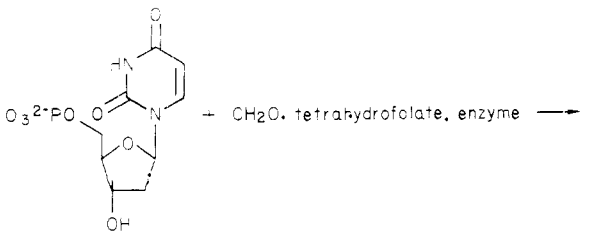
5-[(4-Methyl-1,2,3,4-tetrahydroquinoxalyl)methyl]-2′-deoxyuridine 5′-Phosphate: An Analogue of a Proposed Intermediate in Thymidylate Synthetase Catalysis

Joon Sup Park, Charles T.-C. Chang, and Mathias P. Mertes*

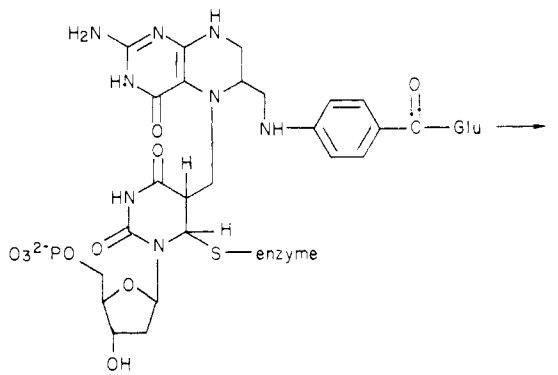
Department of Medicinal Chemistry, School of Pharmacy, University of Kansas, Lawrence, Kansas 66045.
Received March 23, 1979

In a study of the sequence steps involved in the mechanism of thymidylate synthetase catalysis, 5-[(*N*-methylpiperazinyl)methyl]- (**5**) and 5-[(4-methyl-1,2,3,4-tetrahydroquinoxalyl)methyl]-2′-deoxyuridine 5′-phosphate (**6**) were synthesized. Compound **6** has high affinity for the *Lactobacillus casei* enzyme (K_i = 0.75 μM, K_I/K_m = 0.23), which is 50 times stronger than that of the piperazinyl derivative **5**. Compound **6**, a possible multisubstrate inhibitor, is an analogue of a proposed intermediate in the reaction mechanism wherein the enzyme is eliminated from the covalent complex (enzyme–substrate–cofactor) prior to the redox reaction leading to the products 2′-deoxythymidine 5′-phosphate and 7,8-dihydrofolic acid.

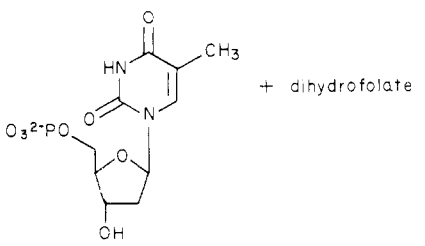
Thymidylate synthetase catalyzes the conversion of 2′-deoxyuridine 5′-phosphate (**1**, dUMP) to 2′-deoxythymidine 5′-phosphate (**3**, dTMP).[1] The chemistry of the reaction can be formulated in two steps: alkylation of the N^5,N^{10}-methylenetetrahydrofolic acid[2] by carbon-5 of the enzyme-activated substrate, followed by rearrangement of the methylene-bridged tetrahydrofolate derivative of the substrate to give the product, dTMP, and 7,8-dihydrofolate. Considerable evidence derived from model studies using 5-fluoro-2′-deoxyuridine 5′-phosphate supports the view that activation of carbon-5 of the substrate is accomplished by addition of a cysteine SH at the active site of thymidylate synthetase to the carbon-6 of the active-site bound substrate.[3]



