This test used 16-week-old male SH rats (Okamoto strain, Taconic Farms) dosed orally by gavage with 100 mg/kg (unless otherwise specified) of test compound dispersed in a starch suspension (3% in normal saline) in a dose volume of 2 mL/kg. Rats were then given an oral load of normal saline (25 mg/kg) and placed in individual metabolism cages, and 0–5-h urine output was collected. Urinary sodium and potassium concentrations were determined by flame photometry. Twenty-four hours after the first dose, rats were redosed with the exception that the 25 mL/kg normal saline load was omitted. Mean arterial blood pressure (MABP) and heart rate (HR) were obtained via direct femoral arterial puncture under local anesthetic 4 h after this second dose.

Additional studies were performed with compound **9d** in a standing colony of chronic phase, two kidney–one clip Goldblatt renal hypertensive dogs. Control MABP and HR were obtained by transdermal femoral arterial puncture and then the compound was given orally in a gelatin capsule in amounts sufficient to deliver 0.1, 0.2, 0.3, and 1.0 mg/kg based on the daily body weight. To insure accurate dosage at the three lowest treatment levels, the compound was extended with lactose to permit weighing of a greater mass. Arterial puncture was repeated at 1-, 3-, and 5-h intervals after dosing to ascertain drug effects. All animals were habituated to the test procedure through their long history of testing.

To investigate the mechanism of antihypertensive action, conscious restrained SHR were used where the caudal artery and vein were catheterized for blood pressure measurement and access for iv injection of test substances. Autonomic agonists were injected at 15-min intervals or longer, if blood pressure did not return to control, and maximum blood pressure changes recorded. Compound **9d** was administered orally by gavage in a 3% starch suspension at a dose of 25 mg/kg and the series of injections repeated after blood pressure had fallen. Pre- and posttreatment responses were compared for significant differences by paired Student's *t* tests. Starch-treated rats were also tested by the same procedure as a negative control.

Registry No. **5**, 609-20-1; **6a**, 7311-34-4; **6b**, 86-81-7; **6c**, 122-85-0; **6d**, 1424-66-4; **6e**, 351-54-2; **6g**, 456-48-4; **6h**, 454-89-7; **6i**, 3218-36-8; **6j**, 3132-99-8; **6k**, 10203-08-4; **6l**, 100-10-7; **6m**, 120-21-8; **6n**, 1199-59-3; **6o**, 55875-47-3; **6p**, 56479-63-1; **6q**, 1524-07-8; **6r**, 84562-48-1; **7a**, 84562-23-2; **7b**, 84562-21-0; **7c**, 92366-56-8; **7d**, 84562-19-6; **7e**, 92366-57-9; **7g**, 85103-57-7; **7h**, 85103-60-2; **7i**, 84562-39-0; **7j**, 84562-44-7; **7k**, 84562-37-8; **7l**, 84562-46-9; **7m**, 84562-29-8; **7n**, 84562-62-9; **7o**, 84562-64-1; **7p**, 84562-58-3; **7q**, 84562-66-3; **7r**, 84562-49-2; **8a**, 92366-58-0; **8b**, 85103-52-2; **8c**, 92366-59-1; **8d**, 85103-54-4; **8e**, 92366-60-4; **8g**, 85103-58-8; **8h**, 92366-61-5; **8i**, 85103-63-5; **8j**, 92366-62-6; **8k**, 85103-67-9; **8l**, 92366-63-7; **8m**, 85103-71-5; **8n**, 85103-73-7; **8o**, 85103-75-9; **8p**, 85103-77-1; **8q**, 85103-79-3; **8r**, 85103-69-1; **9a**, 92366-64-8; **9b**, 85103-51-1; **9c**, 92366-65-9; **9d**, 85103-53-3; **9e**, 85103-55-5; **9f**, 92366-66-0; **9g**, 92366-67-1; **9h**, 92366-68-2; **9i**, 85103-62-4; **9j**, 92366-69-3; **9k**, 92366-70-6; **9l**, 92366-71-7; **9m**, 85103-70-4; **9n**, 85103-72-6; **9o**, 85103-74-8; **9p**, 85103-76-0; **9q**, 85103-78-2; **9r**, 85103-68-0; **10**, 99-30-9; **11**, 2350-60-9; **12**, 84562-31-2; **13e**, 84562-30-1; **13f**, 84562-33-4; **13g**, 84562-32-3; **13h**, 84562-34-5; 3-bromo-*N*,*N*-dimethylaniline, 16518-62-0; 3-fluoro-*N*,*N*-dimethylaniline, 2107-43-9; *N*,*N*-dimethyl-*m*-anisidine, 15799-79-8.

A Potent Multisubstrate Analogue Inhibitor of Human Thymidylate Synthetase[1]

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The synthesis of an 8-deazafolate analogue of the intermediate in the methylation of 2′-deoxyuridylate is described. Alkylation of diethyl 5,6,7,8-tetrahydro-8-deazafolate with 3′-*O*-acetyl-5-(bromomethyl)-2′-deoxyuridine 5′-[bis(trichlorethyl) phosphate], followed by removal of the trichloroethyl groups with a Zn/Cu couple and mild saponification, gave the target inhibitor *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. The free nucleoside and the 5′-(methyl phosphate) diester were similarly prepared. Each of these reactions yielded a pair of diastereoisomers about C-6 of the reduced deazafolate in approximately a 1:1 ratio. These diastereoisomeric mixtures were evaluated as inhibitors of thymidylate synthetase derived from human tumor (HeLa) cells. The 5′-monophosphate was a potent inhibitor, competitive with respect to both 2′-deoxyuridylate (K_i = 0.06 μM) and tetrahydrofolate (K_i = 0.25 μM). In contrast, the nucleoside and the nucleotide methyl ester were poorer inhibitors by more than 3 orders of magnitude, attesting to the importance of the anionic function at the nucleoside 5′-position in the affinity of an inhibitor for the enzyme active site.

The concept of "thymineless death" (loss of cell viability associated with cessation of DNA synthesis resulting from a lack of thymidylate) was put forth by Cohen[4,5] to explain the effects of thymine deprivation upon *Escherichia coli*. Although this concept has for many years been used to justify thymidylate synthetase (TS) as a target for cancer chemotherapy, only recently has it been clearly demonstrated in mammalian cells.[6]

For two decades, the conventional wisdom has been that the antitumor drug 5-fluorouracil acts via its conversion to 2′-deoxy-5-fluorouridylic acid (FdUMP), which inhibits TS and deprives the cell of thymidylate.[7] That view has been challenged by the demonstration that the ribonucleotide (FUMP) is incorporated into RNA and that the incorporation correlates with cytotoxicity in certain human cells line.[8,9] Furthermore, there are recent demonstrations of low-level incorporation and removal of FdUMP from DNA[10–13] and a new mechanism of action of FUdR was

(1) Preliminary accounts of parts of this work have been reported in the following: (a) Srinivasan, A.; Broom, A. D. *Tetrahedron Lett.* **1982**, *23*, 1431. (b) Broom, A. D.; Srinivasan, A. In "Chemistry and Biology of the Pteridines"; Blair, J. A., Ed.; Walter de Gruyter: West Berlin, 1983; p 445.
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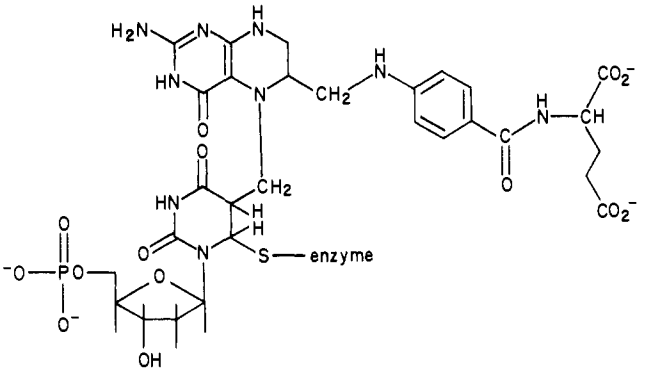
proposed.[12,13] The original mechanism proposed by the late Professor Heidelberger has, however, recently received additional support from the Houghtons[14] and from Washtien[15] in studies involving various human gastrointestinal tumor cell lines. The only thing clear at the present time is that 5-fluorouracil is a multipotent drug, and that the way to get a firm answer to the question of the therapeutic relevance of "thymineless death" is to have available a drug with complete specificity for TS.

