Acta Biochimica Polonica

Vol. 45 No. 1/1998

83–86

QUARTERLY

Synthesis and interaction with thymidylate synthase of 5′-dithiophosphate and 5′-fluorothiophosphate of thymidine[*][o]

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Key words: thymidylate synthase, thymidine 5′-dithiophosphate, thymidine 5′-fluorothiophosphate

Thymidine-5′-fluorothiophosphate, dTMP(S)-F, was synthesized by the oxathiaphospholane, and thymidine 5′-dithiophosphate, $dTMPS_2$, by the dithiaphospholane, method. To estimate the role of 5′-phosphate group ionization in binding of pyrimidine nucleotides by thymidylate synthase, dTMP(S)-F was studied as an inhibitor of mouse tumour (L1210) enzyme, and its inhibitory properties were compared with those of $dTMPS_2$, a close dTMP analogue. While $dTMPS_2$ proved to be an inhibitor, competitive *vs* dUMP, with K_i^{app} = 94 μM, the 5′-fluorothiophosphate congener displayed no activity, indicating that the enzyme requires for binding the presence of a dianionic 5′-phosphate group in a nucleotide.

Thymidylate synthase (EC 2.1.1.45) catalyzes C(5) methylation of 2′-deoxyuridylate (dUMP) in a concerted transfer and reduction of the one-carbon group (at the aldehyde oxidation level) of $N^{5,10}$-methylenetetrahydrofolate, with concomitant production of thymidylate (dTMP) and dihydrofolate [1]. As the sole *de novo* source of thymidylate synthesis in cells, it is a target in chemotherapy [2]. In order to investigate the importance of 5′-phosphate group ionization for binding of pyrimidine nucleotides by the enzyme, we have synthesized thymidine 5′-fluorothiophosphate, dTMP(S)-F, having only one anionic charge at physiological pH. This analogue was studied as an inhibitor of the reaction catalyzed by highly purified thymidylate synthase from mouse leukemia L1210 cells and its inhibitory

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[*] Presented as a poster at the 6th International Symposium on Molecular Aspects of Chemotherapy, July, 1997, Gdańsk, Poland.

[o] Supported by the State Committee for Scientific Research (KBN, grants No. 4/P05F/030/11p02 and 4/P05F/023/10).

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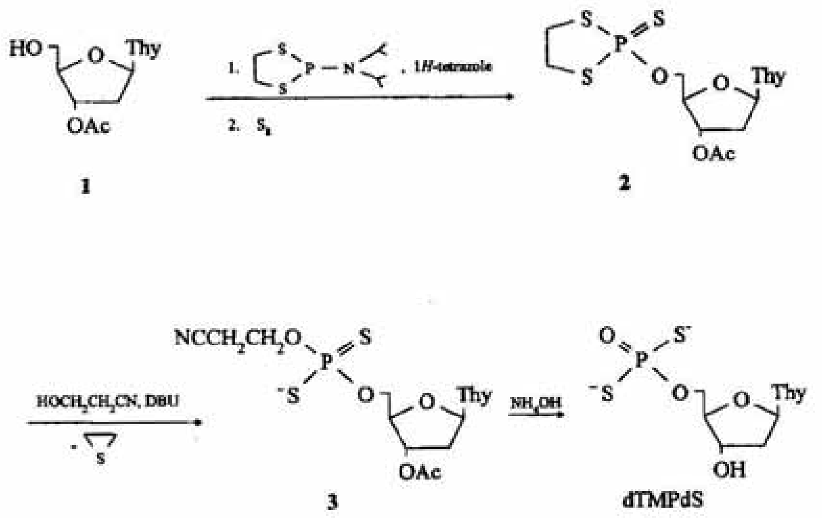
Abbreviations: DBU, 1,8-diazabicyclo[5.4.0]-undec-7-ene; $dTMPS_2$, thymidine 5′-dithiophosphate; dTMP(S)-F, thymidine-5′-fluorothiophosphate.

properties compared with those of thymidine 5′-dithiophosphate (dTMPS$_2$), a close dTMP analogue.

MATERIAL AND METHODS

Syntheses. dTMPS$_2$ was synthesized by the dithiaphospholane method [3, 4] (Scheme 1). Phosphitylation of 3′-*O*-acetylthymidine **1** with *N,N*-diisopropyl-1,3,2-dithiaphospholane in the presence of 1*H*-tetrazole led to the intermediary phosphite which was oxidized by elemental sulfur to give dithiaphospholane **2** in 77% yield. Compound **2** reacted with 2-cyanoethanol in the presence of 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) to yield the protected phosphorodithioate **3** which was deprotected (without isolation) by concentrated ammonia solution giving, after purification on Sephadex A-25, dTMPS$_2$ in 52% yield. A detailed description of the synthesis has been already published [5].