1

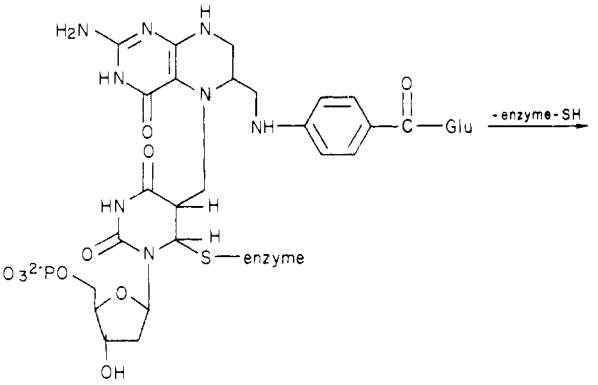


2

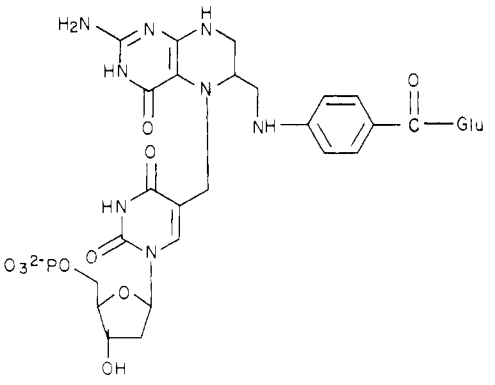


3

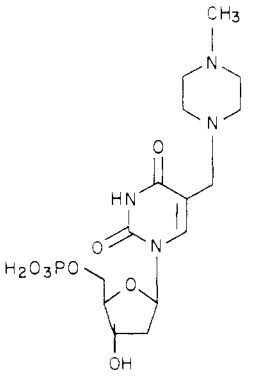
The sequence of reactions beyond the initial covalently bound enzyme–substrate–CH_2–cofactor complex (**2**) is unknown; however, several reasonable pathways can be formulated. Two chief questions addressed in these postulated sequences are the stage at which enzyme is eliminated and the mechanism of the redox rearrangement. If cleavage of the enzyme–complex covalent bond with subsequent re-formation of the sp^2 carbons at positions 5 and 6 of the pyrimidine ring precedes redox rearrangement, then a noncovalently bound enzyme complex such as **4**



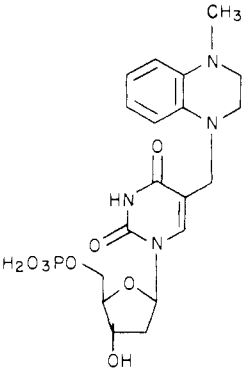
2



4



5



6

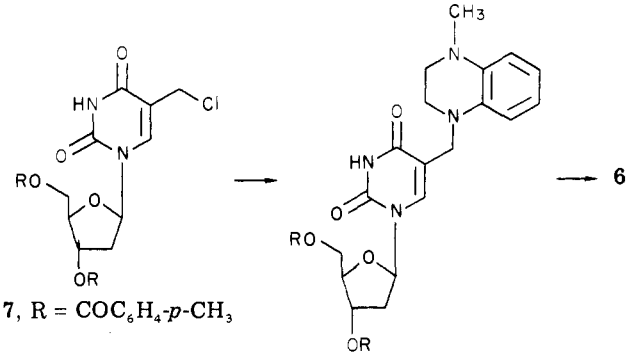
0022-2623/79/1822-1134$01.00/0

would be an intermediate in the reaction.

Compounds **5** and **6** containing the substrate bound through the methyl carbon to amino derivatives having electron-density characteristics of the N_5 and N_8 of the cofactor could act as multisubstrate inhibitors of the enzyme.

The synthesis of 5-[(*N*-methylpiperazinyl)methyl]-2′-deoxyuridine has been described.[4] Conversion to the 5′-phosphate was accomplished by the procedure of Sowa and Ouchi.[5] The product **5** was separated in low yield from the 3′-phosphate[4] by gradient-elution chromatography on DEAE-cellulose.

The starting material for the synthesis of **6** was the ditoluoyl ester of 5-(chloromethyl)-2′-deoxuridine[6] (**7**).



