Krebs solution, maintained at 37 °C and gassed with 95% O_2/5% CO_2 throughout the whole experiment. An initial basal tension of 1 g was applied to each tracheal chain, and the tissue was allowed to stabilize for 90 min. Isometric force was recorded from the preparations by a force-displacement transducer and an Omni-Scribe recorder. A constant level of tone was induced by the addition of carbachol chloride (5.5×10^{-7} M, Sigma) to the bath, and, after 15 min, a control concentration–response curve for isoprenaline was obtained. The tissue was washed thoroughly, and 30 min later a single concentration of antagonist (**3a–h**, **4a–h**) was added to the bath and allowed to act for 30 min. During the last 15 min of antagonist incubation, carbachol chloride (5.5×10^{-7} M) was added to the bath, and the cumulative concentration–response curve for isoprenaline was again determined. All responses to different concentrations of isoprenaline were expressed as percentages of the maximal relaxation recorded for the control curve. β-Blockade was evaluated by determining the pA_2 as described by Van Rossum;[12] the slope of Schild plot was calculated in each case, according to Arunlakshana.[13]

(b) Isolated Guinea Pig Left Atria. Guinea pigs of either sex, weighing 300–350 g, were sacrificed by a blow to the head. The heart was removed as quickly as possible and placed in a dish containing carbogenated Krebs solution. Left atrial preparations were subjected to electric pulses delivered by a Cibertec stimulator through platinum electrodes on the muscle holder. The tissue was mounted under 1.0 g of tension in a 30-mL organ bath containing modified Krebs solution at 37 °C, continuously bubbled with carbogen. The tissue was allowed to equilibrate for 60 min before eliciting responses to drugs. After 45-min equilibration, the tissue was stimulated to contract by pulses of 5 ms and submaximal voltage. Contractions were recorded isometrically with a force-displacement transducer connected to an Omni-Scribe recorder. Cumulative concentration–response curves of isoprenaline were recorded, the solution in the muscle chamber was removed and after a 15 min period, and the preparation was exposed to a single concentration of antagonist for additional 15 min, after which another cumulative concentration–response curve for isoprenaline was reelicited. pA_2 values were calculated as described above.

Acknowledgment. We are grateful to the Comisión Asesora de Investigación Científica y Técnica for financial support.

Registry No. *erythro*-**3a**, 115462-42-5; *threo*-**3a**, 115462-43-6; *erythro*-**3b**, 88624-96-8; *threo*-**3b**, 88624-97-9; *erythro*-**3c**, 115462-44-7; *erythro*-**3c**·HCl, 115462-74-3; *threo*-**3c**, 115462-45-8; *erythro*-**3d**, 115462-46-9; *threo*-**3d**, 115462-47-0; *erythro*-**3e**, 115462-48-1; *threo*-**3e**, 115462-49-2; *erythro*-**3f**, 115462-50-5; *threo*-**3f**, 115462-51-6; *erythro*-**3g**, 115462-52-7; *threo*-**3g**, 115462-53-8; *erythro*-**3h**, 115462-54-9; *erythro*-**3h**·HCl, 115462-75-4; *threo*-**3h**, 115462-55-0; **4a**, 7695-63-8; **4b**, 525-66-6; **4c**, 2007-72-9; **4d**, 2933-94-0; **4e**, 5790-46-5; **4f**, 19343-24-9; **4g**, 80617-74-9; **4h**, 62372-04-7; **6**, 55967-94-7; **7a**, 115462-36-7; **7a**·HCl, 115462-72-1; **7b**, 88624-92-4; **7c**, 115462-37-8; **7c**·HCl, 115462-73-2; **7d**, 115462-38-9; **7e**, 115462-39-0; **7f**, 115462-40-3; **7g**, 115462-41-4; *erythro*-**8a**, 115462-58-3; *threo*-**8a**, 115462-59-4; *erythro*-**8b**, 115462-60-7; *threo*-**8b**, 115462-61-8; *erythro*-**8c**, 115462-62-9; *threo*-**8c**, 115462-63-0; *erythro*-**8d**, 115462-64-1; *threo*-**8d**, 115462-65-2; *erythro*-**8e**, 115462-66-3; *threo*-**8e**, 115462-67-4; *erythro*-**8f**, 115462-68-5; *threo*-**8f**, 115462-69-6; *erythro*-**8g**, 115462-70-9; *threo*-**8g**, 115462-71-0; *erythro*-**9**, 115462-56-1; *threo*-**9**, 115462-57-2; C_6H_5OH, 108-95-2; H_3C-*m*-C_6H_4OH, 108-39-4; H_3C-*p*-C_6H_4OH, 106-44-5; $C_6H_5CH_2OH$, 100-51-6; $C_6H_5(CH_2)_3OH$, 122-97-4; 1-naphthol, 90-15-3; 2-naphthol, 135-19-3.

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A Pyrimidine-Based "Flexible" Bisubstrate Analogue Inhibitor of Human Thymidylate Synthase

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Received December 14, 1987

The synthesis and characterization of two "flexible" bisubstrate analogues of the intermediate in the thymidylate synthase reaction are reported. Steric constraints are minimized and diasteromeric mixtures avoided by using a pyrimidine-based analogue as the folate portion of the inhibitor while retaining all known important binding sites. A preliminary assessment of certain conformational parameters by NMR is presented. The compounds are shown to be potent competitive inhibitors with respect to dUMP or 5,10-CH_2-H_4PteGlu but gave mixed kinetics with respect to 5,10-CH_2-H_4PteGlu$_5$ for human thymidylate synthase.

