METHYLATION OF 4-THIO-2'-DEOXYURIDYLATE BY THYMIDYLATE SYNTHETASE

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Summary: 4-Thio-2'-deoxyuridylate was found to be a substrate for the thymidylate synthetase of a dichloromethotrexate resistant strain of Lactobacillus casei. The enzymic product which contained the carbon derived from formaldehyde, was identified as 4-thiothymidylate by chromatographic analysis. 4-Thio-2'-deoxyuridylate inhibited the methylation of deoxyuridylate with an apparent K_i of 7×10^{-5} M, reflecting its lower affinity for the enzyme as compared to the natural substrate. 4-Thio-2'-deoxyuridylate is, so far, the only substrate analog modified in the base which is methylated by thymidylate synthetase.

4-Thiouridine, a minor component of tRNA (1), is believed to be snythesized in the cells at the macromolecular level (2,3). When added exogenously, it was found to inhibit the growth of various microbial and mammalian cells in culture (4,5). Presumably, the cytotoxic activity of 4-thiouridine is related to its ability to act as an analog of uridine, with which it might compete at the level of their corresponding nucleotides for various enzymes involved in the biosynthesis of RNA or DNA. Results (to be published) of an inhibition analysis in microbial system (6) indicated that one of the possible biochemical targets of the action of 4-thiouridine in the cells is the thymidylate synthetase reaction; this presupposes prior metabolic conversion of this ribonucleoside analog to 4-thio-dUMP which is the corresponding structural analog of the natural substrate of thymidylate synthetase. Therefore, 4-thio-dUMP was synthesized and studied as a substrate and as an inhibitor of a purified thymidylate synthetase from bacterial cells.

MATERIALS AND METHODS

4-Thio-2'-deoxyuridine-5'-phosphate (4-thio-dUMP) was synthesized by two alternative procedures. Thiation of 5'-O-trityl-3'-O-acetyl-2'-deoxyuridine (7) with P_2S_5 in pyridine (8), followed by selective removal of the 5'-O-trityl

group, yielded 3'-O-acetyl-2'-deoxy-4-thiouridine. This was then phosphorylated, using p-nitrophenylphosphorodichloridate, and the 3'-O-acetyl group was removed together with the p-nitrophenyl group in the final hydrolysis step (0.1 N NaOH, 100°, 2 hrs). Alternatively, the 3'-O-acetyl-2'-deoxy-4-thiouridine was deacetylated with $NaOCH_3$/MeOH, yielding 4-thio-2'-deoxyuridine which was phosphorylated with $POCl_3$ in $(EtO)_3PO$ (9). Both 4-thio-dUMP products were purified by preparative TLC on cellulose and gave identical R_f values: 0.56 and 0.38, using BuOH-AcOH-H_2O (5:2:3, v/v) and 1 M NH_4OAc-EtOH (2:3, v/v), respectively, as the solvents. The UV spectra of the two preparations were also identical: λ_{max} 330 (ϵ 20,000) at pH 2. Franke et al. (10) previously reported the synthesis of 4-thio-dUMP by a different route, and gave an R_f value (0.26, with 1 M NH_4OAc-EtOH, 2:5, v/v) but no other data for identification of their product.

Radioactive 4-[^{35}S]thiothymidine (spec. act. 30 μCi/μmole) was prepared by application of the general method of Morávek and Kopecký (11), that is, reacting elemental [^{35}S]sulfur (Amersham/Searle, 50 mCi/mg) with 4-thiothymidine in refluxing pyridine. The product was purified by repeated TLC on cellulose, using the following solvents: iPrOH-NH_4OH-H_2O (7:2:1, v/v) and BuOH-AcOH-H_2O, (5:2:3, v/v).

Radioactive 4-[^{35}S]thiothymidylate (spec. act. 26 μCi/μmole) was prepared by phosphorylation of 4-[^{35}S]thiothymidine with E.coli B thymidine kinase (12) and isolated by preparative TLC on cellulose (1 M NH_4OAc:EtOH, 2:3, v/v).

Thymidylate synthetase of a dichloromethotrexate resistant strain of Lactobacillus casei was purified according to the method of Leary and Kisliuk (13). Enzyme activity was assayed (a) by the spectrophotometric method of Wahba and Friedkin (14), (b) by measuring the incorporation of [^{14}C] formaldehyde into the nucleotide product, and (c) by employing the tritium release assay of Lomax and Greenberg (15), essentially as described by Dunlap et al. (16). In Method (a), the assay mixture contained the following: Tris-acetate, 70 mM, pH 7.4; mercapto-ethanol, 100 mM; $MgCl_2$, 20 mM; d,l-L-tetrahydrofolate (Sigma), 0.3 mM; HCHO, 9 mM: EDTA, 0.3 mM; dUMP (Calbiochem), 0.25 mM and sufficient