7, R = COC_6H_4-*p*-CH_3

8, R = COC_6H_4-*p*-CH_3
9, R = H

Treatment with 1-methyltetrahydroquinoxaline gave a 70% yield of **8**, which was found to be light sensitive during workup. Transesterification of **8** gave the nucleoside **9**, which was converted to the nucleotide **6** by treatment with phosphorus oxychloride in pyridine, water, and acetonitrile.[5]

Thymidylate synthetase from amethopterin-resistant *Lactobacillius casei* was used in the inhibition studies. The substrate K_m for dUMP in these studies was found to be 3.2 μM using a radioisotope assay; this compares to a range of 0.7–5.2 μM reported for this enzyme using a spectrophotometric assay.[7,8] Figure 1 is a double-reciprocal plot showing the inhibition due to two concentrations of the *N*-piperazinyl compound **5**; the K_i calculated from the slope is 37 μM. Similarly, Figure 2 shows the inhibition due to the 1 and 2 μM concentration of the *N*-methyltetrahydroquinoxaline derivative **6**. The K_i calculated from the slopes of the inhibited reaction is 0.75 μM.

Since compound **6**, an analogue of intermediate **4** in the enzyme-catalyzed reaction, could function as a multisubstrate inhibitor, the nature of the inhibition of the cofactor was examined. The K_m for *dl*-tetrahydrofolate, as determined from the double-reciprocal plot (Figure 3), was 5.3 μM using the radioisotope assay. As noted in Figure 3, compound **6** shows noncompetitive or mixed inhibition kinetics for the cofactor, which is not unusual considering the report of Daron and Aull[7] wherein 7,8-dihydrofolate shows a similar pattern. The K_i for **6** as a cofactor inhibitor calculated from the slopes of the lines in Figure 3 is 42 μM.

Thymidylate synthetase from several species has shown tolerance for large substituents in the 5 position of the substrate, dUMP.[9] With few exceptions,[3] those being strong electron-withdrawing groups such as formyl, mercapto, hydroxymethyl, halo, and trifluoromethyl, 5-substituted 2′-deoxyuridine 5′-phosphates have relatively low affinity for the enzyme. Compound **5**, the *N*-methylpiperazinylmethyl derivative of dUMP, also has low affinity. However, the aromatic amino derivative **6** clearly

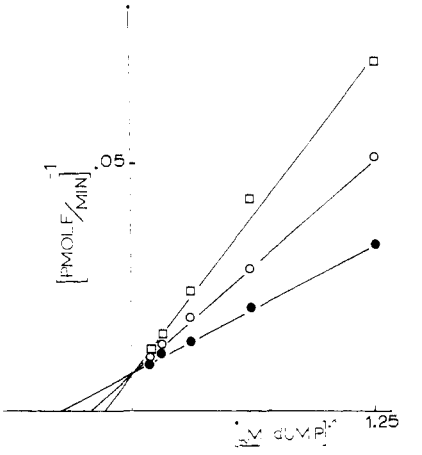


Figure 1. Double-reciprocal plot of the velocity of formation of ^3H_2O vs. concentration of substrate, 2′-deoxyuridine 5′-phosphate (dUMP), with and without 5-[(*N*-methylpiperazinyl)methyl]-2′-deoxyuridine 5′-phosphate (**5**): no inhibitor (●); 25 μM inhibitor (○); 50 μM inhibitor (□).