Thymidylate synthase (EC 2.1.1.45) plays a unique role in cellular biochemistry as the sole de novo source for the production of 2′-deoxythymidylate (dTMP). The reaction utilizes 2′-deoxyuridylate (dUMP) and 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4PteGlu) as substrate and cofactor, respectively. A unique feature of the reaction in comparison with other one-carbon transfer reactions is that 5,10-CH_2-H_4PteGlu acts both as one-carbon donor and as reductant, leading to the formation of dTMP and dihydrofolate. Although some details of the nature of this mechanism remain fuzzy, enough information has been developed about the molecular mechanism that structure 1 can be drawn as representing a ternary complex among substrate, cofactor, and enzyme.[1-5]

Efforts from a number of laboratories over several years[6-8] culminated in the synthesis of the deaza bisubstrate analogue **2** in this laboratory.[9] Compound **2** proved to be a potent competitive inhibitor of thymidylate synthase, and NMR studies suggested that the compound was

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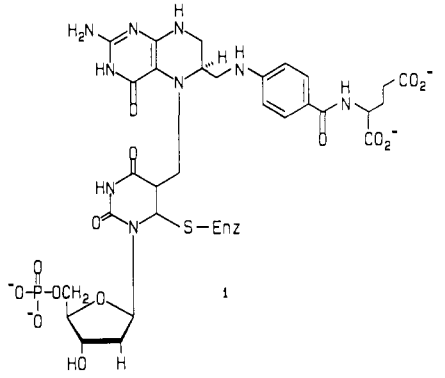
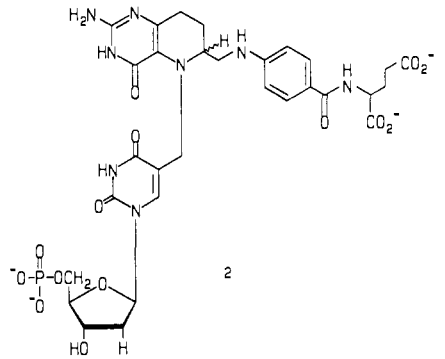
surprisingly rigid.[9,10] It was of considerable interest to explore the conformation of **2** by using such two-dimensional NMR techniques as COSY and NOESY. However, the rigidity of the molecule led to nonequivalence of virtually every proton and carbon signal in the spectra because of the presence of virtually inseparable diastereoisomers generated by chemical reduction of the pyridine ring of the deazafolate.

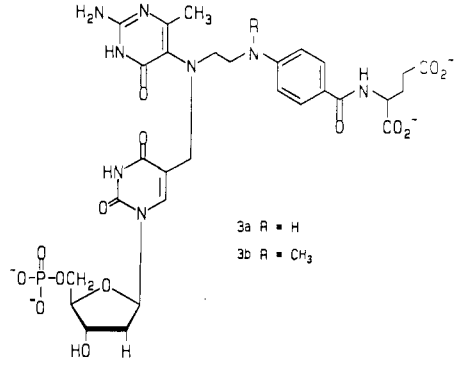


1



2

It was of interest, therefore, to attempt to prepare a bisubstrate analogue that would retain the binding functionalities of **2** but would have greater flexibility and would not exist in diastereoisomeric forms. The present report describes the synthesis and the initial NMR and biological evaluations of such a "flexible" inhibitor, the pyrimidine-based folate analogue **3**. It should be noted that inhibitor **3** is formally derivable from the rigid inhibitor **2** by "excision" of the 7-methylene group, thereby releasing the constraints imposed by the tetrahydropyridine ring in the latter.

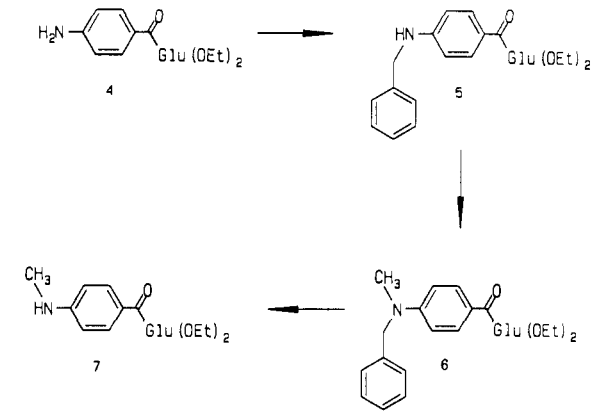


3a R = H
3b R = CH_3

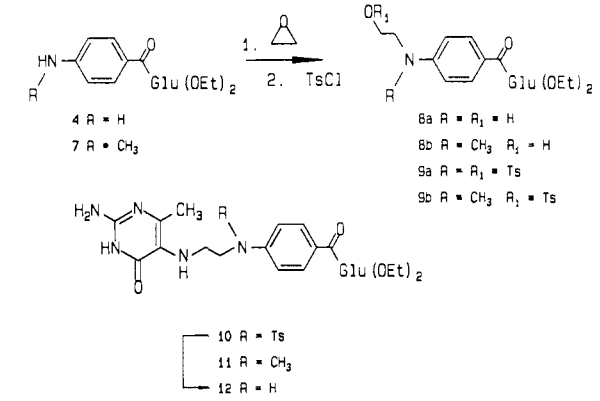
Results and Discussion

In the middle 1960s, there was a flurry of interest in the synthesis and testing of pyrimidine-based folate ana-

Scheme I



Scheme II



logues.[11-15] This interest waned as it became clear that these compounds were inhibitory toward thymidylate synthase only in the 10 μm–2 mmol range. For the sake of those knowledgeable in the folate field, the numbering of the key exocyclic nitrogens in this system will conform to folate nomenclature, i.e., N-5 and N-10 will be used, despite the fact that the folate analogue components of **3a** and **3b** are actually *p*-(aminoethyl)amino derivatives of benzoylglutamate. The key folate analogues were thus **11** and **12**.

The simplest way to approach the synthesis of the N-10 methyl derivative would be to have as starting material diethyl [*p*-(methylamino)benzoyl]glutamate (**7**, Scheme I). Of the various procedures devised for the synthesis of this material, the best to date appears to have been that of Santi.[16] This six-step procedure provided good overall yields of the target compound, but was found to be rather cumbersome in practice. A much simpler procedure was therefore devised—a sequence of reductive benzylation, reductive methylation, and hydrogenolytic debenzylation of commercially available diethyl (*p*-aminobenzoyl)-glutamate gave **7** in 68% overall yield in only three steps.

The key to the alkylation reactions involves maintaining the solution slightly acidic. The procedure is monitored with a bromcresol green indicator and addition of acetic acid as needed to maintain pH slightly on the acidic side.