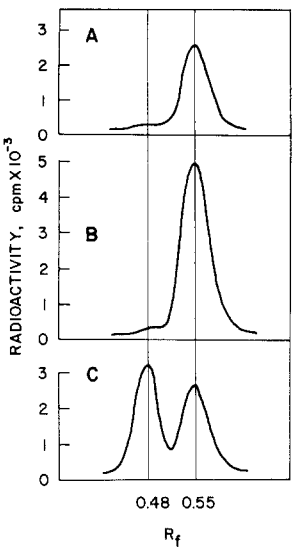


Figure 1. Paperchromatographic analysis of the product formed from 4-thio-dUMP by the action of thymidylate synthetase. 4-Thio-dUMP, 1 mM, was incubated at 30° for 60 min in the assay mixture of Method (b). After cooling to 0°, 25 µl saturated dimedon solution (50% aq. EtOH) was added and the mixture was centrifuged. On Whatman No. 4 paperstrips, 20 µl aliquots of the supernatant were spotted and after horizontal chromatography, using BuOH:AcOH:H_2O (5:2:3, v/v) as solvent, the radioactivity was measured as described in Method (b). A, ^{14}C-product alone; B, ^{14}C-product + 4-[^{35}S]thio-dTMP marker; C, ^{14}C-product + [^{14}C]dTMP marker (New England Nuclear, spec. act. 5 mCi/mmole, 0.02 µCi).

enzyme to produce an absorbance change of 0.010-0.015 unit/min at 340 nm. In Method (b), the assay mixture was the same, except that 2 mM [^{14}C]formaldehyde (spec. act. 6 mCi/mmole) and 14µg of the enzyme (spec. act. 1.7 µmoles/mg/ml) were used in a total volume of 0.1 ml. In Method (c), the unlabelled dUMP in the assay mixture of Method (a) was replaced by 0.01 mM of 5-[^{3}H]dUMP (New England Nuclear, 200 mCi/mmole).

In Method (a), the spectrophotometric measurements were conducted with a Gilford 2000 multiple absorbance recorder. In Method (b), radioactivity was measured with a Nuclear Chicago Actigraph III paperstrip scanner at a 3 mm window slit setting and 30 cm/hr chart speed, and in Method (c), by scintillation spectrometry, using a Packard Model 3320 counter.

RESULTS AND DISCUSSION

Using the spectrophotometric assay according to Method (a), no thymidylate

synthetase activity was detected when dUMP was replaced by 4-thio-dUMP as the substrate, at concentrations of 10^{-6} to $5x10^{-5}$ M. However, these results were considered as uncertain because of the high UV absorbance of the 4-thiopyrimidine chromophore at the 340 nm operating wavelength of the assay, which prohibited measurements at higher concentrations of this compound. Therefore, Method (b) was used in subsequent studies to determine the substrate activity of 4-thio-dUMP by measuring the incorporation of [^{14}C]formaldehyde into the nucleotide product.

When 4-thio-dUMP was incubated with purified thymidylate synthetase in the presence of tetrahydrofolate and [^{14}C] formaldehyde, a radioactive product could be separated from the reaction mixture by paper chromatography, as shown by the results of a typical experiment represented in Fig. 1A. This product had the same mobility as 4-[^{35}S]thio-dTMP prepared by enzymatic phosphorylation of 4-[^{35}S]thiothymidine. As seen in Fig. 1B, the ^{14}C-product of the thymidylate synthetase reaction could not be separated from the 4-[^{35}S]thio-dTMP used as a marker. In contrast, this product readily separated from a [^{14}C]dTMP marker as shown in Fig. 1C, demonstrating that the product is distinctly different from dTMP which, conceivably, could have been formed in the thymidylate synthetase reaction following hydrolysis of the 4-thio-dUMP. (A trace amount of [^{14}C]dTMP was indeed formed in the reaction, and was detectable as a minor radioactive peak at R_f 0.48, as shown in Fig. 1A and 1B). No radioactive substance could be detected in the entire paper chromatographic region shown in Fig. 1 when either 4-thio-dUMP or the enzyme was omitted from the reaction mixture.

In order to further substantiate the identity of the enzymic product formed from 4-thio-dUMP, a chromatographic analysis of the dephosphorylated nucleoside derivative was performed. The enzymic conversion of 4-thio-dUMP was carried out with a 10-fold concentration of tetrahydrofolate and thymidylate synthetase, using unlabelled HCHO, and the reaction mixture was subsequently treated with snake venom 5'-nucleotidase (Sigma), under conditions previously described (17). The final product of these sequential enzymic reactions was identified as 4-

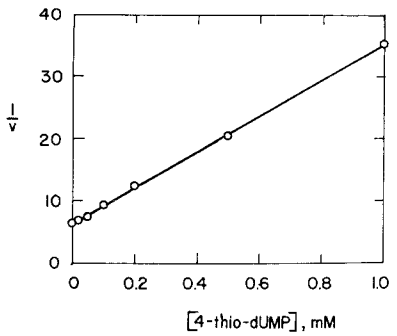
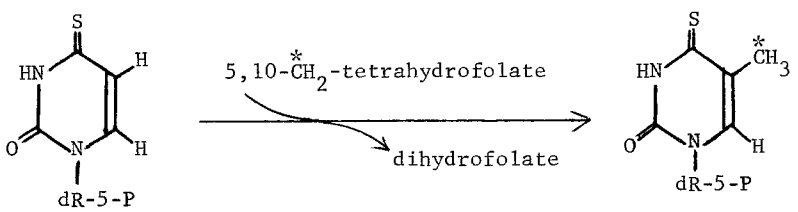