From the foregoing information it seems clear that a highly specific, highly potent inhibitor of TS has potential as a biochemical probe and an antitumor agent. In order to approach the problem rationally, one needs to know a good deal about the molecular mechanism of the enzymatic reaction. Fortunately, sufficient information has been developed in a number of laboratories to enable one to draw the ternary complex formed by the interaction of substrate 2'-deoxyuridylate (dUMP), cofactor $N^{5,10}$-methylenetetrahydrofolate ($N^{5,10}$-CH_2-H_4PteGlu) and TS as the generalized structure 1. The studies leading to the development of this structure relied heavily on the use of FdUMP in the ternary complex and have been well reviewed.[16-18] Particular attention should be accorded the early studies of Santi,[19] the NMR work of James and Santi[20] and of Ellis and Dunlap,[21,22] contributions from the laboratories of Kisliuk[23,24] and Danenberg,[10] and the recent efforts to elucidate the sequence and location of the folate binding site by the Maleys and Baugh.[25]

This information, although fuzzy on the precise position of thymidylate attachment (N^5 vs. N^{10}) and on many details of stereochemistry and conformation of the various components of the ternary complex, is still sufficient to enable the design of compounds having all the right binding functions in, one hopes, all the right places. An initial step in this direction was taken by Mertes,[26] who synthesized thymidylate substituted on the 5-methyl with a simple tetrahydroquinoxaline and found it to inhibit TS with a K_i of 0.75 μM. The present report describes the synthesis, characterization, and enzyme inhibitory activity of multisubstrate analogues **2a-c**.[1]

Results and Discussion

Chemistry. The strategies developed for the synthetic approach were designed to be as convergent as possible, such that more or less readily obtained intermediates could be stockpiled and then reacted in a few steps to give the desired variety of products. The key step in this process



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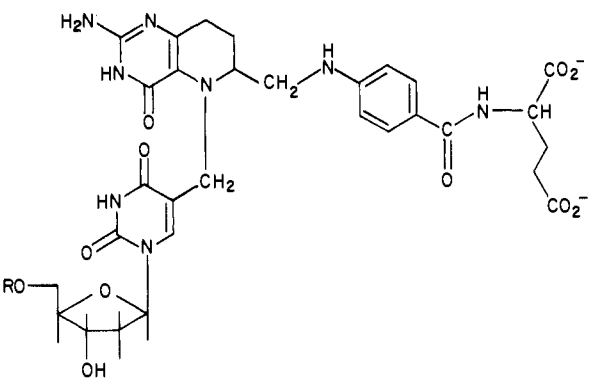

2a, R = H
b, R = PO^{2-}
c, R = $PO_3CH_3^-$

was the linkage of a preformed, suitably protected pyrimidine nucleotide to a protected 8-deaza-5,6,7,8-tetrahydrofolate.

There were several reasons for initially selecting the 8-deazafolate rather than folate itself for study. There is some evidence that the deazafolate analogues have higher affinity for TS than folate.[27,28] DeGraw et al.[29] observed that 8-deazafolate was transported into cells 4 times better than folate. The solubility properties of the simpler deazapteridines are frequently more tractable than those of the corresponding pteridines. Finally, and most important, the reduced 8-deazafolates are stable toward autoxidation, a property not shared by the tetrahydrofolates.[30] Given the number of steps and variety of conditions involved in these syntheses, mixtures resulting from H^4-folate air-oxidation would be most unwelcome.

Several approaches to the 2-amino-4-oxo-6-substituted-pyrido[3,2-*d*]pyrimidines needed as 8-deazafolate precursors have been described.[31-34] Each of these suffers from the difficult accessibility of pyrido[3,2-*d*]pyrimidine starting materials and fairly low overall yields. An alter-

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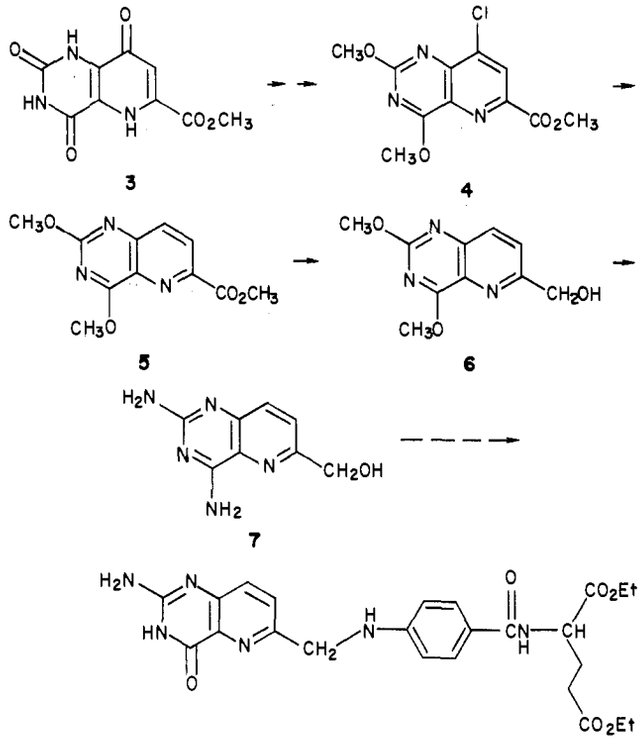
Scheme I