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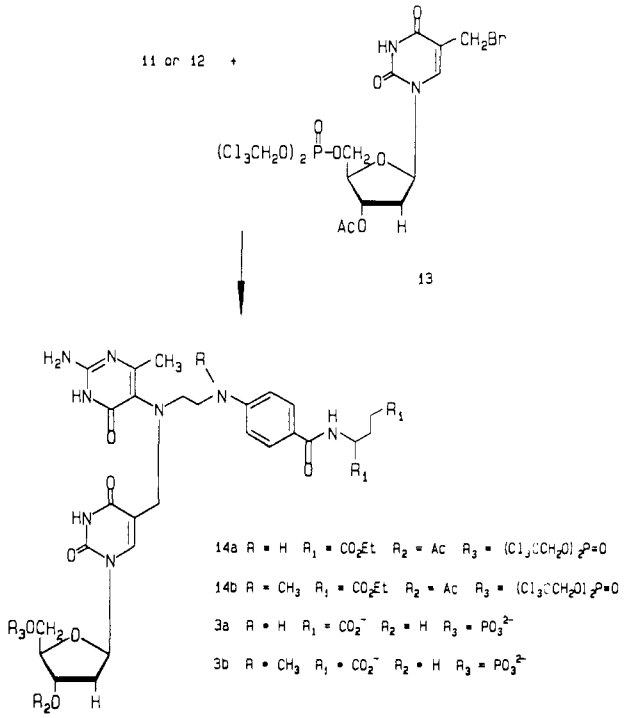
Scheme III



This procedure seems to minimize formation of byproducts and preferential reduction of the aldehyde in favor of formation and reduction of the Schiff's base.

The *N*-methyl derivatives **7** and diethyl (*p*-aminobenzoyl)glutamate itself (**4**) were treated directly with ethylene oxide in acetic acid to form the hydroxyethyl derivatives (Scheme II). As might be expected, the reaction with the *N*-methyl derivative **8b** proceeded cleanly, as judged by TLC, to give an essentially quantitative yield of an oil, which resisted crystallization, but which had the appropriate mass spectral and NMR properties. The reaction with the free *p*-amino group of **4** predictably gave a mixture of dialkyl, monoalkyl, and unalkylated materials. However, these were readily separated by column chromatography on silica gel to give diethyl [*p*-[*N*-(2-hydroxyethyl)amino]benzoyl]glutamate (**8a**) in 40% yield. Compounds **8a** and **8b** were smoothly tosylated to provide the *N,O*-ditosyl and the *O*-tosyl derivatives **9a** and **9b**, respectively. Alternatively, *N*-methyl derivative **8b** could be prepared by reaction of **7** with 2-iodoethanol. Iodoethanol is a more pleasant compound to handle than ethylene oxide, which provides some advantage to this approach. It is interesting to note that hydroxyethylation of the *N*-benzyl compound **5** with either ethylene oxide or iodoethanol proceeded very sluggishly and in poor yield, presumably for steric reasons.

The tosyl derivatives **9a** and **9b** were reacted with 2,5-diamino-6-methyl-4-oxopyrimidine[17] to provide the necessary side chain-to-pyrimidine linkage. Detosylation of **10** with HBr and acetic acid afforded the N-10 deprotected material **12**.

Formation of the fully protected bisubstrate analogues **14a** and **14b** from each of the two folate analogues proceeded smoothly by using the procedures described by Srinivasan et al.[9] (Scheme III). Briefly, 3′-*O*-acetylthymidine 5′-[bis(trichloroethyl) phosphate] was brominated under free radical conditions to give the highly reactive bromomethyl derivative **13**. Alkylation of **11** or **12** with **13** gave the fully protected derivatives, exactly as expected on the basis of earlier studies. Deprotection was carried out by removal of the trichloroethyl functions with a zinc–copper couple and by saponification to remove the three ester functions in the molecules. Both protected and deprotected bisubstrate analogues were characterized by FAB mass spectrometry and by NMR.

Mass Spectral Characterization. Molecules of the size of the inhibitors reported here are not readily analyzed by electron impact mass spectral techniques. The application of fast-atom bombardment (FAB), however, provides useful data for characterization of both protected and deprotected products. Even with fully protected inhibitors **14a** and **14b**, with molecular weights of 1110 and 1124 Da, respectively, clear MH^+ ions may be readily obtained in the FAB mode. Furthermore, the characteristic cluster of chlorine isotope peaks is found in association with MH^+ and with each fragment containing the trichloroethyl protecting groups.

In addition to the useful information that a molecule of the correct mass was formed, examination of the fragmentation pattern was consistent with alkylation of the folate analogue only at N^5. For both **14a** and **14b**, major fragmentation occurred with the loss of 202 (the diethyl glutamate residue); further fragmentation α to each basic nitrogen gave cleavage between the two methylene groups with the loss of 337 for **14a** and 349 for **14b**. Each of the resulting ions gave the usual Cl_6 cluster. These data, coupled with NMR information (vide infra) confirm the site of alkylation at N^5. Finally, MH^+ ions at 753 and 767 for **3a** and **3b**, respectively, confirmed that deprotection had occurred completely to give the target inhibitors, without damage to any of the potentially sensitive bonds in these complex molecules.

NMR Considerations. The mass spectral data demonstrated unequivocally that alkylation had not occurred on the (*p*-aminobenzoyl)glutamate side chain but left open which of several potentially nucleophilic sites on the pyrimidine might be attacked. Although extensive precedent supports the 5-amino group as the likely alkylation site,[7-10] additional confirmation was sought in the NMR spectra.

Other than N^5, the only possible alkylation sites of **11** would be O^4, N^3, N^1, or the 2-amino group. Alkylation at any of the first three positions would eliminate the N-H signal from position 3, whereas 2-amino alkylation would obviously alter the broad 2-proton singlet associated with that functional group. Comparison of the spectrum of **14b** with those of the component folate and nucleotide analogues revealed normal N-H signals for uracil N^3-H (δ 11.5) and folate analogue N^3-H (δ 10.8) and 2-amino (δ 6.36). Clearly, then, the mass spectral and NMR data conclusively identify the alkylation site as N^5.

As indicated above, one of the major motivations for entering into this study was to obtain potent bisubstrate analogue inhibitors of thymidylate synthase by procedures that afforded a single pure compound rather than a diastereoisomeric mixture. In addition, it was thought to be desirable that the inhibitors be somewhat more flexible than the initial prototype in order that they might conform more readily to the enzyme active site, perhaps resulting in enhanced affinity for the enzyme. It came as a considerable surprise, therefore, to obtain NMR spectra on **3a** and **3b** in D_2O and to find that such proton signals as thymidine H-6 and the pyrimidine 6-methyl group existed as pairs of signals, strongly reminiscent of the situation with the prototype compound.[9,10] In keeping with the anticipation that compounds **3a** and **3b** would be more flexible than **2**, this nonequivalence was found to coalesce

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Table I. Inhibition of Human Thymidylate Synthase[a]

compd	IC_{50},[b] μM: 5,10-CH_2-H_4PteGlu (600 μM)	5,10-CH_2-H_4PteGlu$_5$ (40 μM)
2	0.39 ± 0.00	0.20 ± 0.01
3a	7.3 ± 0.3	1.18 ± 0.11
3b	1.0 ± 0.0	0.35 ± 0.00

[a] The experimental conditions are given in the text. [b] The concentration of other substrate, dUMP, used was 28 μM.

as the temperature was increased, and by 50 °C complete coalescence had occurred. This phenomenon was fully reversible, suggesting that two conformers exist that are magnetically nonequivalent and that interconvert slowly on the NMR time scale at or below room temperature.

