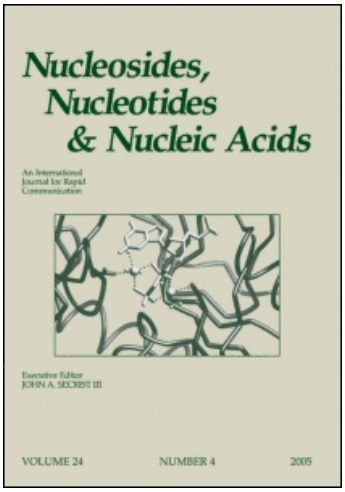

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Said M. M. Bayomi[a]; Diana I. Brixner[a]; Hassan Eisa[a]; Arthur D. Broom[a]; Takamori Ueda[b]; Yung-Chi Cheng[b]
[a] Department of Medicinal Chemistry, College of Pharmacy University of Utah, Salt Lake City, Utah [b] Department of Pharmacology, School of Medicine University of North Carolina, Chapel Hill, NC

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PROBING THE THYMIDYLATE SYNTHASE ACTIVE SITE WITH BISUBSTRATE ANALOG INHIBITORS

Said M.M. Bayomi, Diana I. Brixner, Hassan Eisa and Arthur D. Broom*

Department of Medicinal Chemistry, College of Pharmacy
University of Utah, Salt Lake City, Utah 84112

Takamori Ueda and Yung-Chi Cheng

Department of Pharmacology, School of Medicine
University of North Carolina, Chapel Hill, NC 27514

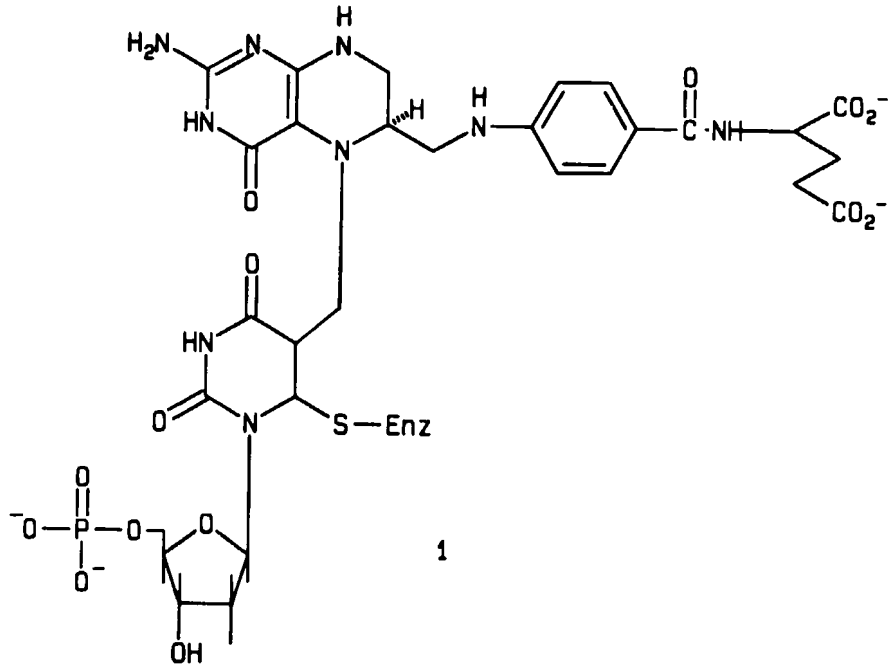
Abstract. Earlier reports on the enhancement of folate analog binding to thymidylate synthase (TS) by N^{10}-propargylation prompted the synthesis of a bisubstrate analog containing an 8-deaza-N^{10}-propargyl-5,6,7,8-tetrahydrofolate moiety coupled through a methylene linkage to 2'-deoxyuridylate.