Figure 2. Dixon plot of the inhibition of thymidylate synthetase by 4-thio-dUMP. The release of ^{3}H from the substrate 5-[^{3}H]dUMP was measured in the presence of varying concentrations of 4-thio-dUMP according to Method (c); v = mμmoles of 5-[^{3}H]dUMP converted/30 min at 30°.

thiothymidine by TLC on cellulose, made visible by its characteristic bright yellow fluorescence under long wavelength UV-light. Its mobility was identical with that of authentic 4-thiothymidine: R_f 0.84 and 0.92, in the two solvent systems, BuOH:AcOH:H_2O (5:2:3, v/v) and 1M NH_4OAc:EtOH (2:3,v/v), respectively. In a parallel control experiment, omitting the thymidylate synthetase from the initial reaction mixture, the treatment with 5'-nucleotidase yielded only the dephosphorylated starting material, 4-thio-2'-deoxyuridine: R_f 0.75 and 0.87, respectively, in the same two solvent systems.

These results demonstrate that thymidylate synthetase catalyses the incorporation of the ^{14}C-label of formaldehyde into 4-thio-dUMP to yield 4-thio-dTMP. Thus, we conclude that 4-thio-dUMP is utilized as a substrate by thymidylate synthetase in a reaction which, by analogy with the conversion of the natural substrate (18), may be represented by the following scheme:



It should be pointed out that 4-thio-dUMP is so far the only dUMP analog containing a modified base which can serve as a substrate for thymidylate syn-

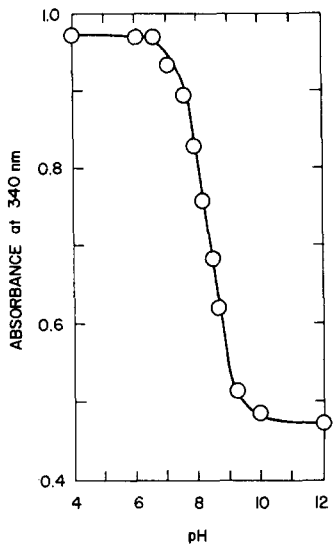


Figure 3. Spectrophotometric titration curve of 4-thio-dUMP. The absorbance values (340 nm) of 0.05 mM 4-thio-dUMP in 0.1 M EDTA solutions (containing 1.0 mM dithiothreitol) were determined at 25° in a Beckman DU spectrophotometer.

thetase. This substrate specificity is compatible with the proposed nucleophilic addition - elimination mechanism of the thymidylate synthetase - catalyzed reaction (19, 20) which requires a particular electronic configuration of the participating - CH = CH - C = X portion of the substrate.

In order to determine the effect of 4-thio-dUMP on the methylation of dUMP by thymidylate synthetase, the tritium release assay was conveniently employed, as described in Method (c). Fig. 2 shows the decrease of the rate of methylation of dUMP with increasing concentration of 4-thio-dUMP. From the data presented in the form of a Dixon plot, an apparent K_i of 7×10^{-5} M was calculated fro 4-thio-dUMP, using the spectrophotometrically determined K_m of 5×10^{-6} M for dUMP. Thus, the 4-thio analog has a substantially lower affinity for the enzyme than the natural substrate dUMP, as reflected by the difference between their apparent dissociation constants.

It has been proposed (21) that the ionization of the N-3 proton has a significant role in the binding of pyrimidine nucleotides to thymidylate synthetase, and a lower pK_a was believed to correlate with a greater affinity. The "anom-

alously" high activity of 5-mercapto-dUMP (in which the N-3 proton has a pK_a of 10.5) as an inhibitor of thymidylate synthetase was explained by the contribution of the ionized 5-mercapto group to the binding (22). Therefore, it was of interest to determine the pK_a of the N-3 proton of 4-thio-dUMP which has no additional ionizable group. The spectrophotometric titration curve of the latter analog is shown in Fig. 3; from the data points a pK_a of 8.28 was calculated. This is significantly lower than that of dUMP (pK_a 9.45) and, in fact, indicates that the N-3 proton of 4-thio dUMP is partially ionized at the pH of the assay. Thus, in the present case, there is actually an inverse relationship between the extent of ionization of these nucleotides and their relative strength of binding to thymidylate synthetase.

With respect to the mode of action of 4-thiouridine, the results of the present study are compatible with thymidylate synthetase being one of the target sites, provided that this nucleoside is metabolically converted to 4-thio-dUMP. The latter could contribute to the over-all cytotoxicity of 4-thiouridine (a) by inhibiting the synthesis of dTMP, and (b) by being further converted to 4-thio-dTMP in which form it could then inhibit subsequent steps of DNA synthesis. However, there are other aspects of the mode of action of 4-thiouridine which will be reported elsewhere.

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