In an attempt to learn a bit more about this odd phenomenon, the apparent pH of the solution was raised from the value of 5.6 in the original sample to 7.4. The nonequivalence largely, but not completely, disappeared. It seems clear from the information currently available that restricted rotation about the pyrimidine 5-amino group, resulting from the attachment to that nitrogen of three bulky substituents, leads to a sufficiently low flip rate that the position appears on the NMR time scale as asymmetric. Warming or raising the pH removes all or most of this apparent asymmetry, resulting in spectra that are those originally predicted for these compounds.

One can draw a variety of "stacked" conformers that show the sort of nonequivalence described. The effect of pH is at present mystifying; the only pK_a in this molecule associated with the change of pH from 5.6 to 7.4 is the secondary phosphate ionization. In order to attempt to understand this conformational problem, molecular graphics studies involving energy minimization and two-dimensional NMR studies are in progress and will be reported elsewhere.

Biological Studies

Compounds **3a** and **3b** together with prototype inhibitor **2** have been evaluated as inhibitors of thymidylate synthase derived from human leukemia cells. The assay solution contains 28 μM dUMP and either 600 μM 5,10-CH_2-H_4PteGlu or 40 μM 5,10-CH_2-H_4PteGlu$_5$ as substrates. The slopes of the inhibition of the enzyme activity by these three compounds were similar. This suggests the mode of interaction of these compounds with the enzyme was similar. The IC_{50} of each of these compounds is shown in Table I. It should be noted that IC_{50} values were quite different when either 5,10-CH_2-H_4PteGlu or 5,10-CH_2-H_4PteGlu$_5$ was employed as substrate. The K_m values were 137 ± 17 and 18.3 ± 14.4 μM, respectively. Detailed kinetic analysis of the inhibitory activity of compound **2** and **3b** was performed. Results are presented in Table II. The K_i values for compound **2** obtained by varying either 5,10-CH_2-H_4PteGlu or dUMP concentration were slightly different from those reported previously. This is due to the difference in fixed substrate concentration used in those assays. The mode of interaction of compounds **2** and **3b** with TMP synthase was apparently identical. The mode of inhibition was altered when 5,10-CH_2-H_4PteGlu$_5$ was used as substrate instead of 5,10-CH_2-H_4PteGlu. The compounds are very potent competitive inhibitors in both enzyme systems, having K_i values versus dUMP in the order of 0.03 μM for the human enzyme versus dUMP. With 5,10-CH_2-H_4PteGlu as variable substrate, the K_i for **3b** was 0.23 μM versus 0.09 for **2**, and with the pentaglutamate as the variable substrate, the inhibition changed to noncompetitive for both compounds, and the K_i values were similar (Table I).

Summary and Conclusions

Bisubstrate analogue inhibitors of thymidylate synthase that were formally derivable from prototype inhibitor **2** by excision of the 7-methylene group have been synthesized, characterized, and evaluated as inhibitors of thymidylate synthase. Although they are indeed much more flexible than **2**, as judged by their NMR behavior, these compounds are not much different from **2** as inhibitors of thymidylate synthase. The change of inhibitory mode from competitive type to mixed type for this group of compounds when 5,10-CH_2-H_4PteGlu$_5$ was used as substrate instead of 5,10-CH_2-H_4PteGlu was observed previously for 10-ethyl-10-deazaaminopterin tetraglutamate.[18] This further supports the notion that 5,10-CH_2-H_4PteGlu$_5$ could alter the enzyme conformation in a way different from 5,10-CH_2-H_4PteGlu.

Comparison of the structures of compounds **3a** and **3b** with **1** led to the hypothesis that the 6-position of the pyrimidine might undergo attack by the active site sulfhydryl group in order to give a ternary complex resembling **1**. However, no time-dependent inactivation was observed, suggesting that covalent modification did not occur or that it was freely reversible. These studies suggest that these inhibitors fit the active site well, but may be acting more as product–substrate analogues than as bisubstrate analogues. In other words, once the carbon is attached to thymidylate C-5, the compound formed may resemble product more than it does substrate. Since product is readily released from the enzyme and binds more weakly to it,[19–22] this may account for the lack of covalent bond formation and time-dependent inactivation of the enzyme by these analogues. Studies are in progress to explore the value of putting an "electron sink" at the thymine 5-position in order to ascertain whether this will facilitate nucleophilic attack at C-6.

Experimental Section

^{1}H NMR spectra were determined with a JEOL FX-270 or an IBM AF200 FT-NMR spectrometer with TMS or DSS as internal standards. UV spectra were obtained on a Beckman DU-8 spectrometer. Melting points were determined on a Thomas-Hoover capillary apparatus and are uncorrected. Mass spectra were performed with a Varian MAT 112S or Varian MAT 731 mass spectrometer. Elemental analyses were performed by MicAnal (now Desert Analytics), Tucson, AZ. Thin-layer chromatography (TLC) was performed on precoated TLC sheets (Silica 60F-254, Malinckrodt).

Diethyl *N*-[*p*-(Benzylamino)benzoyl]-L-glutamate (5). Sodium cyanoborohydride (1.5 g, 24 mmol) was added in small portions to a stirred ethanolic (50 mL) solution of **4** (3.23 g, 10 mmol), benzaldehyde (1.5 g, 14 mmol), and bromocresol green (10 drops) at room temperature. After complete addition, acetic acid was added dropwise to adjust the solution to slightly acidic as determined by the indicator. The reaction solution was stirred for 48 h at room temperature, and during this time, acetic acid was added dropwise as necessary to maintain a slightly acidic solution. The acidic solution was diluted with EtOAc (25 mL) and Et_2O (25 mL) and was washed with 10% NaOH (2 × 25 mL), H_2O (2 × 25 mL), and brine (1 × 30 mL). The collected organic portions were dried (Na_2SO_4), filtered, and evaporated in vacuo to afford a light amber oil. The oil was dissolved in Et_2O (40 mL)

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(20) Galivan, J. H.; Maley, G. F.; Maley, F. *Biochemistry* **1976**, *15*, 356.
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(22) Dolnick, B. J.; Cheng, Y.-C. *J. Biol. Chem.* **1977**, *252*, 7697.