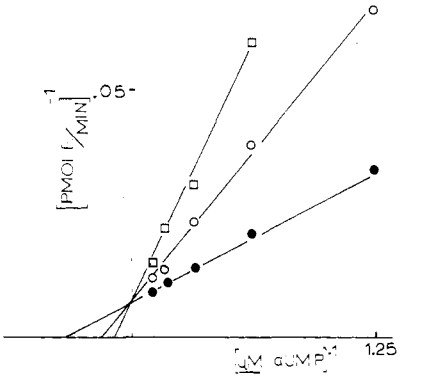
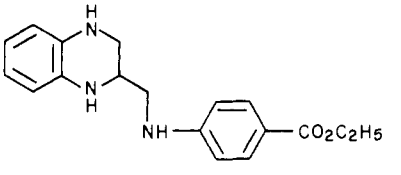
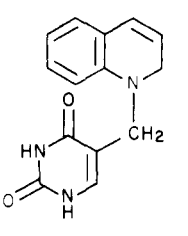


Figure 2. Double-reciprocal plot of the velocity of formation of ^3H_2O vs. concentration of substrate, 2′-deoxyuridine 5′-phosphate (dUMP), with and without added inhibitor 5-[(4-methyl-1,2,-3,4-tetrahydroquinoxalyl)methyl]-2′-deoxyuridine 5′-phosphate (**6**): no inhibitor (●); 1 μM inhibitor (○); 2 μM inhibitor (□).

is an effective inhibitor with enzyme affinity greater than that of the substrate. The binding free-energy difference between **5** and **6** is approximately 2.3 kcal/mol in favor of **6** which could be accounted for by an additional binding site contributing to complex formation. Piperazine has a pK_a of 9.6, and in compound **5** the piperazine ring would be protonated at the pH of the enzyme assay (pH 6.8). In contrast, the pK_a of the quinoxaline ring nitrogen in the tetrahydrofolate analogue **10** is 4.35.[10] Thus, ring nitrogens



10



11

in the quinoxaline analogue **6** would be expected to be unprotonated at pH 6.8 and simulate the charge density of the N_5 nitrogen in tetrahydrofolate, which is reported to have a pK_a of 4.82.[11] Accordingly, the strong affinity

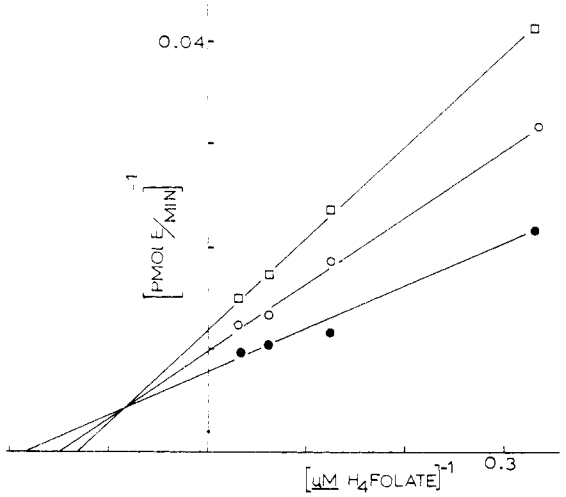


Figure 3. Double-reciprocal plot of the velocity of formation of ^3H_2O vs. concentration of cofactor, *dl*-tetrahydrofolic acid (H_4-folate), with and without added inhibitor 5-[(4-methyl-1,2,3,4-tetrahydroquinoxalyl)methyl]-2′-deoxyuridine 5′-phosphate (**6**): no inhibitor (●); 25 μM inhibitor (○); 50 μM inhibitor (□).

of compound **6** for the enzyme and the low affinity of compound **5** are consistent with the sequence of thymidylate catalysis as proceeding through intermediate **4** by elimination of the enzyme nucleophile from intermediate **2**. Subsequent steps in the reaction would be the redox rearrangement of **4** leading to the products dTMP and dihydrofolate.

A chemical model demonstrating the feasibility of the redox reaction has been reported wherein compound **11**, on heating, gives thymine.[12] When compound **6** was heated to the melting point, we could not detect any thymidine 5′-phosphate.

Experimental Section

IR spectra are measured with a Beckman IR-33, UV spectra with a Cary 219 recording spectrophotometer, and NMR spectra with a Varian Model EM-360 or T-60. Microanalyses were obtained from a Hewlett-Packard 185B at the Department of Medicinal Chemistry, University of Kansas. DEAE-cellulose was the product of Whatman Biochemicals Ltd.