Introduction

Thymidylate synthase (TS, E.C. 2.1.1.45) serves a unique role in cellular biochemistry as the catalyst for the conversion of 2'-deoxyuridylate (dUMP) to 2'-deoxythymidylate (dTMP). Since this reaction is the sole **de novo** source of dTMP, and since dTMP is critically required for the maintenance of cell viability in a proliferating cell population,[1] TS is considered an important target of potential antitumor antimetabolites.[2]

The prototype of these antimetabolites has been 2'-deoxy-5-fluorouridylate (fdUMP), an anabolic product of 5-fluorouracil (FUra).[2] Although FdUMP is a potent inhibitor of TS, and has been invaluable in elucidating the molecular mechanism of dUMP methylation, it is but one of several products derived from 5-fluorouracil. Thus, in addition to its role in TS inhibition, FUra-derived products are incorporated into RNA,[3] into 5-fluorouridyl diphospho sugars[4] and into DNA.[5,6] Very recently, it was reported that FdUMP inhibits the incorporation of dTMP into DNA in a manner more important to cell growth than simple inhibition of dTMP synthesis.[7]

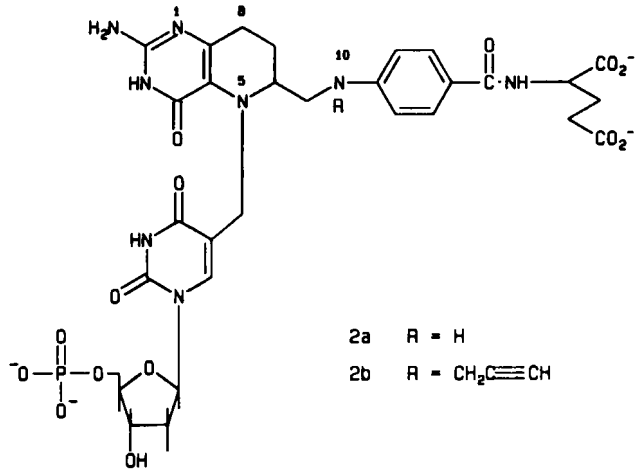
The use of FdUMP as a probe by a number of investigators has partially defined the ternary complex among enzyme, substrate and cofactor as illustrated in 1; these studies have been thoroughly reviewed.[8-12] It is clear from examination of 1 that a compound such as 2 should retain excellent enzyme binding properties while affording an entirely new level of specificity for TS; that expectation was realized with the synthesis of 2a and some related compounds.[13-14]



1

Studies directed toward the development of folate analog TS inhibitors culminated in the discovery by Jones and his colleagues that N^{10}-propargyl-5,8-dideazafolate is an extraordinarily potent inhibitor of the enzyme.[9,15] It has subsequently been demonstrated that N^{10}-propargylation enhances the TS inhibitory activity of folate and its 8-deaza analog, although neither derivative approaches 5,8-dideaza-10-propargylfolate in affinity.[16,17]

Enhancement of folate analog binding by N^{10}-propargylation and the potent (Ki = 0.058 μM) and specific inhibition of TS by bisubstrate analog 2a[13] suggested that the synthesis of 2b might enhance affinity and provide additional information about the TS active site. The synthesis, characterization and evaluation of 2b form the basis of this report.