Table II. Inhibitory Parameters of the Compounds Examined

	variable substrates						
	[dUMP][a]		[5,10-CH_2-H_4PteGlu][b]		[5,10-CH_2-H_4PteGlu$_5$][b]		
compd	inhib type	K_i, μM	inhib type	K_i, μM	inhib type	K_{is},[c] μM	K_{ii},[c] μM
2	C[d]	0.027 ± 0.002	C	0.085 ± 0.007	M[d]	0.33 ± 0.09	0.11 ± 0.03
3b	C	0.037 ± 0.006	C	0.23 ± 0.11	M	1.19 ± 0.01	0.20 ± 0.03

[a] The fixed concentration of 5,10-CH_2-H_4PteGlu used as 600 μM. [b] The fixed concentration of dUMP used was 28 μM. The other conditions were the same as described in the text. [c] K_{is} and K_{ii} are K_i(slope) and K_i(intercept), respectively. [d] C is competitive; M is mixed.

and EtOAc (2 mL) followed by the dropwise addition of petroleum ether until the upper layer had a persistent cloudiness. The title compound (3.88 g, 94%) was collected as white needles: mp 118–120 °C (lit.[23] mp 117–118 °C); ^{1}H NMR (Me_2SO-d_6) δ 1.13–1.195 (m, 6 H, OCH_2CH_3), 1.9–2.25 (m, 2 H, $CHCH_2CH_2$), 2.39 (t, 2 H, CH_2CH_2CO), 4.02–4.115 (m, 4 H, OCH_2CH_3), 4.33 (br d, t, 3 H, $NHCHCH_2$, $NHCH_2Ar$), 6.58 (d, 2 H, Ar *H*), 6.85 (t, 1 H, $ArNHCH_2Ar$), 7.24–7.36 (m, 5 H, $NHCH_2ArH$), 7.62 (d, 2 H, Ar *H*), 8.22 (d, 1 H, CHN*H*CO).

Diethyl *N*-[*p*-(*N*-Benzyl-*N*-methylamino)benzoyl]-L-glutamate (6). Sodium cyanoborohydride (660 mg, 10.5 mmol) was added in small portions to a stirred solution of **5** (2.75 g, 6.7 mmol), formaldehyde (37% aqueous, 5.6 mL, 67 mmol), and bromocresol green (10 drops) in dry acetonitrile (50 mL) at room temperature. After complete addition, acetic acid was added dropwise to give an acidic solution as determined by the indicator. The reaction solution stirred for 1 h at room temperature with a consistent acidic pH maintained by the addition of acetic acid as needed. The reaction solution was diluted with EtOAc (50 mL) and Et_2O (50 mL) followed by washing with 10% NaOH (2 × 20 mL), H_2O (2 × 20 mL), and brine (2 × 15 mL). The collected organic portions were dried (Na_2SO_4), filtered, and evaporated to dryness in vacuo to afford an oil. The oil was crystallized from Et_2O/petroleum ether to yield the title compound as flocculent white crystals (2.61 g, 91%): mp 67–70 °C; *m/z* 426; UV λ_{max} (ϵ_{max}) (ethanol): 307 nm (24 250); ^{1}H NMR (Me_2SO-d_6) δ 1.13–1.2 (m, 6 H, OCH_2CH_3), 1.95–2.14 (m, 2 H, $CHCH_2CH_2$), 2.41 (t, 2 H, CH_2CH_2CO), 3.09 (s, 3 H, NCH_3), 3.97–4.12 (m, 4 H, OCH_2CH_3), 4.38 (d of t, 1 H, $NHCHCH_2$), 4.66 (s, 2 H, NCH_2Ar), 6.74 (d, 2 H, Ar *H*), 7.17–7.24 (m, 5 H, NCH_2ArH), 7.7 (d, 2 H, Ar *H*), 8.3 (d, 1 H, CH*NH*CO). Anal. ($C_{24}H_{30}N_2O_5$) C, H, N.

Diethyl *N*-[*p*-(Methylamino)benzoyl]-L-glutamate (7). An ethanolic (55 mL) solution of **6** (850 mg, 2 mmol), 10% palladium on carbon (85 mg), and 1 N HCl (5 mL) was subjected to an atmosphere of hydrogen at 48 psi for 3 h. The hydrogenation mixture was then filtered through Celite, and the filtrate was diluted with EtOAc (50 mL). The organic solution was washed with saturated $NaHCO_3$ (2 × 20 mL) and H_2O (1 × 30 mL). The collected organic portion was dried (Na_2SO_4), filtered, and evaporated in vacuo to afford an oil. The oil was dissolved in a minimal amount of EtOAc, and petroleum ether was added dropwise until the upper layer had a persistent cloudiness. The title compound (538 mg, 80%) was collected as white needles, mp 86–88 °C (lit.[16] mp 90–91 °C); ^{1}H NMR (Me_2SO-d_6) δ 1.11–1.2 (m, 6 H, OCH_2CH_3), 1.95–2.15 (m, 2 H, $CHCH_2H_2$), 2.42 (t, 2 H, CH_2CH_2CO), 2.71 (s, 3 H, NCH_3), 4.0–4.13 (m, 4 H, OCH_2CH_3), 4.38 (d of t, 1 H, $NHCHCH_2$), 6.33 (br s, 1 H, $NHCH_3$), 6.53 (d, 2 H, Ar *H*), 7.68 (d, 2 H, Ar *H*), 8.24 (d, 1 H, *NH*CO).