Thymidylate synthetase purified from methotrexate-resistant *Lactobacillus casei* was purchased from the New England Enzyme Center, Tufts University, at a specific activity of 1.1 μmol of TMP formed min^{-1} (mg of protein)$^{-1}$. The substrate 2′-deoxy[5-^{3}H]-uridine 5′-monophosphate at a specific activity above 15 Ci/mmol was purchased from Moravek Biochemicals, Industry, Calif., and diluted with cold substrate purchased from Sigma Chemical Co., St. Louis. The cofactor *dl*-tetrahydrofolic acid was also purchased from Sigma Chemical Co.

Triethylammonium Salt of 5-[(*N*-Methylpiperazinyl)-methyl]-2′-deoxyuridine 5′-Phosphate (5). The same procedure as described in the synthesis of compound **6** was used, with the exception that the product is not light sensitive and workup can be done in light. The reaction temperature was 0 °C using 5-[(*N*-methylpiperazinyl)methyl]-2′-deoxyuridine dihydrochloride[4] (205 mg, 0.5 mmol), acetonitrile (500 μL), water (25 μL), phosphorus oxychloride (200 μL), and pyridine (190 μL). The reaction mixture was stirred for 6 h at 0 °C. The product was obtained after resolution on two DEAE-cellulose columns to yield 35 mg (12%) of **5** as a white hygroscopic powder: UV (0.1 N HCl) λ_{max} 266 nm (ε 9000), λ_{min} 237 (2800); UV (H_2O) λ_{max} 264 nm (ε 9300), λ_{min} 237 (2800); UV (0.1 N NaOH) λ_{max} 264 nm (ε 6500), λ_{min} 243 (4700); NMR (Me_2SO-d_6) δ 8.0 (s, 1, H-6), 6.2 (t, 1, H-1′), 4.7 (s, 2, CH_2N), 4.6–2.0 (m, unresolved), 1.9 (m, 2, H-2′), 1.1 (t, 9, CH_2CH_3). Anal. ($C_{15}H_{25}N_4O_8P \cdot C_6H_{15}N \cdot H_2O$), M_r 539.6, C, H, N.

1-(3,5-Di-*O*-*p*-toluoyl-2-deoxy-β-D-ribofuranosyl)-5-[(4-methyl-1,2,3,4-tetrahydroquinoxalyl)methyl]uracil (8). A mixture of 1-(3,5-di-*O*-*p*-toluoyl-2-deoxy-β-D-ribofuranosyl)-5-(chloromethyl)uracil (**7**; 0.94 g, 1.8 mmol), 1-methyl-1,2,3,4-tetrahydroquinoxaline (0.5 g, 2.4 mmol), and triethylamine (0.18 g, 1.8 mmol) in 5 mL of dry dioxane was stirred for 48 h at room temperature under nitrogen gas in the dark. The filtrate, after removing the hydrochloride salt of triethylamine, was concentrated in vacuo to leave an oily substance. Purification was done with an alumina column using 10% methanol in ethyl acetate as eluent. The column was run under nitrogen gas in the dark. Fractions containing the product were combined and evaporated to give a gummy material, which was triturated with water to give 0.8 g (70%) of **8** as a solid material: NMR (Me_2SO-d_6) δ 7.9 (two sets of d, 4), 7.5 (s, 1, H-6), 7.3 (two sets of d, 4), 6.5 (br s, 4), 6.3 (t, 1, H-1′), 5.6 (br, 1, H-3′), 5.5 (br, 3, CH_2N and H-4′), 4.0 (br s, 2, H-5′), 3.2 (br two sets of d, 4, NCH_2CH_2), 2.7 (s, 3, NCH_3), 2.3 (s, 6, CH_3); mass spectrum *m/e* 624. Anal. ($C_{35}H_{36}N_4O_7$), M_r 624.7, C, H, N.