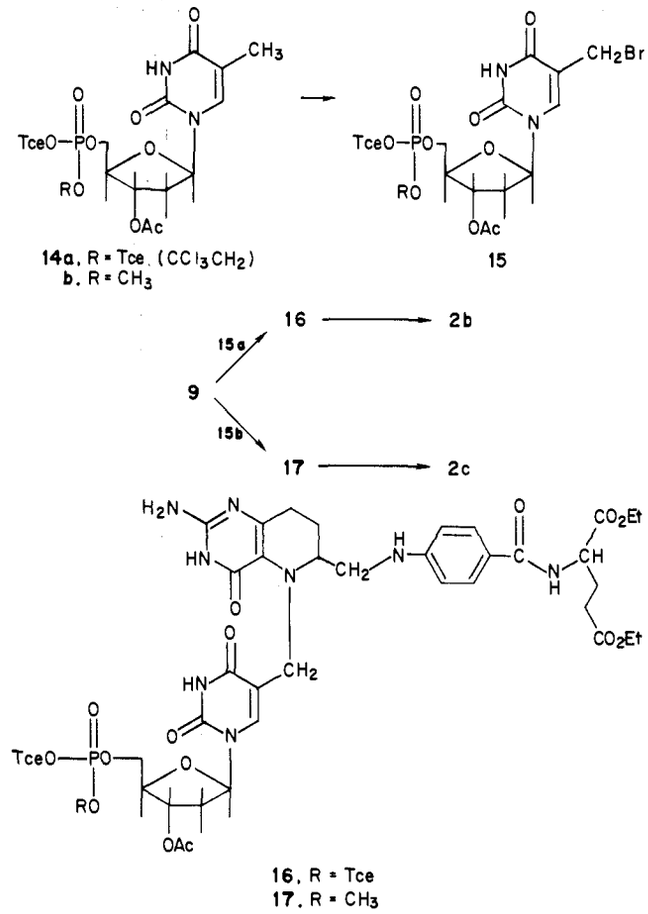


native, simpler, higher yield procedure has now been developed on the basis of conversions of the readily accessible (from commercially available starting materials) 6-carbomethoxy-2,4,8-trioxopyrido[3,2-*d*]pyrimidine (**3**).[35] Chlorination of **3** as previously described[35] gave crude 6-carbomethoxy-2,4,8-trichloropyrido[3,2-*d*]pyrimidine, which was treated directly with methanolic sodium methoxide to give 6-carbomethoxy-8-chloro-2,4-dimethoxypyrido[3.2-*d*]pyrimidine (**4**) according to the previously described substitution pattern (Scheme I).[35] Hydrogenolysis over 5% Pd/C gave dimethoxy derivative **5**, $LiBH_4$ reduction of the ester gave hydroxymethyl compound **6**, and amination afforded the previously described 2,4-diamino-6-(hydroxymethyl)pyrido[3,2-*d*]pyrimidine (**7**) in 30% overall yield from **3**. The diamino derivative **7** was then converted to diethyl 8-deazafolate as previously described.[32] This reaction sequence enables routine synthesis of multigram quantities of **8**.

The next critical step, and still the worst step in the entire synthetic scheme, was reduction of **8** to its tetrahydro derivative. As also observed by Temple et al.,[33] hydrogenolysis of the "benzylic" side-chain amine competes rather too well with nuclear reduction. Use of 1.0 equiv of 0.1 N aqueous HCl in ethanol over Adams catalyst at 42 psi hydrogen pressure gave a 60% yield of the tetrahydro-8-deazafolate **9**. Alkylation of the reduced pyridopyrimidine with 3′,5′-di-*O*-acetyl-5-(bromomethyl)-2′-deoxyuridine[36] gave, after deprotection, nucleoside **2a** (Scheme II).

It was necessary to establish unequivocally that alkylation had occurred on N^5 rather than N^{10}. A modification of the procedure of Cosulich and Smith[37] for the nitrosation of **8** gave a good yield of diethyl N^{10}-nitroso-8-deazafolate (**10**). Reduction afforded diethyl 8-deaza-N^{10}-nitroso-5,6,7,8-tetrahydrofolate (**11**), which was smoothly alkylated to give **13**. Alkylation of **9** gave **12**, the di-*O*-acetyl derivative of **2a**. Nitrosation of **12** gave **13**, identical in all respects with the compound obtained from the unambiguously prepared N^{10}-nitroso derivative **10**.

There remained, then, only the introduction of a 5′-phosphate or methyl phosphate group on nucleoside **2a**. To do this directly, however, was not practicable. Yields of nucleoside phosphorylation reactions are usually modest even with simple nucleosides, and a molecule such as **2a** has too many nucleophilic sites to be considered a "simple" nucleoside. In any event, as noted above, a convergent approach was to be utilized whenever possible. Ways of preparing a suitably protected 5-(bromomethyl)-2′-deoxyuridylate were therefore sought. It was found that 3′-*O*-acetylthymidine 5′-[bis(2,2,2-trichloroethyl) phosphate][38,39] was smoothly brominated to give the key bromomethyl derivative **15a**. Reaction of **14a** with the 5,6,7,8-tetrahydro derivative (**9**) of **8**, removal of the trichloroethyl groups using a Zn/Cu couple, and saponification gave **2b**. The same procedures applied to 3′-*O*-acetylthymidine 5′-(methyl 2,2,2-trichloroethyl phosphate)[40,41] (**14b**) afforded **2c**.



14a, R = Tce (CCl_3CH_2)
b, R = CH_3

15

16 → **2b**

9

17 → **2c**

16, R = Tce
17, R = CH_3

It was previously demonstrated,[42] using model systems bearing a methyl group at position 6 of the deazapteridine rather than the [methylene(*p*-aminobenzoyl)]glutamate side chain, that the N^5 and C^6 groups were trans disposed,

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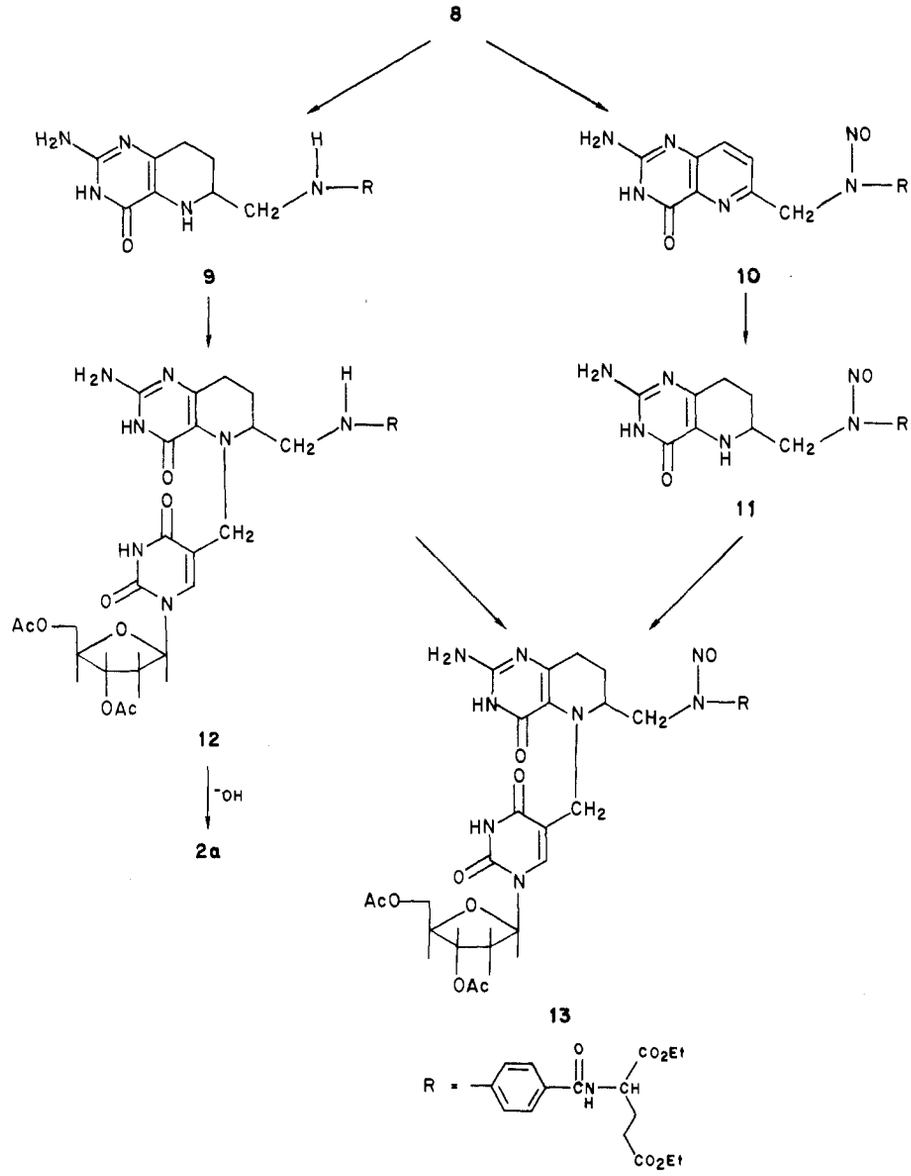
Scheme II



