1.88 (s, 3H), 1.90 (d, J = 1.1 Hz, 3H), 2.28 (m, 4H), 3.78 (m, 2H), 3.84, 3.85 (pair of d, J = 11.4 Hz, 11.3 Hz, 3H), 4.33 (m, 4H), 4.40 (m, 1H), 5.06 (m, 1H), 6.28 (m, 2H) 7.54 (m, 1H), 7.78 (m, 1H); ^{31}P NMR (CH_3OD): 0.22, 0.06; MS (−FAB) m/z 559.3 (M−H)

Dithymidylyl-3′,5′-*O*-methyl phosphorothioate (19). To a solution of dithymidylyl-3′,5′-phosphorothiofluoridate (**14**) (0.037 g, 0.07 mmol) in methanol (0.6 mL) was added triethylamine (0.05 mL, 0.36 mmol). ^{31}P NMR spectrum showed the reaction was completed after 2.5 h. The crude product was purified by preparative TLC (developing system: chloroform: ethanol, 4:1 (v/v)). Product **19** was obtained with 45% yield (0.018 g): TLC (chloroform/ethanol 4:1) R_f 0.75, ^{1}H NMR (CD_3OD) δ 1.88 (s, 3H), 1.90 (d, J = 1.1 Hz, 3H), 2.28 (m, 4H), 3.78 (m, 2H), 3.84, 3.85 (pair of d, J = 11.4 Hz, 11.3 Hz, 3H), 4.33 (m, 4H), 4.40 (m, 1H), 5.06 (m, 1H), 6.28 (m, 2H) 7.54 (m, 1H), 7.78 (m, 1H); ^{31}P NMR (CD_3OD): 70.01, 69.89; MS (−FAB) m/z 575.0 (M−H), (+FAB) m/z 577.6 (M+H)

Dithymidylyl cyclic-3′,5′-phosphate (20). To a solution of dithymidylyl-3′,5′-phosphorofluoridate (**7**) (0.020 g, 0.036 mmol) in anhydrous acetonitrile (0.5 mL) was added DBU (0.027 mL, 0.182 mmol). ^{31}P NMR spectrum showed completition of the reaction after 1 h. The crude product was purified by preparative TLC (developing system: chloroform/ethanol, 4:1 (v/v)) Product **20** was obtained with 52% yield (0.010 g): TLC (chloroform/ethanol 4:1) R_f 0.51, ^{1}H NMR (CD_3CN) δ 1.26 (s, 6H), 2.50 (m, 4H), 3.52 (m, 1H), 4.01 (m, 2H), 4.35 (m, 1H), 4.54 (m, 1H), 4.92 (m,1H), 6.21 (m, 2H), 7.36 (m,2H); ^{31}P NMR (CD_3CN) δ −3.17, −4.62 (1:2.2), MS (−FAB) m/z 527.3 (M−H).

Dithymidylyl cyclic-3′,5′-phosphorothioate (21). To a solution of dithymidylyl-3′,5′-phosphorothiofluoridate (**14**) (0.041 g, 0.072 mmol) in anhydrous acetonitrile (0.6 mL) was added DBU (0.055 mL, 0.37 mmol). ^{31}P NMR spectrum showed completition the reaction after 2.5 h. The crude product was purified by preparative TLC (developing system: chloroform:ethanol, 4:1 (v/v)) Product **21** was obtained with 23% yield (0.009 g): TLC (chloroform/ethanol 4:1) R_f 0.80, ^{1}H NMR (CD_3CN) δ 1.28 (s, 6H), 2.53 (m, 4H), 3.50 (m, 1H), 4.02 (m, 2H), 4.32 (m, 1H), 4.54 (m, 1H), 4.92 (m,1H), 6.23 (m, 2H), 7.38 (m,2H); ^{31}P NMR (CD_3CN) δ 66.30, 63.66 (2.9:1), MS (−FAB) m/z 543.4 (M−H).

Enzyme inhibition studies. Dithymidylyl-3′,5′-phosphate (**16**) (0.1 mM) and svPDE (0.005 U/mL) in 0.1 M Tris–HCl pH 8.0 containing 20 mM $MgCl_2$, with or without **7** or **14** (both 0.05, 0.1, or 0.2 mM) added (total volume 1000 μL), were incubated at 37 °C for 20, 40, and 60 min. After each time samples were analysed on HPLC. In an analogous way, experiments with sPDE (0.15 U/mL) in 0.05 M acetate buffer pH 5.0 were performed.

Adenosine 5′-*O*-monophosphate (0.1 mM) and AP (150 U/mL) in 0.05 M Tris–HCl pH 8.0 containing 5 mM $MgCl_2$ with or without **7** or **14** (both 0.05, 0.1, or 0.2 mM) added (total volume 1000 μL), were incubated at 37 °C for 20, 40, and 60 min. After each time samples were analysed on HPLC.

Mice toxicity studies. Compounds **7** and **14** were dissolved and diluted in the Hanks buffer immediately before injections. Doses 2.5, 5.0 and 10.0 mg/kg were given intraperitoneally to male mice CD2F1 (groups of 5). No mortality was observed during 3 weeks. In treated mice the loss of weight was dose-dependent and was not more then 10% in the highest dose.

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