5-[(4-Methyl-1,2,3,4-tetrahydroquinoxalyl)methyl]-2′-deoxyuridine (9). A suspension of **8** (624 mg, 1 mmol) and 360 mg (2.6 mmol) of potassium carbonate was stirred in 50 mL of dry methanol at room temperature for 1.5 h under a nitrogen stream in the dark. The resulting clear solution was neutralized with Dowex 50 (H^+) and evaporated in vacuo. The residue was treated with *n*-hexane to leave a gummy material. Purification was done with a silica gel column using ethyl acetate–acetone (1:1) as eluent under a nitrogen stream in the dark. Fractions containing the product (R_f 0.3) were combined and evaporated to leave an oily substance, which was crystallized from absolute ethanol to give 250 mg (64%) of the product **9**: mp 188–189 °C; NMR (Me_2SO-d_6) δ 7.7 (s, 1, H-6), 6.4 (s, 4), 6.1 (t, 1, H-1′), 4.0 (s, 2, NCH_2), 3.3 [two sets of br d, 4, $N(CH_2)_2$], 2.8 (s, 3, NCH_3), 2.0 (m, 2, H-2′); mass spectrum *m/e* 338. Anal. ($C_{19}H_{24}N_4O_5$), M_r 388.4, C, H, N.

Bis(triethylammonium) Salt of 5-[(4-Methyl-1,2,3,4-tetrahydroquinoxalyl)methyl]-2′-deoxyuridine 5′-Phosphate (6). Using the method of Sowa and Ouchi,[5] 194 mg (0.5 mmol) of **9** was added to a cold (0 °C) solution of acetonitrile (500 μL), water (25 μL), phosphorus oxychloride (200 μL), and pyridine (190 μL) prepared in the above sequence. The reaction mixture was stirred for 3 h at 0 °C under nitrogen stream in the dark. Cold water (6 mL) was added and stirred for another 1 h at 0 °C. The entire mixture after neutralization with triethylamine was applied to a DEAE-cellulose column (2.5 × 40 cm); gradient elution was performed using 0.01 and 0.3 M triethylammonium bicarbonate buffer (pH 7.5). Fractions containing the product were collected and lyophilized to leave a white material. This was rechromatographed in the dark on a longer DEAE-cellulose column (2.5 × 70 cm) with the 0.01–0.3 M triethylammonium bicarbonate as a gradient eluent. The product fractions were collected, lyophilized, and dried under the high vacuum to yield 45 mg (13%) of **6**: UV (0.1 N HCl) λ_{max} 220 nm (ε 17 000) sh, 270 (9200), λ_{min} 228 (4800); UV (H_2O) λ_{max} 250 nm (ε 19 100), 262 (9200), λ_{min} 241 (7600); UV (0.1 N NaOH) λ_{max} 220 nm (ε 19 100), 262 (9600), λ_{min} 243 (7700); NMR (D_2O) δ 7.9 (s, 1, H-6), 6.0 (m, 4), 6.1 (t, 1, H-1′), 3.2 (two sets of br d, 4, N-CH_2CH_2), 2.7 (s, 3, NCH_3), 2.1 (m, 2, H-2′), 1.1 (t. 18, CH_2CH_3). Anal. ($C_{19}H_{25}N_4O_8P \cdot 2(C_6H_{15}N) \cdot 1.5H_2O$), M_r 697.8.