Diethyl *N*-[4-[*N*-(Hydroxyethyl)amino]benzoyl]-L-glutamate (8a). Ethylene oxide (5 mL, cooled to <0 °C) was added to a solution of **4** (4.83 g, 15 mmol) in 120 mL of 50% aqueous acetic acid cooled by an ice bath. *Note: Ethylene Oxide is a gas at room temperature and a known carcinogen. It should be handled at ice bath temperatures in a hood. Appropriate protective garb should be used.* The solution was stirred in a stoppered/flask at room temperature for 17 h. The reaction solution was concentrated in vacuo to a volume of 20 mL and then neutralized with 10% NH_4OH at ice-bath temperature. The resultant solution was extracted with $CHCl_3$ (3 × 100 mL), and the collected organic layers were washed with H_2O (2 × 100 mL). The organic portion was dried over Na_2SO_4 and filtered, and the filtrate was evaporated in vacuo to a syrup. The syrup was dissolved in a minimal amount of EtOAc and purified by column chromatography (silica gel, 3.5 × 60 cm; EtOAc), and the fractions containing **8a** (TLC, R_f 0.3, EtOAc) were collected. Evaporation of the solvent in vacuo followed by trituration with anhydrous Et_2O gave 2.1 g (40%) of **8a**: mp 74 °C; *m/z* 366 (M^+); ^{1}H NMR (Me_2SO-d_6) δ 1.1 (t, 6 H, CH_3), 2.0 (m, 2 H, CH_2CH_2CO), 2.4 (t, 2 H, CH_2CO_2), 3.1 (q, 2 H, NCH_2), 3.5 (q, 2 H, OCH_2), 4.0 (q, 4 H, $COOCH_2$), 4.4 (q, 1 H, C*H*), 4.7 (t, 1 H, O*H*), 6.2 (t, 1 H, N*H*), 6.6 and 7.6 (d, 4 H, Ar *H*), 8.2 (d, 1 H, CON*H*). Anal. ($C_{18}H_{26}N_2O_6$) C, H, N.

Diethyl *N*-[4-[*N*-(2-Hydroxyethyl)-*N*-methylamino]benzoyl]-L-glutamate (8b). Method A. Compound **8b** was prepared in the manner described for **8a**. Compound **8b** was obtained in quantitative yield to give an oil, which resisted crystallization: *m/z* 380 (M^+); ^{1}H NMR (Me_2SO-d_6) δ 3.0 (s, 3 H, NCH_3), 3.4 and 3.5 (t and q, 4 H, CH_2CH_2), 4.7 (t, 1 H, O*H*).

Method B. 2-Iodoethanol (140 mg, 0.81 mmol) was added to a stirred solution of **7** (250 mg, 0.74 mmol) in DMAC (3 mL). The reaction solution was stirred at 100 °C, protected from moisture, for 5 h. The cooled solution was diluted with EtOAc (30 mL) and washed with H_2O (3 × 15 mL) followed by brine (2 × 10 mL). The collected organic portion was dried over Na_2SO_4 and filtered, and the filtrate was evaporated in vacuo to a dark oil. The oil was purified on a flash column (EtOAc; 1.5 cm/min; 10 mL fractions; 60 mL void) to give the desired product in fractions 6–13. Evaporation of the solvent in vacuo afforded 241 mg (86%) of **8b** as an oil identical in every respect with the product prepared by method A.

Diethyl *N*-[4-[*N*-(2-Tosyloxyethyl)-*N*-tosylamino]benzoyl]-L-glutamate (9a). *p*-Toluenesulfonyl chloride (4.45 g, 23.3 mmol) was added to a stirred solution of **8a** (3.88 g, 10.6 mmol) in dry pyridine (10 mL) at room temperature. The solution was stirred for 1 h and was then poured in to 30 mL of 1 N HCl. The acidic solution was extracted with $CHCl_3$, and the organic portion was collected and dried over Na_2SO_4. The desiccant was removed by filtration, and the filtrate was evaporated in vacuo to afford an oil. The oil was triturated with anhydrous ether (10 mL) to afford **9a** as a white solid (6.50 g): mp 105–107 °C; *m/z* 674 (M^+); ^{1}H NMR (Me_2SO-d_6) δ 2.39 (s, 3 H, Ts CH_3), 2.41 (s, 3 H, Ts CH_3), 7.40–7.77 (m, 8 H, Ts Ar *H*). Anal. ($C_{32}H_{38}N_2O_{10}S_2$) C, H, N.

Diethyl *N*-[4-[*N*-(2-Tosyloxyethyl)-*N*-methylamino]benzoyl]-L-glutamate (9b). *p*-Toluenesulfonylchloride (267 mg, 1.4 mmol) was added to a stirred solution of **8b** (540 mg, 1.4 mmol) in dry pyridine (5 mL) at room temperature. The solution was stirred for 1 h and was then poured in to 15 mL of 1 N HCl. The acidic suspension was extracted with $CHCl_3$ (3 × 15 mL), and the collected organic portions were dried over Na_2SO_4. The desiccant was removed by filtration, and the filtrate was evaporated in vacuo to afford an oil. The oil was triturated with anhydrous Et_2O to give 595 mg (80%) of **9b** as a white solid: mp 120–122 °C; *m/z* 534 (M^+); ^{1}H NMR (Me_2SO-d_6) δ 2.4 (s, 3 H, Ts CH_3), 2.9 (s, 3 H, NCH_3), 3.7 and 4.2 (t, 4 H, CH_2CH_2), 6.6, 7.4, 7.6 and 7.7 (d, 8 H, Ar *H*). Anal. ($C_{26}H_{34}N_2O_8S$) C, H, N.

Diethyl *N*-[4-[*N*-[2-[*N*-(2-Amino-4-oxo-6-methylpyrimidin-5-yl)amino]ethyl]-*N*-tosylamino]benzoyl]-L-glutamate (10) and Its Detosylated Derivative (12). 2,5-Diamino-4-oxo-6-methyl pyrimidine[17] (3.6 g, 25.9 mmol) in dry DMF (20 mL) under a static atmosphere of N_2 was heated at 85–90 °C (oil bath) to cause a homogeneous solution. After complete dissolution had taken place, **9a** (5.8 g, 8.64 mmol) was added, and the reaction mixture was stirred at this temperature for 48 h. The cooled solution was evaporated in vacuo, and the resulting residue was coevaporated with methanol (30 mL). The residue was

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suspended in H_2O (25 mL) and extracted with $CHCl_3$ (2 × 200 mL). The collected organic portions were dried over Na_2SO_4 and filtered, and the filtrate was concentrated in vacuo to a small volume. The concentrated solution was applied to a silica gel column ($CHCl_3$/MeOH, 95:5) and the fractions containing **11a** (TLC, CH_3CN/H_2O, 96:4, R_f 0.2) were combined and evaporated in vacuo. Trituration of the residue with anhydrous ether afforded the title compound **10** (1.0 g, 18%): mass spectrum (FAB) 643 (MH^+); ^1H NMR (Me_2SO-d_6) δ 1.9 (s, 3 H, 6-CH_3), 2.4 (s, 3 H, Ts CH_3), 6.1 (s, 2 H, 2-NH_2), 7.4 (dd, 4 H, Ts Ar *H*), 10.8 (s, 1 H, 3-N*H*).