Scheme 1.

dTMP(S)-F was synthesized by the oxathiaphospholane method [6], presented on Scheme 2. The 3′-hydroxy moiety of thymidine was protected with a methoxyacetyl group to allow deprotection under conditions milder than those required to remove the acetyl group. 3′-*O*-Methoxyacetylthymidine **4** was phosphitylated by *N,N*-diisopropylamino-1,3,2-xathiaphospholane in the presence of 1*H*-tetrazole. The resulting phosphite was oxidized *in situ* by elemental sulfur to give oxathiaphospholane **5** as a mixture of diastereomers (1:1) in 70% yield. Compound **5** reacted with triethylammonium fluoride in the presence of DBU. A fast oxathiaphospholane ring-opening process, with subsequent elimination of episulfide, leading to protected phosphorothiofluoridate **6** (1:1 mixture of diastereomers), was observed. Compound **6** was deprotected (without isolation) by its exposure to concentrated ammonia solution for 2 h at room temperature. Crude dTMP(S)-F was purified by chromatography on Sephadex A-25 using a 0.02–0.4 M linear gradient of triethylammonium bicarbonate buffer, pH 7.5, as an eluent. DTMP(S)-F (1:1 mixture of diastereomers) was obtained in 83% yield. Its 99% purity was confirmed by ^{19}F- and ^{31}P-NMR, and RP-HPLC.

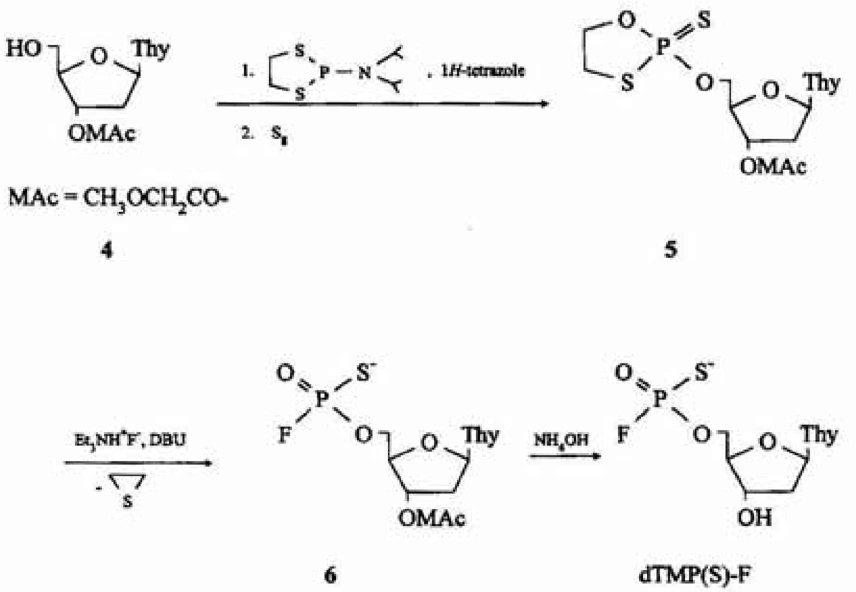
Thymidylate synthase. Highly purified preparation of thymidylate synthase from mouse leukemia L1210 cells [7] was used.

Enzyme assays. The [5-^{3}H]dUMP tritium release assay was employed [7].

Kinetic studies. Identification of the type of inhibition involved was performed as previously described [7].

RESULTS AND DISCUSSION

The one-pot dithiaphospholane method of dTMPS$_2$ synthesis [5] seems to be superior to another approach described earlier [8], involving synthesis and isolation of appropriately protected thymidine 5′-*O*-dithio-*H*-phosphonate and its oxidative phosphorylation with fluorenemethanol in the presence of iodine. The fluorenemethyl diester intermediate has to be isolated and then hydrolyzed with ammonia to give dTMPS$_2$ in 22% total yield, while our method provides the product in 52% yield without the necessity of purification of the intermediary phosphodiester.



Scheme 2.

The oxathiaphospholane method was also used for synthesis of dTMP(S)-F. 3′-*O*-Dimethoxytrityl-protected dTMP(S)-F was recently synthesized by phosphoramidite [9] and *H*-phosphonate [10] approaches but no attempts to get the final dTMP(S)-F were undertaken. The oxathiaphospholane method of synthesis of dTMP(S)-F is simple (2 steps) and efficient (58% yield starting from 3′-*O*-protected thymidine).

Inhibition of thymidylate synthase by dTMPS$_2$, due to competition *vs* dUMP, was examined by varying the dUMP concentration at different concentrations of the inhibitor, added simultaneously to the reaction mixture. dTMPS$_2$ proved to be an inhibitor, competitive *vs* dUMP (reflected by intersection at the ordinate of Lineweaver-Burk plots), with K_i^{app} = 94 μM, as compared to 12 μM for dTMP and 32 μM for thymidine 5′-monothiophosphate [11]. By contrast, the 5′-fluorothiophosphate analogue (dTMP(S)-F), tested at concentrations up to 1 mM and in the presence of only 5 μM dUMP (its potential competitor) in the reaction mixture, displayed no inhibitory activity. Thus, while dTMP with dithio substitution in the 5′-phosphate inhibits the enzyme 8-fold weaker than the parent compound, a single fluoro substitution abolishes the interaction, pointing to its dependence on the dianionic form of the phosphate group. The same conclusion was suggested by the results of studies on thymidylate synthase interaction with *H*-phosphonates of dUrd and its analogues, and on pH-dependence of the enzyme inhibition by 5′-thiophosphate of 5-fluorodeoxyuridine [11].

A similar requirement for a nucleotide with a dianionic phosphate group has been described for the nucleotide activator site of glycogen phosphorylase *b* [12].

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