Enzyme Assay. The enzyme was assayed by modification of the radioisotope assays described by Roberts[13] and Lomax and Greenberg.[14] The solution, 0.1 mL, contained 25 mM mercaptoethanol; 0.22 mM *dl*-tetrahydrofolic acid; 6.75 mM formaldehyde; 5 mM sodium bicarbonate; 1.5 mM magnesium chloride; 0.06 mM EDTA, 3 mM Tris–acetate buffer, pH 6.8; 5 μL of the diluted enzyme solution (0.1 μg); substrate; and, when indicated, inhibitor. Control reactions lacked the cofactor, tetrahydrofolic acid. The substrate 2′-deoxy[5-^{3}H]uridine 5′-monophosphate was used at a specific activity of 500 μCi/μmol. The assays were started by the addition of the enzyme to the complete mixture and then incubated at 30 °C. Incubation was stopped after 30 s by the addition of 50 μL of 20% trichloroacetic acid. A 20% aqueous suspension of charcoal (0.25 mL) was added, and the solution was vortexed and allowed to stand for 15 min. The suspension was filtered through a glass-wool-plugged Pasteur pipet, and 0.1 mL of the filtrate was counted in Beckman scintillation fluid (Ready Solv HP). Counting efficiency was 33%; control samples lacking the cofactor were found to have less than 5% of the respective sample counts.

When the cofactor concentration was varied, the substrate concentration was maintained at saturating levels, 100 μM.

Acknowledgment. This work was supported by a research grant (CA 7522) from the National Cancer Institute of the National Institutes of Health.

References and Notes

(1) M. Friedkin, *Adv. Enzymol.*, **38**, 235 (1973).
(2) See S. J. Benkovic, *Acc. Chem. Res.*, 11, 315 (1978), for a review of this topic.
(3) See P. V. Danenberg, *Biochim. Biophys. Acta*, **473**, 73 (1977), for a review of thymidylate synthetase inhibition and mechanism.
(4) M. S. Edelman, R. L. Barfknecht, R. Huet-Rose, S. Boguslawski, and M. P. Mertes, *J. Med. Chem.*, **20**, 669 (1977).
(5) T. Sowa and S. Ouchi, *Bull. Chem. Soc. Jpn.*, **48**, 2084 (1975).
(6) R. Brossmer and E. Rohm, *Hoppe-Seyler's Z. Physiol. Chem.*, **348**, 1431 (1967).
(7) H. H. Daron and J. L. Aull, *J. Biol. Chem.*, **253**, 940 (1978).
(8) T. C. Crusberg, R. Leary, and R. L. Kisliuk, *J. Biol. Chem.*, **245**, 5292 (1970).
(9) A. Kampf, R. L. Barfknecht, P. J. Shaffer, S. Osaki, and M. P. Mertes, *J. Med. Chem.*, **19**, 903 (1976).
(10) S. J. Benkovic, P. A. Benkovic, and D. R. Comfort, *J. Am. Chem. Soc.*, **91**, 5270 (1969).
(11) R. G. Kallen and W. P. Jencks, *J. Biol. Chem.*, **241**, 5845 (1966).
(12) R. S. Wilson and M. P. Mertes, *J. Am. Chem. Soc.*, **94**, 7182 (1972).
(13) D. Roberts, *Biochemistry*, **5**, 3546 (1966).
(14) M. I. S. Lomax and G. R. Greenberg, *J. Biol. Chem.*, **242**, 109 (1967).

5-Cyano-2′-deoxyuridine 5′-Phosphate: A Potent Competitive Inhibitor of Thymidylate Synthetase

Charles T.-C. Chang, Michael W. Edwards, Paul F. Torrence, and Mathias P. Mertes*[1]

Department of Medicinal Chemistry, School of Pharmacy, University of Kansas, Lawrence, Kansas 66045, and the Laboratory of Chemistry, National Institute of Arthritis, Metabolism and Digestive Diseases, U.S. National Institutes of Health, Bethesda, Maryland 20014. *Received March 1, 1979*