2a R = H

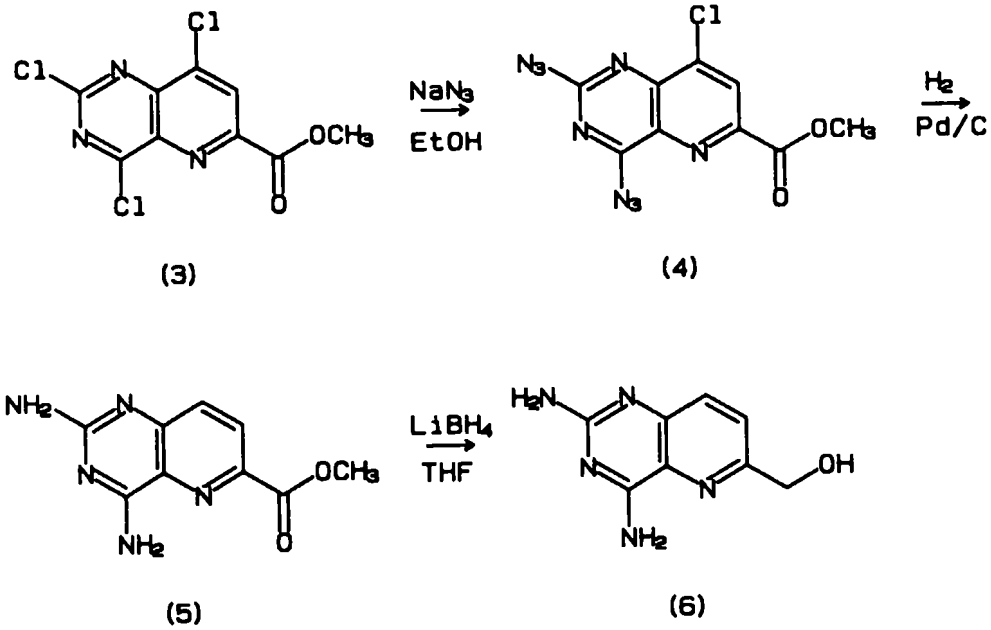
2b R = $CH_2C{\equiv}CH$

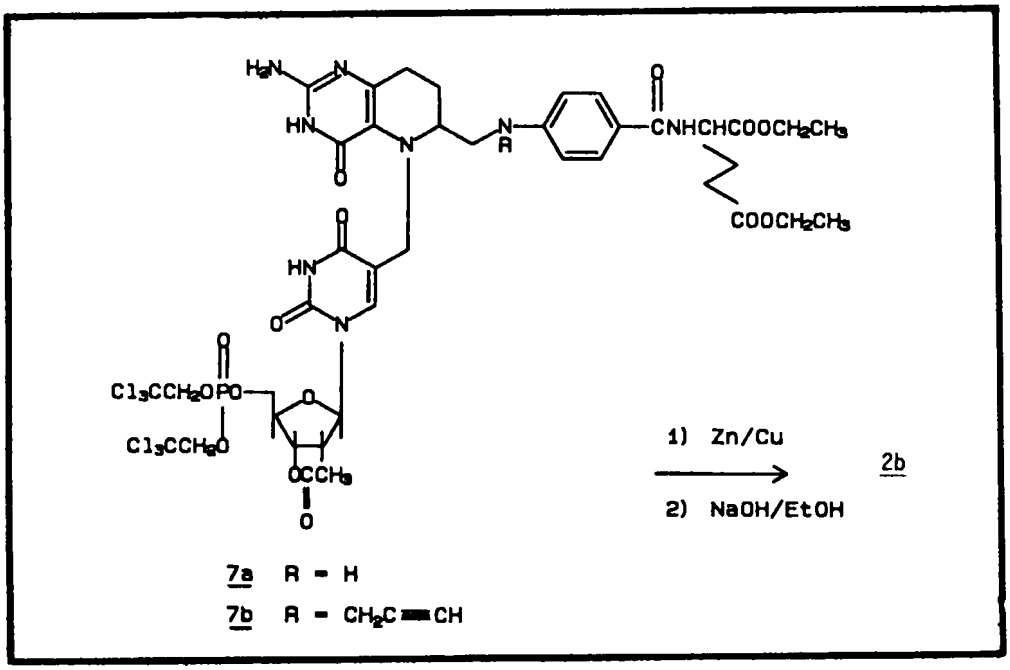
Results and Discussion

Several approaches to the synthesis of 8-deazafolate, a key precursor to **2a**, have been described.[13,18-20] Each has suffered from one or more difficult steps and poor to modest yields. A simpler and much higher yield procedure has now been devised (Scheme 1). Treatment of 6-carbomethoxy-2,4,8-trichloropyrido[3,2-d]pyrimidine (**3**)[21] with sodium azide gave the 2,4-bis-azido derivative **4** in 82% yield. Treatment with hydrogen over palladium on charcoal at 42 psi gave simultaneous reduction of both azide functions to amines and hydrogenolysis of the 8-chloro substituent to provide 6-carbomethoxy-2,4-diaminopyrido[3,2-d]pyrimidine (**5**), which was converted to the known hydroxymethyl derivative **6** using $LiBH_4$. The previously reported procedure[13] was used to obtain bisubstrate analog **2a** as its fully protected derivative **7a**.