A portion of **10** (707 mg, 1.1 mmol) was stirred with 3 mL of 30% HBr in acetic acid containing phenol (10–50 mg) for 3.5 h. The dark brown reaction mixture was swirled with Et_2O (20 mL). The resultant mixture was decanted, and the remaining solid was dissolved in $CHCl_3$ (30 mL). The $CHCl_3$ solution was washed with $NaHCO_3$ (saturated 3 × 50 mL) followed by H_2O (50 mL). The collected organic portion was dried over Na_2SO_4 and filtered, and the filtrate was evaporated in vacuo. The residue was triturated with anhydrous ether to afford **12** (362 mg, 67%), mp 90 °C dec; mass spectrum (FAB) 489 (M^+); UV λ_{max} (ϵ_{max}) (EtOH) 218 (sh) 301 (24 700), (pH 2) 215 (15 200) 292 (16 800), (pH 7) 296 (18 000), (pH 12) 293 nm (19 200); ^1H NMR (Me_2SO-d_6) δ 2.0 (s, 3 H, 6-CH_3), 6.1 (s, 2 H, 2-NH_2), 6.3 (s, 1 H, N*H*). Anal. ($C_{23}H_{32}N_6O_6$) C, H, N.

Diethyl *N*-[4-[*N*-[2-[*N*-(2-Amino-4-oxo-6-methylpyrimidin-5-yl)amino]ethyl]-*N*-methylamino]benzoyl]-L-glutamate (**11**). 2,5-Diamino-4-oxo-6-methylpyrimidine (200 mg, 15 mmol) in dry DMF (20 mL) and under a static atmosphere of N_2 was heated at 90–100 °C (oil bath) to cause a homogeneous solution. After complete dissolution had taken place, **9b** (250 mg, 5 mmol) was added, and the reaction mixture was stirred at this temperature for 24 h. The cooled solution was evaporated in vacuo, and the residue was suspended in H_2O (15 mL) followed by extraction with $CHCl_3$ (3 × 50 mL). The collected $CHCl_3$ portions were combined and washed with saturated aqueous $NaHCO_3$ (2 × 15 mL) and H_2O (2 × 10 mL) and then dried over Na_2SO_4. The desiccant was removed by filtration, and the filtrate was concentrated to a volume of 3 mL. The concentrated solution was applied to a flash column ($CHCl_3$/MeOH, 95:5; 2 cm/min; 5 mL fractions; 45 mL void), and fractions 18–23 contained the desired product **11b**. Evaporation of the solvent and trituration with anhydrous Et_2O yielded 495 mg (20%) of **11** as a white solid: mp 95–97 °C; FAB 503 (M^+); UV λ_{max} (ϵ_{max}) (EtOH) 306 (19 200), (pH 2) 303 (11 000), (pH 7) 311 (15 700), (pH 12) 306 nm (16 200); ^1H NMR (Me_2SO-d_6) δ 2.0 (s, 3 H, 6-CH_3), 3.0 (s, 3 H, NCH_3), 6.1 (s, 2 H, 2-NH_2), 6.7 and 7.7 (d, 4 H, Ar *H*), 11.0 (s, 1 H, 3-N*H*). Anal. ($C_{24}H_{34}N_6O_6 \cdot 0.5H_2O$) C, H, N.

General Procedure for Alkylation of Folate Analogues. A solution of **13**[9] in dry DMF (10 mL) was added to the folate derivative **11** or **12** (1.0 mmol) and $NaHCO_3$ (100 mg) in dry DMF (10 mL), and the reaction was stirred at room temperature. The reaction was monitored by TLC ($CHCl_3$/MeOH, 90:10). After the base had completely reacted (2 h), the solution was evaporated in vacuo, and the residue was suspended in H_2O and extracted with $CHCl_3$. The organic portion was dried over $NaSO_4$ and filtered, and the filtrate was concentrated in vacuo. The concentrated solution was applied to a silica gel column to elute the unreacted **13** ($CHCl_3$/MeOH, 97:3) and then the desired product ($CHCl_3$/MeOH, 94:6).

Diethyl *N*-[4-[*N*-[2-[*N*-(2-Amino-4-oxo-6-methylpyrimidin-5-yl)-*N*-[(3′-*O*-acetyl-2′-deoxyuridin-5-yl)methyl]amino]ethyl]amino]benzoyl]-L-glutamate 5′-[Bis(trichloroethyl) phosphate] (**14a**). Prepared in 41% yield: mp 128–130 °C; mass spectrum (FAB) 1111 (MH^+); UV λ_{max} (ϵ_{max}) (EtOH) 295 (13 500), (pH 2) 268 (13 300) 300 nm (sh); ^1H NMR (Me_2SO-d_6) δ 1.9 (s, 3 H, CCH_3), 2.0 (s, 3 H, COCH_3), 4.8 (t, 4 H, OCH_2CCl$_3$), 6.0 (t, 1 H, *p*-N*H*), 6.1 (t, 1 H, 1′-*H*), 7.5 (s, 1 H, uracil C_6-*H*), 11.5 (s, 1 H, uracil N_3-*H*). Anal. ($C_{39}H_{49}N_8O_{15}Cl_6P \cdot H_2O$) C, H, N.

Diethyl *N*-[4-[*N*-[2-[*N*-(2-Amino-4-oxo-6-methylpyrimidin-5-yl)-*N*-[(3′-*O*-acetyl-2′-deoxyuridin-5-yl)methyl]amino]ethyl]-*N*-methylamino]benzoyl]-L-glutamate 5′-[Bis(trichloroethyl) phosphate] (**14b**). Prepared in 42% yield: FAB 1125 (MH^+); UV λ_{max} (ϵ_{max}) (EtOH) 306 (12 200), (pH 2) 262 (12 200), 313 (9800), (pH 12) 268 (sh), 316 nm (20 800); ^1H NMR (Me_2SO-d_6) δ 2.0 (2 s, 6 H, COCH_3, CCH_3), 2.9 (s, 3 H, NCH_3), 6.2 (t, 1 H, 1′-*H*), 6.3 (br s, 2 H, 2-NH_2), 10.7 (s, 1 H, pyrimidine N*H*), 11.4 (s, 1 H, uracil N_3-*H*). Anal. ($C_{40}H_{51}N_8O_{15}PCl_6$) C, H, N.