pseudodiaxial, and rather rigidly held in this relationship by steric constraints. This conformational assignment was based upon an analysis of $J_{6,7}$ values, examination of space-filling models, and the magnetic nonequivalence of virtually every carbon and proton signal in each of the two C-6 diastereoisomers. A particularly useful probe for this nonequivalence was found to be H-6 of the pyrimidine moiety. This proton signal appeared in an unencumbered region of the spectrum (δ 7.5–8.0) and was found to consist of a pair of roughly equivalent intensity singlets separated by about 0.06 ppm at 270 MHz. Identical behavior has now been observed for **2a–c** and their fully protected precursors in $(CD_3)_2SO$ or D_2O. These data and molecular model analysis support the view that, like the model, the heterocyclic bases are rigidly held in a V-shaped geometry and that the major flexibility in the molecules residues in the sugar phosphate and (*p*-aminobenzoyl)glutamate moieties.

Final confirmation of the structures of **16**, **17**, and **2a–c** was obtained by means of FAB mass spectrometry. Protected nucleotides **16** and **17** contain six and three chlorine atoms, respectively. The molecular ion (MH^+) cluster at 1123, 1125, 1127, 1129 for **16** and 1007, 1009, 1011 for **17** had the ratios expected. In each case, major fragment clusters were observed corresponding to loss of $NH_2CH(CO_2Et)CH_2CH_2CO_2Et$ (203) and $CH_3NHC_6H_4CONHCH(CO_2Et)CH_2CH_2CO_2Et$ (336) from the deazafolate side chain. For deprotected nucleotide **2b**, a molecular ion MH^+ was observed at *m/e* 765; for methyl ester **2c**, both MH^+ (779) and MNa^+ (801) were observed. Finally, treatment of **2b** with alkaline phosphatase gave rapid and complete (1 h) dephosphorylation to nucleoside **2a**.

Biology. Each of the potential multisubstrate analogues **2a–c** was evaluated by means of a double-reciprocal plot with two concentrations of inhibitor. The data for compound **2b**, the molecule containing all known important binding sites of both nucleotide and folate portions of the ternary complex, are presented in Figure 1 and Table I. The double-reciprocal plots clearly reveal that the inhibition is competitive against both dUMP (Figure 1a) and $N^{5,10}$-$CH_2(H_4PteGlu)$ (Figure 1b). Apparent inhibition constants derived from the slopes of the inhibited reactions are 0.058 and 0.25 μM, respectively (Table I). The K_m value for dUMP, as determined from the double-reciprocal plot, is 0.0 μM and that for $N^{5,10}$-$CH_2(H_4PteGlu)$ is 50 μM. Thus, the affinity of **2b** for thymidylate synthetase is about 50-fold higher than that of dUMP and about 200-fold higher than that of the cofactor. The apparent K_i value determined against dUMP is quite consistent with values obtained for the reversible inhibition of the *Lactobacillus*

Table I. Inhibition of Human Thymidylate Synthetase[a]

compd	variable [dUMP] inhib type	K_i,[b] μM	K_i/K_m	K_i/K_i(2b)	variable [$N^{5,10}$-CH_2-H_4PteGlu] inhib type	K_i, μM	K_i/K_m	K_i/K_i(2b)
dUMP		3.0[c]		50[d]				
2b	C	0.058	0.02	1.0	C	0.25	0.005	1.0
2c	C	120	40	2068	NC	700	14	2800
2a	NC	150	50	2586	NC	240	4.8	960
$N^{5,10}$-CH_2-H_4PteGlu						50[c]		200[d]
11	NC	240	80	4137	NC	150	3	600

[a] The experimental conditions are given in the text. [b] All K_i values are actually apparent K_i's. [c] K_m. [d] K_m/K_i(2b).

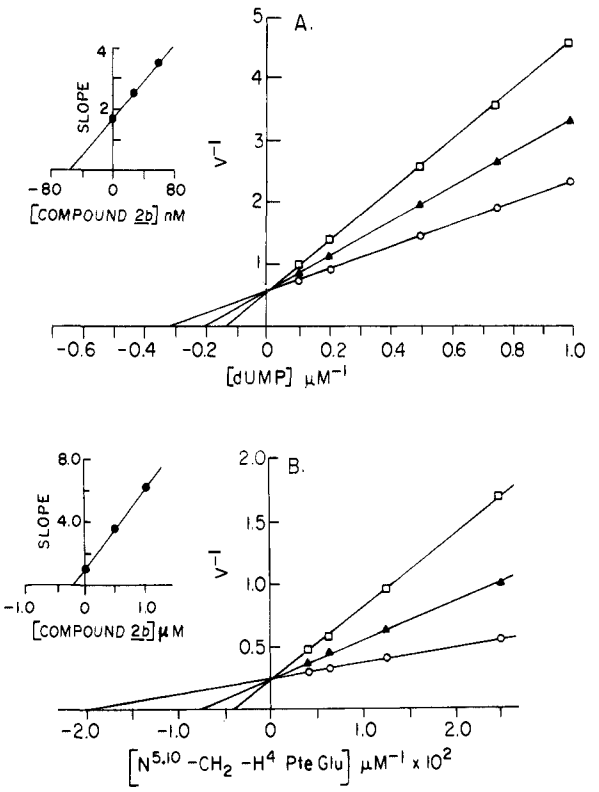


Figure 1. (A) Double-reciprocal plot of the velocity of formation of TMP vs. concentration of substrate dUMP with and without **2b**: no inhibitor (○), 30 nM (▲), 60 nM (□). (B) Double-reciprocal plot of the velocity of formation of TMP vs. concentration of cofactor, $N^{5,10}$-CH_2-H_4PteGlu, with and without added inhibitor **2b**: no inhibitor (○), 0.5 μM (▲), 1.0 M (□).

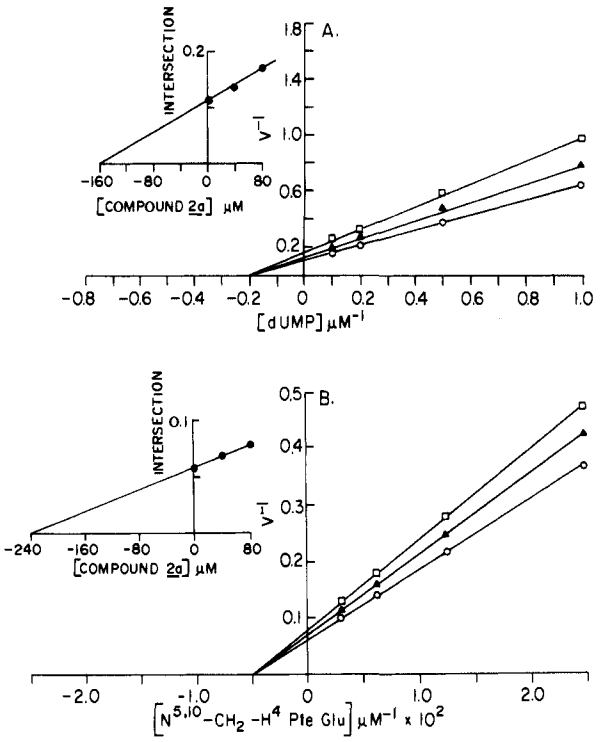


Figure 2. (A) Double-reciprocal plot of the velocity of formation of TMP (nmol min^{-1} mL^{-1}) vs. concentration of dUMP with and without **2a** as an inhibitor: no inhibitor (○), 40 μM (▲), 80 μM (□). (B) Double-reciprocal plot of the velocity of formation of TMP (nmol min^{-1} mL^{-1}) vs. concentration of $N^{5,10}$-CH_2-H_4PteGlu with and without **2a** as an inhibitor: no inhibitor (○), 40 μM (▲), 80 μM (□).

casei enzyme by a number of potent pyrimidine nucleotide inhibitors.[43] The potent competitive inhibition against both substrate and cofactor establishes that **2b** is acting as a true multisubstrate analogue, binding tightly to both the dUMP and reduced folate binding sites.