Alkylation of **7a** to give the N^{10}-propargyl derivative proved to be a delicate reaction. General alkylation procedures in aprotic polar solvents such as DMF, DMAC, or DMSO led to many products. Refluxing in EtOH with 1.1 equivalents of propargyl bromide and base, with or without KI as a catalyst, would only proceed to about 50% completion. In refluxing EtOH, with a large excess of propargyl bromide (2.5 equiv.), the reaction was found to proceed further before other products appeared, making it possible to isolate compound **7b** by silica gel chromatography in 42% yield based on unrecovered **7a**.

Scheme I





Assignment of the site of alkylation as N^{10} in this complex molecule was made using FAB mass spectrometry. A molecular ion (MH^+) was evident at 1161 together with the expected complex of Cl-containing ions. Fragment ions gave unequivocal proof of the position of the propargyl group. Two strong fragment ions were observed at 787 and 373 (Figure 1). Examination of the FAB mass spectrum of the fully protected unsubstituted multisubstrate analog **7a** also shows a strong fragment ion at 787 corresponding to cleavage between folate C^6 and C^9. Hence, the propargyl group is attached to the p-aminobenzoylglutamic acid portion of the molecule with substitution at the amine being the only logical assignment.

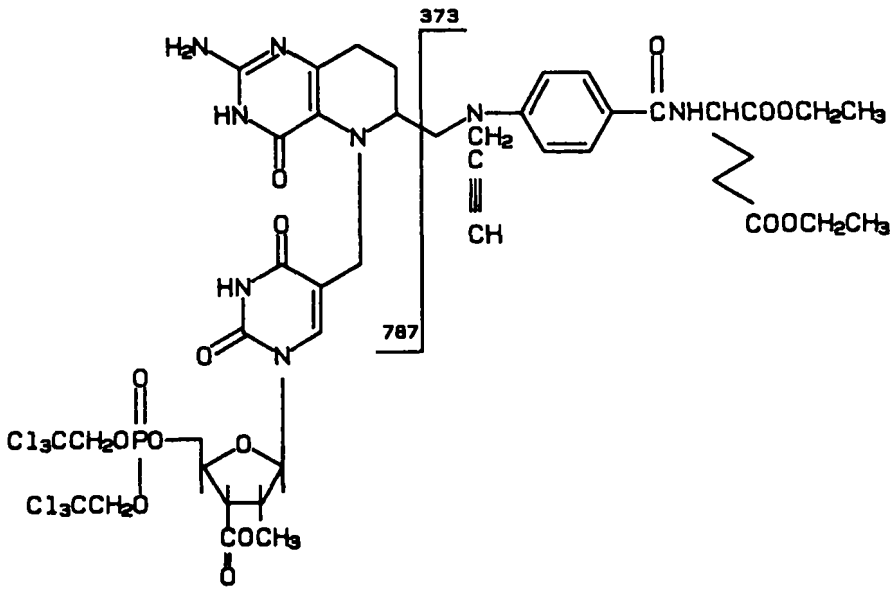


Figure 1. Major cleavage in the FAB mass spectrum of 7b.