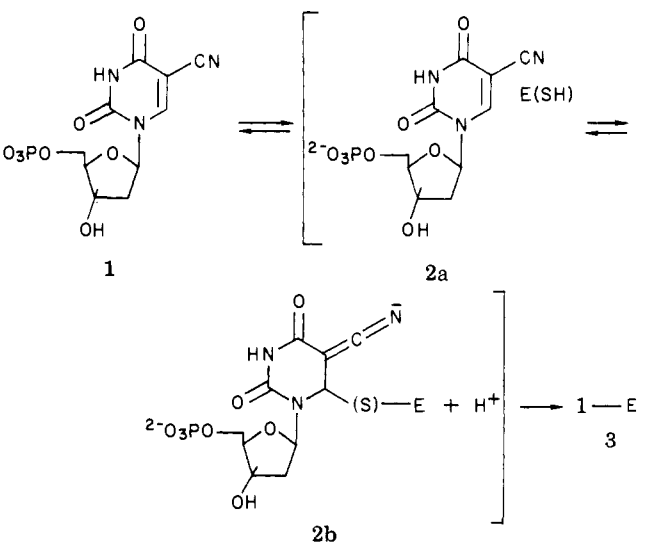
The 5′-phosphate (1) of the antiviral nucleoside 5-cyano-2′-deoxyuridine was synthesized and evaluated for inhibition of thymidylate synthetase purified from methotrexate-resistant *Lactobacillus casei*. Compound 1 was a potent competitive inhibitor with a K_I of 0.55 μM. Irreversible enzyme inhibition by this compound could not be detected.

Thymidylate synthetase (EC 2.1.1.45) catalyzes a two-step reductive alkylation of 2′-deoxyuridine 5′-phosphate (dUMP) to give thymidine 5′-phosphate (dTMP). Inhibition of this enzyme by 5-substituted derivatives of the substrate has been clinically useful in the control of cancer and viral infections.[2a,b] Two of the most effective compounds in this regard, the 5-fluoro and 5-(trifluoromethyl) derivatives, are notably electron withdrawing. A quantitative assessment of the electronic and polar effects on inhibition by various 5-substituted 2′-deoxyuridine 5′-phosphates has been reported by Hansch and co-workers[3] who concluded that the electronic effect (electron withdrawal) is the major factor contributing to enzyme affinity. Since the inductive and resonance effects of the cyano group are great, the introduction of this group at the 5 position of 2′-deoxyuridine 5′-phosphate should result in potent inhibition.

5′-Cyano-2′-deoxyuridine is able to arrest the multiplication of vaccinia virus in cell cultures,[4] albeit not as effectively as the halogenated analogues 5-bromo-2′-deoxyuridine and 5-iodo-2′-deoxyuridine. Evidence has been presented elsewhere that suggests that 5-cyano-2′-deoxyuridine must first be phosphorylated to the corresponding 5′-monophosphate[5] and that this may then block thymidylate synthetase.[6] This latter hypothesis was based largely on the observation that 5-cyano-2′-deoxyuridine inhibited labeled 2′-deoxyuridine incorporation into DNA but had no effect on labeled deoxythymidine incorporation. For this reason and because of the wealth of literature relating to the biological activity of the 5-halo-2′-deoxyuridines to inhibition of thymidylate synthetase, it was of considerable interest to examine the effect of the 5-cyano derivative 1 on the enzyme.

A further aspect of this study was the potential irreversible k_{cat} type inhibition that would be expected if an

Scheme I



enzyme nucleophile (cysteine SH) at the active site added to carbon-6 of the pyrimidine ring. Under similar experimental conditions, enzyme inactivation has been noted[2] for the 5-fluoro-, 5-(trifluoromethyl)-, 5-carboxyaldehyde-, and 5-nitro-2′-deoxyuridine 5′-phosphate.[7,8] The enzyme–inhibitor complex which requires cofactor for inactivation has been characterized[9,10] only for the 5-fluoro derivative. Santi and Sakai,[11] on the basis of model studies, formulated a two-step mechanism for the inactivation of thymidylate synthetase by 5-(trifluoromethyl)-2′-deoxyuridine 5′-phosphate.

As seen in Scheme I, if the enzyme nucleophile adds to carbon-6 of 5-cyano-2′-deoxyuridine 5′-phosphate (1) the resultant complex (2) would be a ketenimine,[12] a strong

0022-2623/79/1822-1137$01.00/0