Since the 5′-phosphate of dUMP is generally considered to be critical in binding of the substrate to TS, it was of considerable interest to examine the effect of removing or modifying the phosphate in **2b**. In view of the multiplicity of other functional groups potentially involved in binding, one might think that the loss of only one (presumably ionic) binding site would weaken binding only slightly. However, removal of the phosphate (nucleoside **2a**, Figure 2, Table I) or modification to the methyl phosphate diester (**2c**, Figure 3, Table I) reduced affinity for the enzyme by 3 orders of magnitude. In addition, the kinetics of the inhibition became either completely (**2a**, Figure 2) or partially (**2c**, Figure 3b) noncompetitive. These data might be explained by postulating that binding of the nucleoside 5′-phosphate dianion produces a specific conformational change in the active site that facilitates the binding of the ternary complex. Alternatively, the presence of the 5′-phosphate may be necessary to initiate a conformational change of the nucleoside moiety secondary to initial electrostatic binding and required for tight binding. It is not presently possible to rule out one of these alternatives; indeed, both may play a role. There is evidence for sequential addition of substrate followed by cofactor to the active site resulting in a conformational change in the enzyme and opening up a second dUMP binding site.[44] Although evidence of a substantial conformational change in the active site resulting from substrate–enzyme binary complex formation is lacking, one might speculate that the phosphate dianion in **2b** could bind and open up the cofactor site to facilitate binding of the folate analogue portion of the molecule. The phosphate monoanion in **2c** might, then, be attracted to the anion binding site but fail to induce the conformational change required to facilitate binding of the folate portion. This could explain the ob-

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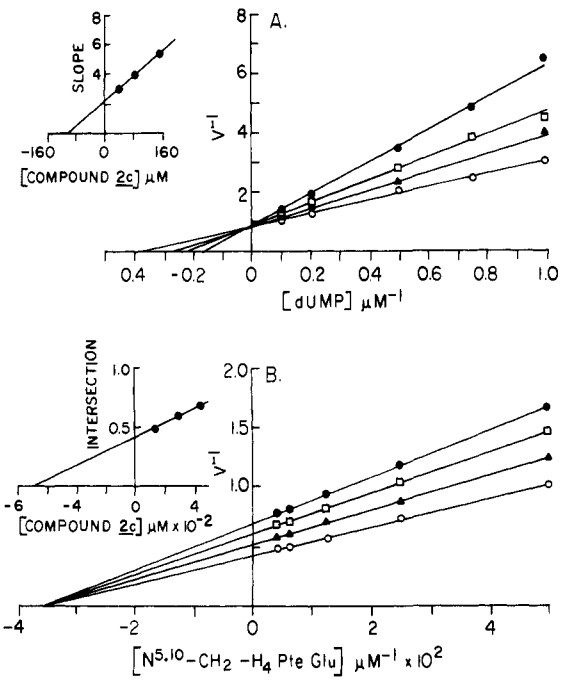

Figure 3. (A) Couble-reciprocal plot of the velocity of formation of TMP (nmol min^{-1} mL^{-1}) vs. concentration of dUMP with and without **2c** as an inhibitor: no inhibitor (○), 40 μM (▲), 80 μM (□), 150 μM (●). (B) Double-reciprocal plot of the velocity of formation of TMP (nmol min^{-1} mL^{-1}) vs. concentration of $N^{5,10}$-CH_2-H_4PteGlu with and without **2c** as an inhibitor: no inhibitor (○), 150 μM (▲), 300 μM (□), 450 μM (●).

served kinetics—competitive against dUMP and noncompetitive against CH_2-H_4PteGlu—for methyl phosphate **2c**. Nucleoside **2a** probably binds nonspecifically, perhaps even to another site on the enzyme, accounting for the weak binding and noncompetitive kinetics observed. It has been proposed that initial binding involves the interaction of the phosphate dianion with a protonated arginine residue in the active site.[22,45] Support for a role of conformational change in the inhibitor rather than (or in addition to) the active site may be found in the observation that an anti to syn conformational change about the glycosidic bond appears to be associated with dUMP binding to thymidylate synthetase.[46] Of course, failure of tight binding of methyl ester **2c** may be related to steric inhibition of the electrostatic interaction by the methyl group rather than to an absolute requirement for a dianion. Analogues designed to resolve this problem are currently being prepared.

Experimental Section

The ^1H NMR spectra were obtained with a JEOL FX-270 operating at 269.65 MHz in the FT mode with $(CD_3)_2SO$ as solvent and tetramethylsilane as internal standard or D_2O with DSS as external reference. A Beckman DU-8 UV/vis spectrophotometer was used to record UV spectra. Electron-impact mass spectra were obtained with a Varian 112S instrument. FAB spectra were produced by a Varian 731 mass spectrometer. Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are not corrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. Triethylammonium bicarbonate buffer, pH 8.0 (TEAB), was obtained by dilution of 2 M stock solution.

6-Carbomethoxy-8-chloro-2,4-dimethoxypyridol[3,2-*d*]pyrimidine (4). 6-Carbomethoxy-2,4,8-trichloropyrido[3,2-*d*]pyrimidine[35] (5.87 g, 20 mmol; crude product from the chlorination of 6 g of **3**) in methanol (100 mL) was treated with a solution of sodium methoxide (from 0.97 g of sodium) in methanol and the mixture was stirred for 2 h. Cold water (200 mL) was added, and the precipitate was filtered, washed with water (40 mL), and dried. Crystallization from ethyl acetate gave 4.5 g (80%) of **4**: mp 214–215 °C; mass spectrum, *m/e* 283 (M^+); UV λ_{max} (ϵ_{max}) (CH_3OH) 319.8 nm (7000), 288 (8500); ^1H NMR δ 3.94, 4.07, and 4.16 (s, 3 each, CH_3), 8.48 (s, 1, C-7 H). Anal. ($C_{11}H_{10}ClN_3O_4$) C, H, N.

6-Carbomethoxy-2,4-dimethoxypyrido[3,2-*d*]pyrimidine (5). Compound **4** (6.0 g) in DMF (180 mL) containing sodium acetate (2.4 g) was hydrogenated in the presence of palladium on activated charcoal (5%, 0.6 g) with a Parr hydrogenator (42 psi, 1 h). The catalyst was removed by filtration and extracted with cold DMF (40 mL) and hot ethyl acetate (150 mL). The filtrate and the extracts were combined and evaporated. The residue was triturated with cold water (25 mL), filtered, and washed with cold water: 4.15 g (88%). An analytical sample was obtained through recrystallization from ethyl acetate: mp 193–194 °C; mass spectrum, *m/e* 249 (M^+); UV λ_{max} (ϵ_{max}) (CH_3OH) 314.8 nm (7760), 275.6 (8630); ^1H NMR δ 3.93, 4.02, and 4.14 (s, 3 each, CH_3), 8.19 and 8.34 (d, 1 each, C-7 H and C-8 H). Anal. ($C_{11}H_{11}N_3O_4$) C, H, N.