The NMR spectrum of fully protected **7b** was taken in $(CD_3)_2SO$. As expected from earlier studies on **2a**,[13] the existence of two diastereoisomers at deazafolate C^6 gave rise to substantial nonequivalence in the spectrum. Because of major overlap at 200 or 270 MHz in the region of δ1 to 5, many assignments were made possible only by the application of 2D COSY techniques. Thus, it was possible to assign the glutamate signals as (CH_α, 4.4δ; $(CH_2)_\beta$, 2.0; $(CH_2)_\gamma$, 2.4 beginning with the glu NH signal at 8.25. Similarly, beginning with the nonoverlapped nucleotide 1'-H, the application of COSY clearly demonstrated that the 2'-H signals occurred in a complex set of signals at 2.35δ, the 3'-H at 5.2, 4'-H at 4.3 and the 5'-proton signals at about 4.0. The propargyl CH and CH_2 signals were tentatively assigned as at 3.1 and 4.0δ, respectively, on the basis of a weak cross-peak in the COSY spectrum and by comparison with 8-deaza-10-propargylfolate.[17]

The protecting groups on **7b** were removed by sequential treatment with a freshly prepared Zn/Cu couple and with ethanolic sodium hydroxide. After purification on DEAE Sephadex, inhibitor **2b** was isolated in 56% yield. FAB mass spectrometry afforded the appropriate molecular ion, MH^+ 803.

The NMR spectrum of **2b** in D_2O showed the expected complexity arising from the presence of two diastereoisomers. One feature of the spectrum was, however, remarkable. In the case of **2a**, the separation of the two pyrimidine 6-H signals was 0.06 ppm at 270 MHz.[13] In **2b**, however, the separation was 0.25 ppm at 200 MHz, or 50 Hz. Examination of the CPK space-filling models reveals that several conformers stabilized by hydrophobic stacking interactions[22] may exist for each diastereoisomer. It was of interest, therefore, to examine the spectrum in the presence of a denaturing solvent such as $(CD_3)_2SO$. Most of the identified proton signals were deshielded in $(CD_3)_2SO/D_2O$(3:1) relative to D_2O, as expected on the basis of destacking and loss of anisotropic shielding in the denaturing solvent; the Δδ values ranged from 0.1 to 0.3 ppm. However, the pyrimidine H-6 signals showed a remarkable upfield shift (Δδ 0.30 and 0.45), actually shifting from the low field side of the downfield benzene aromatic signal to between the two sets of benzene signals. Since it is difficult to imagine a mechanism for the establishment of a specific anisotropically shielding conformation in a denaturing solvent, it seems likely that in the stacked aqueous solution conformation, pyrimidine H-6 is constrained in the deshielding region of some functional group, probably a carbonyl. Such an interpretation is consistent with molecular model conformations, but must await detailed nuclear Overhauser enhancement studies for confirmation.

Compounds **2a** and **2b** were evaluated in parallel as inhibitors of TS obtained from human leukemia cells; the results are illustrated in Table 1. Both compounds inhibited the enzyme competitively with either dUMP or 5,10-CH_2-H_4PteGlu as variable substrate; the presence of the propargyl group seemed marginally to enhance the affinity. Both compounds clearly act as bisubstrate analogs. When 5,10-methylenetetrahydropteroyl pentaglutamate was the variable substrate, the kinetics changed to noncompetitive, indicating that the pentaglutamate alters the binding behavior of the bisubstrate analogs.

The high affinity for and competitive nature of the inhibition of TS observed for **2a** and **2b** suggest that these compounds fit well into both nucleotide and folate binding sites. The preordained geometry of these compounds makes it likely, although by no means certain, that the enzyme-inhibitor complex resembles the normal reaction ternary complex. The biological data for the multisubstrate analogs may be compared to those of 8-deazafolate and N^{10}-propargyl-8-deazafolate as TS inhibitors.[17] In the case of the folate analogs, propargylation at the N-10 position led to roughly a 20-fold increase in activity. When comparing the corresponding multisubstrate analogs, propargylation led to little increase in activity. The relatively small impact of N^{10}-propargylation, viewed in this context, suggests that there is neither a "hydrophobic pocket" nor a steric constraint in the N^{10}-region of the folate binding site. Further evaluation of this suggestion must await more detailed cystallographic studies of the type very recently reported by Stroud and his colleagues.[23]

Experimental: Chemistry

The ^1H NMR spectra were recorded on an IBM FT-200 or a JEOL FX 270 NMR spectrometer in $(CD_3)_2SO$ or D_2O. Melting points were determined on a Thomas-Hoover melting point apparatus and were not corrected. Mass spectra were taken on a Varian 112S or LKB-GCMS 9000S spectrometer. UV spectra were taken on a Beckman DU-8 spectrometer. Elemental analysis was performed by MicAnal Laboratories, Tucson, Arizona. All thin-layer chromatography was done on precoated TLC sheets, silica gel 60 F254, layer thickness 0.2 mm.