6-(Hydroxymethyl)-2,4-dimethoxypyrido[3,2-*d*]pyrimidine (6). To a suspension of **5** (5 g, 20 mmol) in dry THF (125 mL) cooled in water (20 °C) was added lithium borohydride (0.55 g, 25 mmol) in small portions with stirring. The suspension became a clear solution in 15 min. Methanol (100 mL) was added, and after 10 min, the solvents were removed. The residue was triturated with 25 mL of cold water, filtered, and washed with cold water (15 mL) to give **6**: 3.7 g (84%). The solid was crystallized from water: mp 152–153 °C; mass spectrum, *m/e* 221 (M^+); UV λ_{max} (α_{max}) (pH 2) 311.4 nm (7270), (pH 7) 313 (5980), 250.6 (6850), 244.8 (6920), (pH 12) 314 (5870); ^1H NMR δ 3.99 and 4.10 (s, 3 each, OCH_3), 4.69 (d, 2, CH_2), 5.68 (t, 1, OH), 7.92 and 8.10 (d, 1 each, C-7 H and C-8 H). Anal. ($C_{13}H_{11}N_3O_3$) C, H, N.

2,4-Diamino-6-(hydroxymethyl)pyrido[3,2-*d*]pyrimidine (7). Compound **6** (3.98 g, 18 mmol) was heated with methanol saturated with ammonia (at −5 °C, 100 mL) at 180 °C in a sealed vessel for 8 h. Ammonia and methanol were removed, and the yellow residue was crystallized from water: yield, 2.1 g (61%). It was identical with a previously prepared sample.[32]

Diethyl N^{10}-Nitroso-8-deazafolate (10). A slightly modified procedure of Cosulich and Smith[37] was used. To a solution of 1.25 g (2.5 mmol) of diethyl 8-deazafolate in 10 mL of 6 N HCl cooled to 0–5 °C was added dropwise a solution of $NaNO_2$ (0.18 g, slight excess). The temperature was maintained between 0 and 5 °C during addition.

After 3–5 min, the solution was allowed to come to room temperature and diluted with water and solid bicarbonate was added in small amounts to pH 7. The resulting suspension was extracted with $CHCl_3$ (3 × 50 mL). The combined extracts were washed with water, dried over anhydrous Na_2SO_4, and evaporated to give 1.05 g (80%) of **10**. An analytical sample from ethanol melted at 148–149 °C; NMR δ 2.05 (m, 2 CH_2COOEt), 2.43 (m, 2, $CHCH_2$), 4.44 (m, 1, $CHCH_2$), 5,41 (s, 2, $N(NO)CH_2$), 6.52 (br, 2, $2\text{-}NH_2$), 7.37 (d, 1, C-7 H), 7.54 (d, 1, C-8 H), 7.88 and 8.01 (d each, 4, C_6H_4), 8.30 (d, 1, glu NH), 11.24 (br, 1, 3H); UV i_{max} (ϵ_{max}) (pH 1) 250 nm (24 500), 285 (15 000), (pH 7) 272 (22 500), 294 (s, 13 000), (pH 13) 274 (21 000), 334 (s, 7800). Anal. ($C_{24}H_{27}N_7O_7$) C, H, N.

Diethyl 5,6,7,8-Tetrahydro-8-deazafolate (9). Diethyl 8-deazafolate **8** (496 mg, 1 mmol) was dissolved in warm ethanol (75 mL) containing 10 mL of 0.1 N HCl. After cooling, the solution was hydrogenated in the presence of 35 mg of PtO_2 at 42 psi. The reduction was followed by TLC (silica gel) in CH_3CN/H_2O (90:10). After 3 h, the deazafolate completely disappeared and TLC revealed three compounds, diethyl (*p*-aminobenzoyl)-L-glutamate, 2-amino-4-oxo-6-methyl-5,6,7,8-tetrahydropyrido[3,2-*d*]pyrimidine, and **9**. The former two were identified by TLC with authentic samples (silica gel, CH_3CN/H_2O (90:10), $CHCl_3$/MeOH (80:20)). The catalyst was removed by filtration through a bed of Celite, and the filtrate was neutralized with 5% $NaHCO_3$ and evaporated.

The residue was stirred in warm $CHCl_3$ (100 mL) and filtered. The filtrate was evaporated, stirred with warm toluene (50 mL), and filtered to give a 0.3 g (60%) of **9**: mp 165–167 °C; NMR δ 1.6 (m, 1, C-7 H), 2.00 (m, 3, $CHCH_2$ and C-7 H), 2.37 (m, 4,

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CH_2COOEt and 8-CH_2), 5.86 (br, 2, 2-NH_2), 6.61 (t, 1, N^{10} H), 6.63 and 7.68 (d each, 4, C_6H_4), 8.24 (d, 1, glu NH,); UV i_{max} (ϵ_{max}) (pH 1) 294 nm (13150), (pH 7) 300 (27000), (pH 13) 197 (27000). Anal. ($C_{24}H_{32}N_6O_6$) C, H, N.

Diethyl N^{10}-Nitroso-5,6,7,8-tetrahydro-8-deazafolate (11). A solution of 1.04 g (2 mmol) of N^{10}-nitroso-8-deazafolate diethyl ester in 50 mL of ethanol containing 20 mL of 0.1 HCl was hydrogenated at 42 psi. The hydrogenation was complete in 3–4 h. The catalyst was removed by filtration. The filtrate was neutralized by adding saturated sodium bicarbonate solution. The cloudy suspension was evaporated to dryness. The residue was suspended in water and extracted with $CHCl_3$. The chloroform layer was washed with water, dried over Na_2SO_4, and concentrated to a small volume (ca, 10 mL). The solution was applied to a column (silica gel, 60–200 mesh) and elution with $CHCl_3$/MeOH (96:4) followed by $CHCl_3$/MeOH (90:10) gave N^{10}-nitroso-5,6,7,8-tetrahydro-8-deazafolate (11): 0.76 g (72%); mp 177–178 °C; NMR δ 1.57 (m, 1, C-7 H), 1.66 (m, 1, C-7 H), 2.03 (m, 2, $CHCH_2$), 2.43 (m, 2, CH_2COOEt), 2.46 (m, 2, 8-CH_2), 4.4 (m, 3, $CHCH_2$, NCH_2), 5.7 (br, 2, NH_2), 7.84 and 8.02 (d each, 4, C_6H_4), 8.84 (d, 1, glu NH), 10.03 (br, 1, N-3 H); UV λ_{max} (ϵ_{max}) (pH 1) 273 nm (19000), (pH 7) 282 (16000), (pH 13) 285 (16500). Anal. ($C_{24}H_{31}N_7O_7$) C, H. N.

Preparation of Thymidine Derivatives. 3′-*O*-Acetylthymidine 5′-[bis(2,2,2-trichloroethyl) phosphate] was prepared from 3′-*O*-acetylthymidine according to the procedure of Eckstein and Sheit[38] or by acetylation of thymidine 5′-[bis(trichloroethyl) phosphate].[39]

3′-Acetylthymidine 5′-(Methyl 2,2,2-trichloroethyl phosphate) (14b). The procedure of Ogilvie[40,41] was used. A solution of 5.85 g (10 mmol) of thymidine 5′-[bis(2,2,2-trichloroethyl) phosphate][38] in 200 mL of absolute methanol containing 15.1 g of CsF (100 mmol) was stirred at room temperature for 3 days. The solvent was removed in vacuo, the residue was suspended in water, and the suspension was extracted with EtOAc. The organic layer was washed with water, dried over Na_2SO_4, and evaporated. Trituration with petroleum ether gave 3.64 g (78%) of mixed triester: mp 176–177 °C; ^{1}H NMR δ 1.79 (s, 3, 5-CH_3), 2.15 (m, 2, 2′-CH_2), 3.78 (d, J = 9.1 Hz, 3, $POCH_3$), 3.94 (m, 1, 3′-CH), 4.72 (t, 2, CCl_3CH_2), 5.46 (m, 1, 4′-CH), 6.21 (t, J = 6.4 Hz, 1,1′-CH), 7.49 (s, 1, 6-CH), 11.32 (br, 1, NH).

The above mixed triester (3.50 g, 7.5 mmol) in 75 mL of dry pyridine was stirred with 25 mL of acetic anhydride with the exclusion of moisture for 24 h. The solvent was evaporated to dryness and the residue was evaporated with ethanol several times to give a viscous oil (3.3 g, 88%). The compound did not solidify; mass spectrum, m/e 508; ^{1}H NMR δ 2.01 (s, 3, $COCH_3$).

General Procedure for the Bromination of Thymidine Derivatives. To a refluxing solution of protected thymidine derivative (1.25 mmol in 40–50 mL) in dry CCl_4, irradiated with a sunlamp, was added dropwise 0.22 g of Br_2 (1.1 equiv) in 3–4 mL of CCl_4. The addition rate was adjusted so that each drop was consumed before the addition of the next drop. The reaction was followed by TLC (silica gel, EtOAc). When the addition was complete, approximately 75–80% of the starting compound had been reacted. The solution was cooled to room temperature and evaporated to dryness, and the residue was used without further purification.

General Procedure for Alkylation of Diethyl 8-Deaza-5,6,7,8-tetrahydrofolate by 5-(Bromomethyl)-2′-deoxyuridine Derivatives. A solution of the bromo compound (75–80%; from 1.25 mmol of the corresponding protected thymidine derivative) in 20 mL of dry DMF containing 1 mmol of folate derivative and 100 mg of solid $NaHCO_3$ (dried at 100 °C in vacuo) was stirred at room temperature. The reaction was followed by TLC (silica gel, $CHCl_3$/MeOH (80:20)). After the base has completely reacted (6 h), a few drops of methanol were added to convert any unreacted bromo compound to the methoxymethyl derivative.

After 2 h, the solvent was removed in vacuo, and the residue was suspended in water and extracted with $CHCl_3$. The organic layer was washed with water, dried over Na_2SO_4, concentrated to a small volume, and applied to a silica gel column. Initial elution with $CHCl_3$/MeOH (98:2) gave thymidine derivatives and elution with $CHCl_3$/MeOH (96.4) gave the N-5 alkylated product.

(i) **Diethyl *N*-[4-[[[2-amino-3,4,5,6,7,8-hexahydro-4-oxo-5-[(3′,5′-diacetyl-2′-deoxyuridin-5-yl)methyl]pyrido[3,2-*d*]pyrimidin-6-yl]methyl]amino]benzoyl]-L-glutamate** (12): prepared in 61% yield, mp 140–142 °C (prior softening); NMR (partial) δ 1.90 and 1.95 (s each, 3, 3′-$COCH_3$), 2.0, 2.02 (s each, 3, 5′-$COCH_3$), 5.22 (m, 1, 4′-CH), 6.05 (br, 2, 2-NH_2), 6.15 (m, 1-CH, two sets of anomeric), 6.50 and 7.3 (d each, 4, C_6H_4), 8.0 and 8.2 (s each, pyrimidine C-6 H), 8.24 (d, glu NH), 10.01 (br, NH); UV λ_{max} (ϵ_{max}) (pH 1) 269 nm (21000), 294 (s, 14100), (pH 7) 270 (25400), 299 (25500), (pH 13) 292 (25000). Anal. ($C_{38}H_{48}N_8O_{13}\cdot H_2O$) C, H, N.

(ii) **Diethyl *N*-nitroso-*N*-[4-[[[2-amino-3,4,5,6,7,8-hexahydro-4-oxo-5-[(3′,5′-diacetyl-2′-deoxyuridin-5-yl)methyl]pyrido[3,2-*d*]pyrimidin-6-yl]methyl]amino]benzoyl]-L-glutamate** (13): (a) prepared as described above in 66% yield: mp 133–137 °C; NMR (partial) δ 1.99 and 2.00 (s each, 3, 3′-$COCH_3$), 2.06 (s, 3, 5′-$COCH_3$), 7.10 and 7.80 (s each, 1, C-6 H pyrimidine), 7.63 and 7.89 (m, 4, C_6H_4); UV λ_{max} (ϵ_{max}) (pH 1) 269 nm (21800), (pH 7) 269 (21500) 282 (s, 18000), 269 (19000), 285 (16600). Anal. ($C_{38}H_{47}N_9O_{14}\cdot 0.5H_2O$) C, H, N.

(b) To an ice cold solution of 12 in 6 N HCl (0.068 g, 0.5 mL) was added 8 mg of $NaNO_2$ in two portions (bath temperature, 0 °C). The solution was stirred for 3 min, diluted to 2 mL with cold water, and neutralized with 5% HCO_3^- solution.

The solution was extracted with $CHCl_3$, washed with water, dried with Na_2SO_4, and evaporated (ca. 40 mg). Silica gel TLC in $CHCl_3$/MeOH (80:20) and CH_3CN/H_2O (90:10) showed N^{10}-nitroso compound 13 as the major product. ^{1}H NMR spectra were identical for the two compounds.

(iii) **Diethyl *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(trichloroethyl) phosphate]** (16): prepared in 62% yield; mp 176–179 °C; ^{1}H NMR (partial) δ 2.05 and 2.06 (s each, 3′-$COCH_3$), 4.6 (m, CCl_3CH_2, ×2), 5.99 (2, NH_2, br) 7.90 and 7.97 (s each, C-6 H), 8.19 (d, glu NH) 6.46 and 7.61 (d each, C_6H_4).

(iv) **Diethyl *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′-(trichloroethyl methyl phosphate)** (17): prepared in 56% yield; mp 157–159 °C; ^{1}H NMR δ 2.00 and 2.03 (3 each, $COCH_3$), 3.73 (t, $POCH_3$), 7.90 and 7.97 (s each, 1, C-6 H).

***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 (2a).** To a solution of 0.21 g (0.25 mmol) of 12 in 1 mL of ethanol was added 1 mL of 1 N NaOH, and the solution was stirred overnight. A slightly turbid solution was observed. Addition of one drop of 1 N NaOH gave a clear solution. After the solution was stirred for an additional 3 h, the solvent was removed in vacuo. The residue was dissolved in 1 mL of H_2O and cooled in an ice bath, and cold 0.1 N HCl was added with stirring to pH 3.5–4. The precipitated solid was washed with cold water and dried: mp >280 °C; yield 95 mg (55%); a FAB mass spectrum gave 685 (M^+ + H); UV λ_{max} (ϵ_{max}) (pH 1) 269 nm (17000), (pH 7) 278 (22800), 299 (21500); (pH 13) 291 (26000). Anal. ($C_{30}H_{36}N_8O_{11}\cdot 2.5H_2O$) C, H, N.