6-Carbomethoxy-2,4-diazido-8-chloropyrido[3,2-d]pyrimidine (**4**)

6-Carbomethoxy-2,4,8-trichloropyrido[3,2-d]pyrimidine[9] (**3**) (5.87 g, 20 mmol) was added to 100 mL of dry EtOH. To this mixture was added 3.12

g (48 mmol) of NaN_3 and the reaction was stirred at ambient temperature for 2 hours. Then 200 mL of cold H_2O were added. The precipitate was filtered and rinsed with cold water. The crude product was stirred in 50 mL EtOH, filtered and dried to yield 4.9 g (82%) of (**4**). TLC, Hex/EtOAc (6:4) showed the product to be pure: mp 150-151°C; mass spectrum, m/e 305 (M^+); ^1H NMR, ($CDCl_3$) δ4.06 (s,3,OCH_3), 8.6 (s,1,C-7H; $UV_{\gamma\ max}$ (ϵ_{max}) (pH 1), 331 nm (11,000), 316 nm (12,300), 307 nm (13,000), 258 nm (16,500), (pH 7) 294 nm (7,400), 247 nm (9,200); (pH 11) 289 nm (8,800), 258 nm (8,300); anal. ($C_4H_9ClN_9O_2$) C, H, N. Calc. 35.36, 1.31, 41.24. Obs. 35.59, 1.25, 40.87.

6-Carbomethoxy-2,4-diaminopyrido[3,2-d]pyrimidine (**5**)

Compound **4** (6.0 gm) was dissolved in 200 mL DMF. To this solution 3 g NaOAc and 2 g palladium on activated charcoal (10%) were added. The reaction mixture was shaken on a Parr hydrogenator at 42 psi at room temperature for 48 hours. The catalyst was removed by filtration through celite. The celite was rinsed with DMF (2 x 50 mL). The washings were combined with the filtrate and all solvent was removed. The solid was washed with cold water, filtered and dried: 4.2 g (quantitative). Recrystallization from MeOH/H_2O (8:2) gave light brown crystals: mp 240-242°C with decomposition; mass spectrum, m/e 219 (M^+); ^1H NMR, δ3.90 (s,3,OCH_3), 6.72 (s,2,2-NH_2), 7.63 and 8.13 (d each 2,C-7 and C-8 Hs), 7.74 (s,2,4-NH_2); $UV_{\gamma\ max}$ (ϵ_{max}) (pH 1), 318 nm (13,700), 258 nm (19,400); (pH 7) 345 nm (9,800), 302 nm (9,900), 260 nm (16,200); (pH 11) 340 nm (6,800), 292 nm (9,000), 254 nm (14,000); anal. ($C_9H_9N_5O_2 \cdot H_2O$) C, H, N. Calc. 47.36, 4.41, 30.72. Obs. 47.42, 3.99, 30.59.

2,4-Diamino-6-(hydroxymethyl)pyrido[3,2-d]pyrimidine (**6**)

Compound **5** (4.2 g, 19 mmol) was added to 200 mL dry THF. This mixture was cooled in an ice water bath and 1.23 g (57 mmol) of lithium borohydride were added. The ice bath was removed after 30 minutes and the reaction mixture was stirred at ambient temperature for 24 hours. Methanol was added (50 mL) and after 20 minutes all solvents were removed. The solid was taken up in 500 mL H_2O and the pH was adjusted to 7 with 1N HCl. The solution was boiled until clear and then filtered. The filtrate was concentrated to 50 mL and allowed to cool to room temperature. After refrigeration the solid was filtered and air dried. Yield 2.98 g (79%). It was identical to a previously prepared sample.[9]

Diethyl N^{10}-propargyl-N-[4-[[[2-amino-3,4,5,6,7,8-hexahydro-4-oxo-5-[2'-deoxy-3'-O-acetyluridin-5-yl)methyl)]pyrido[3,2-d]pyrimidin-6-yl]methyl]amino]benzoyl]-L-glutamate 5'-(bis-trichlorethyl phosphate) (7b).

0.2 g (0.17 mmol) of 7a, synthesized as previously described,[9] was added to 3 mL of EtOH. To this solution was added 2.5 mL (large excess) of propargyl bromide 80 wt% in toluene. The solution was refluxed for 2 hours. TLC, CH_3CN/H_2O (9:1) showed the reaction to be about 80% complete. The reaction was stopped at this point to prevent formation of additional products. The solvents were removed in vacuo. Water was added and the pH brought to 7 with 5% $NaHCO_3$. The suspension was extracted with $CHCl_3$ (3 x 10 mL, dried over $Na2_SO_4$ and the solvent was evaporated. The compound was dissolved in $CHCl_3$ and applied to a deactivated (6% H_2O silica gel (60-200 mesh) column packed in $CHCl_3$/MeOH (96:4) containing 2% triethylamine. Elution with the same solvent system yielded 62 mg (62% based on 60 mg recovered starting material): mp 132°C; FAB mass spectrum gave 1161 (MH^+) with fragment ions at 787 and 373; ^1H NMR, δ 11.2 (2s,1,3-NH), 8.25 (2d,1,glu NH), 7.7 (s,1,thymidylyl C-6 H), 7.7 and 6.65 (d and 2d,4,C_6H_4), 6.5 (br,2-NH_2), 5.85 (q,1,1'-H), 5.2 (m,1,3'-H), 4.8 (m,4,CCl_3CH_2 x 2), 4.0 (m,4,$COOCH_2CH_3$ x 2), 2.35 (br,6,2'-H,C-8 H_2,CH_2COOEt), 2.1 (d,3,3'-$COCH_3$) 2.0 (br,3,C-7 H and $CHCH_2$), 1.6 (br,1,C-7 H), 1.1 (m,6,CH_2CH_3; $UV_{Y\ max}$ (ε_{max}) (pH 1) 306 nm (16,700); (pH 7) 307 nm (13,600); (pH 12) 305 nm (14,700); anal. ($C_{43}Cl_6H_{51}N_8O_{15}P$) C, H, N. Calc. 44.48, 4.40, 9.65. Obs. 44.42, 4.42, 9.28.

N^{10}-propargyl-N-[4-[[[2-amino-3,4,5,6,7,8-hexahydro-4-oxo-5-[2'-deoxyuridin-5-yl)methyl]pyrido[3,2-d]pyrimidin-6-yl]methyl]amino]benzoyl]-L-glutamic acid 5'-monophosphate (2b)

Compound 12 (0.1 g, 0.09 mmol) was dissolved in 3 mL of dry DMF. About 50 mg Zn/Cu couple were added. The mixture was stirred at 55°C for 20 minutes. TLC, CH_3CN/H_2O (9:1) showed absence of starting material. TLC in isopropanol/NH_4OH/H_2O (7:1:2) revealed a major spot at Rf 0.2 with minor impurities (possible partial cleavage of 3'-acetyl group). The solution was cooled and filtered. H_2S was bubbled through after dilution with 10-15 mL H_2O. Additional precipitated sulfides were removed by filtration. Nitrogen was bubbled to removed H_2S and solvent was removed in vacuo. The green residue was transferred to a small flask using 2 mL EtOH and 1 mL 1N NaOH. The solution was stirred for 48 hours. The reaction appeared complete in the 7:1:2 TLC system. The pH was adjusted to 5 with 1N HCl and diluted to 100 mL.