***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 16 (0.225 g, 0.2 mmol) was dissolved in 5 mL of dry DMF, and 0.2 g (2 mmol) of acetylacetone and 0.26 g of Zn/Cu couple were added. The mixture was stirred at 50–55 °C in an oil bath. The deprotection was followed by TLC (silica gel, 2-isopropanol/NH_4OH/H_2O (7:1:2)). When the reaction was judged complete (approximately 1 h), the solution was cooled and diluted to 10 mL with water and H_2S was bubbled through for about 10 min. The precipitated sulfides were removed by filtration. Nitrogen was bubbled to remove H_2S and solvent was removed in vacuo (bath temperature, ~30 °C). The residue was suspended in 1 mL of ethanol, and 5 mL of N NaOH was added. The solution was stirred for 48 h. The solution was diluted to ca. 15 mL, the pH was adjusted to ca. 4.5 with 0.5 N HCl, and the solution was diluted to ca. 300 mL with water.

The solution was applied to a DEAE-Sephadex 25 (HCO_3^-) column (0.9 × 10 cm). The column was washed with 100 mL of water followed by a linear gradient (200 mL/200 mL) of 0.2 M TEAB/0.8 M TEAB (pH 8). Chromatographically pure fractions were combined and evaporated to dryness. The residue was

coevaporated with ethanol several times and redissolved in EtOH (~5 mL).

This fraction was added to a stirred solution of sodium perchlorate (0.2, g, 2 mmol) in 15–20 mL of dry acetone. Stirring was continued for 10–15 min. The solid was collected by centrifugation (washed three times with acetone) and dried in high vacuum. **2b**: yield 110 mg (3050 AU_{278}, 0.132 mmol; 66%); ^1H NMR (D_2O) δ 6.22 (t, C-1 H), 6.62, 7.59 (d each, C_6H_4), 7.82, 8.06 (s each, pyrimidine C-6 H); FAB mass spectrum, MH^+ 765.

Enzymatic Phosphate Cleavage from 2b. A mixture containing 50 μL of **2b** (2 mg/mL), 10 μL of M Tris-HCl (pH 9.0), and 5 μL of alkaline phosphatase (*E. coli*, EC 3.1.3.1; Sigma) was incubated at 37 °C. In 1 h, TLC (silica, 2-propanol/NH_4OH/water (7:1:2)) indicated the complete loss of phosphate in **2b** to give **2a**.

***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′-(Methyl phosphate) (2c).** Compound 17 (0.21 g, 0.2 mmol) in 5 mL of dry DMF containing 0.2 g (2 mmol) of acetylacetone and 0.2l g of Zn/Cu couple was stirred for 3 h at room temperature. The methyl ester was isolated by essentially the procedure described above. After removal of metals by H_2S and a similar workup, the final volume was ca. 300 mL. The solution was applied to a DEAE-Sephadex 25 (HCO_3^-) column (0.9 × 10 cm) which was washed with water and then eluted with a linear gradient (200 mL/200 mL) of water –0.2 M TEAB (pH 8). Chromatographically pure fractions were combined and precipitated from an acetone solution of sodium perchlorate (see above): yield 0.11 g; 3200 AU_{278}, 0.14 mmol, 70%; ^1H NMR (D_2O) 3.53 (d, $POCH_3$), 6.20 (t, C-1 H), 6.66, 7.60 (d each, C_6H_4), 7.80, 7.94 (s each, pyrimidine C-6 H); FAB mass spectrum, MH^+ 779, MNa^+ 801.

Biological Evaluation. Chemicals. dUMP and [5-^{3}H]dUMP (1 mCi/mmol) were purchased from Sigma; CH_2-H_4PteGlu was prepared by treating tetrahydrofolate with formaldehyde in 0.1 M phosphate buffer (pH 7.5), and the wavelength of maximum absorbance of the solution and the concentration were checked spectrophotometrically. All other chemicals were of reagent grade or better.

Enzyme Preparation. Pellets of HeLa-S_3 cells (5 mL) were used as the source of thymidylate synthetase. The enzyme was purified by methotrexate–Sepharose column chromatography as described previously[47] except that gel filtration on a Sephadex G-25 column (0.5 × 20 cm) was employed instead of overnight dialysis. The purified enzyme was stored at –20 °C in the presence of 0.1% (w/v) bovine serum albumin and 40% glycerin; no substantial loss of activity was found after 3 months of storage.

Enzyme Assay. The activity of thymidylate synthetase was assayed by the tritium release procedure of Roberts.[48] The reaction mixture contained in a volume of 40 μL the following: dUMP (a mixture of dUMP and [5-^{3}H]dUMP in a molar ratio of 10:1) at concentrations indicated in each experiment; CH_2-H_4PteGlu at concentrations as indicated in each assay; 150 mM Tris-HCl, pH 7.5; 0.125 mM dithiothreitol; 50 mM NaF; 50 mM formaldehyde; 0.15% BSA; 32 mM sucrose; and enzyme. Reactions were initiated by adding the enzyme, and then the mixture was incubated at 37 °C for 60 min. Reactions were terminated by the addition of 20 μL of 2 N trichloroacetic acid. Then 0.5 mL of aqueous charcoal suspension (Darco G-60, 100 mg/mL) was added and the mixture spun in a table-top centrifuge at 4000 rpm for 5 min. The supernatant (200 μL) was counted in 7.5 mL of ASC scintillant.

Apparent K_i Determination. With respect to dUMP as a substrate, apparent K_i determination was performed by keeping CH_2-H_4PteGlu at 200 μM and varying the concentration of dUMP. Enzyme activities were assayed in the presence of the examined inhibitor at variable concentrations. Other ingredients in the assay mixture were the same as those in the standard reaction mixture described above. Data obtained were presented in the Lineweaver–Burk format. Then, for competitive inhibition, the slopes of the lines in the double-reciprocal plot were replotted against the concentrations of the examined compound; for noncompetitive inhibition, the intersections on the vertical axis were used to replot. Similarly, with CH_2-H_4PteGlu as a substrate, apparent K_i determination was conducted by keeping dUMP at 100 μM and varying the concentration of CH_2-H_4PteGlu.

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Registry No. **2a** (isomer I), 92007-98-2; **2a** (isomer II), 92007-99-3; **2b** (isomer I), 92008-00-9; **2b** (isomer II), 92008-01-0; **2c** (isomer I), 92008-02-1; **2c** (isomer II), 92008-03-2; **4**, 91900-19-5; **5**, 91900-20-8; **6**, 91900-21-9; **7**, 76822-61-2; **8**, 76807-65-3; **9** (isomer I), 92007-90-4; **9** (isomer II), 92007-91-5; **10**, 82855-87-6; **11** (isomer I), 92007-92-6; **11** (isomer II), 92007-93-7; **12** (isomer I), 92007-94-8; **12** (isomer II), 92007-95-9; **13** (isomer I), 92007-96-0; **13** (isomer II), 92007-97-1; **14a**, 88543-88-8; **14b**, 88543-90-2; **15a**, 88543-89-9; **15b**, 88543-91-3; **16** (isomer I), 92008-88-3; **16** (isomer II), 92075-79-1; **17** (isomer I), 92008-89-4; **17** (isomer II), 92008-90-7; 6-(carbomethoxy)-2,4,8-trichloropyrido[3,2-*d*]pyrimidine, 68409-26-7; sodium methoxide, 124-41-4; thymidine 5′-[bis(2,2,2-trichloroethyl phosphate], 20744-74-5; thymidylate synthetase, 9031-61-2; thymidine 5′-[(methyl 2,2,2-trichloroethyl)phosphate], 91900-22-0.

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