The solution was applied to a DEAE Sephadex 25 (HCO_3^-) column (0.9 x 10 cm). The column was washed with 100 mL of water followed by a linear gradient (300 mL/300 mL) of 0.1 M TEAB/0.8 M TEAB (pH 8). Chromatographically pure fractions were combined and evaporated to dryness. The solid was coevaporated with EtOH (3 x 10 mL). The residue was then suspended in 1 mL EtOH and transferred with EtOH to a test tube containing 10 mL dry acetone (3 x 10 mL) and dried over nitrogen. Yield: 38 mg (56%); ^1H NMR ($D_2O/(CD_3)_2SO$) δ 7.7 and 6.75 (q and t,4,C_6H), 7.47 and 7.35 (s each, 1,6-H), 6.09 (m,1,1′-H), 4.34 (s,1,CH_2CCH); FAB mass spectrum, MH^+ 803; UV γ_{max} (ε_{max}) (pH 1) 303 nm (19,200), (pH 7) 305 nm (27,500); (pH 12) 303 nm (23,300).

Biological Evaluation

Chemicals. [5-^{3}H]dUMP (22 Ci/mmol) and dUMP were obtained from Moravek Biochemicals, Inc., Brea, CA, and Sigma Chemical Company, St. Louis, MO, respectively. H_4PteGlu and H_4PteGlu$_5$ were prepared by the chemical synthesis as described.[24,25] Purity of the bisubstrate analogs was established by HPLC and their concentrations were determined spectrophotometrically. All other chemicals used were of reagent grade or higher.

Enzyme Purification Assays. TS was purified approximately 1350-fold to a specific activity of 17.4 units/mg of protein by procedures described previously[26] from human leukemic cells (obtained by leukophoresis) of an untreated patient. These cells were provided by North Carolina Memorial Hospital. The enzyme assay was performed by the tritium release procedure of Roberts[27] as reported by Dolnick and Cheng,[26] except that the concentration of the substrate, dUMP, 5,10-CH_2-H_4PteGlu or 5,10-CH_2-H_4PteGlu$_5$ was changed as indicated (Table 1). One unit of enzyme activity was defined as the conversion of 1 nmole of substrate (dUMP) per minute. The reaction was started by the addition of the enzyme and the incubation time was either 45 or 60 minutes. All assays were conducted in duplicate or triplicate and repeated at least once with similar results. Ki determinations were performed by assaying the enzyme at varying levels of dUMP, 5,10-CH_2-H_4PteGlu or 5,10-CH_2-H_4PteGlu$_5$ in the presence of several fixed concentrations of each bisubstrate analog. Ki values for competitive inhibitors were determined from both slope and intercept replots.

TABLE 1- Kinetic Properties of the Bisubstrate Analogs in the TS Assay

	5,10-CH_2-H_4PteGlu		dUMP		5,10-CH_2-H_4PteGlu$_5$		
Compound	Inhibition	K_{is}[a](μM)	Inhibition	K_{is}(μM)	Inhibition	K_{is}(μM)	K_{ii}[c](μM)
2a	C[b]	0.09	C	0.027	NC[d]	0.33	0.11
2b	C	0.09	C	0.013	NC	0.27	0.07

In apparent K_i determination, 5,10-CH_2-H_4PteGlu and dUMP were used as the varying substrates. The concentration of TS used in each assay was 0.072 U/mL. The fixed concentration of [5-^{3}H]dUMP was 28 μM (1.07 mCi/μmol) and the fixed concentration of 5,10-CH_2-H_4PteGlu was 840 μM. The reaction was started by the addition of the enzyme and the incubation time was 60 minutes or 45 minutes with 5,10-CH_2-H4PteGlu$_5$. K_m values for 5,10-CH_2-H_4PteGlu, for 5,10-CH_2-H_4PteGlu$_5$, and for dUMP were 137 μM, 18.3 μM, and 1.3 μM, respectively.

a: K_i slope; b: competitive inhibition; c: intercept; d: noncompetitive

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