***N*-[4-[*N*-[2-[*N*-(2-Amino-4-oxo-6-methylpyrimidin-5-yl)-*N*-[(2′-deoxyuridin-5-yl)methyl]amino]ethyl]amino]benzoyl]-L-glutamic Acid 5′-Monophosphate** (**3a**). Freshly prepared Zn–Cu couple (0.26 g) was added to a stirred solution of **14a** (222 mg, 0.2 mmol) in dry DMF (5 mL) and acetylacetone (0.2 g, 2 mmol). The mixture was stirred at 50–55 °C (oil bath), and the deprotection process was monitored by TLC (2-propanol; NH_4OH/H_2O, 7:2:1). When the reaction was judged complete (1.5 h), the cooled solution was diluted with H_2O (50 mL) and H_2S was bubbled through the solution for 10 min. The precipitated sulfides were removed by filtration. Nitrogen was bubbled through the filtrate for 12 h to remove excess H_2S followed by evaporation in vacuo to give a gummy residue. The residue was suspended in ethanol (1 mL) and 1 N NaOH (2 mL) and stirred for 48 h. The basic solution was diluted with H_2O (100 mL), the pH was adjusted to 4.5 with HCl (0.5 N), the solution was further diluted with H_2O to a volume of 300 mL and applied to a DEAE-Sephadex 25 (HCO_3^-) column (0.9 × 10 cm). The column was washed with H_2O (100 mL) followed by a linear gradient (200 mL of 0.1 M TEAB/200 mL of 0.5 M TEAB pH 8). Chromatographically pure fractions were combined and evaporated to dryness in vacuo. The residue was coevaporated with ethanol (3 × 10 mL) and redissolved in ethanol (5 mL). The solution was added to a stirred solution of sodium perchlorate (0.28 g, 2 mmol) in dry acetone (10–20 mL). The resulting suspension was stored at 4 °C overnight, and the precipitate was collected by centrifugation (washed three times with acetone), dissolved in H_2O, and freeze-dried. Collected 60 mg (40%) of the title compound: FAB 753 (MH^+); UV λ_{max} (ϵ_{max}) (pH 7) 281 nm (14 700); ^1H NMR (D_2O) (20 °C) δ 1.93 and 1.96 (2 s, 3 H, CH_3), 6.3 (t, 1 H, 1′-*H*), 7.30 (2 s, 1 H, uracil C_6*H*), 6.7 and 7.7 (2 d, 4 H, Ar *H*), (50 °C) 1.94 (s, 3 H, CH_3), 7.2 (s, 1 H, uracil C_6*H*).

***N*-[4-[*N*-[2-[*N*-(2-Amino-4-oxo-6-methylpyrimidin-5-yl)-*N*-[(2′-deoxyuridin-5-yl)methyl]amino]ethyl]-*N*-methylamino]benzoyl]-L-glutamic Acid 5′-Monophosphate** (**3b**). Prepared as described above in 44% yield: FAB 767 (MH^+); UV λ_{max} (ϵ_{max}) (pH 7) 310 nm (15 200); ^1H NMR (D_6O) (21 °C) δ 1.86 and 1.98 (2 s, 3 H, CH_3), 2.88 and 2.90 (2 s, 3 H, NCH_3), 6.3 (m, 1 H, 1′-H), 7.40 and 7.45 (s each, 1 H, uracil C_6-*H*), 6.6 and 7.6 (2 d, 4 H Ar *H*), (50 °C) 1.90 (s, 3 H, CH_3), 2.9 (s, 3 H, NCH_3), 6.2 (t, 1 H, 1′-H), 7.37 (s, 1 H uracil C_6-*H*), 6.7 and 7.6 (2 d, 4 H, Ar *H*).

Biological Evaluation. Chemicals. [5-^{3}H]dUMP (22 Ci/mmol) and dUMP were obtained from Moravek Biochemicals, Inc., Brea, CA, and Sigma Chemical Co., St. Louis, MO, respectively. 5,10-CH_2-H_4PteGlu and 5,10-CH_2-H_4PteGlu$_5$ were prepared by the chemical synthesis as described.[24,25] Purity of the bisubstrate analogues 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[22] from a pellet of human acute myeloblastic leukemia cells. These cells were provided by North Carolina Memorial Hospital. The enzyme assay was performed by the tritium release procedure of Roberts[26] as reported by Dolnick and Cheng,[22] 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 (Tables I and II). One unit of enzyme activity was defined as the conversion of 1 nmol of substrate (dUMP) per minute. The reaction was started by the addition of the enzyme, and the incubation time was either 45 or 60 min. All assays were conducted in duplicate or triplicate and repeated at least once with similar results. K_i 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 analogue. The

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fixed concentration of dUMP used was 28 μM when either 5,10-CH_2-H_4PteGlu or 5,10-CH_2-H_4PteGlu$_5$ was used as variable substrate. The fixed concentration of 5,10-CH_2-H_4PteGlu was 600 μM when dUMP was used as variable substrate.

Registry No. **3a**, 107716-41-6; **3b**, 107716-42-7; **4**, 13726-52-8; **5**, 70280-70-5; **6**, 115827-03-7; **7**, 2378-95-2; **8a**, 107716-29-0; **8b**, 107716-30-3; **9a**, 107716-31-4; **9b**, 107716-32-5; **10**, 107745-48-2; **11**, 107716-34-7; **12**, 107716-33-6; **13**, 88543-89-9; **14a**, 107716-37-0; **14b**, 107716-38-1; PhCHO, 100-52-7; H_2CO, 50-00-0; ICH_2CH_2OH, 624-76-0; ethylene oxide, 75-21-8; 2,5-diamino-4-oxo-6-methylpyrimidine, 4214-86-2; thymidylate synthase, 9031-61-2.

Topical Nonsteroidal Antipsoriatic Agents. 2. 2,3-(Alkylidenedioxy)naphthalene Analogues of Lonapalene[1]

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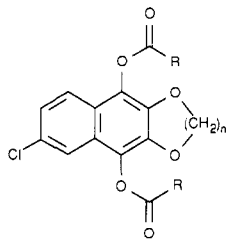
A series of 2,3-(alkylidenedioxy)naphthalene analogues (**5a–p**) of lonapalene (RS-43179, 1), a 5-lipoxygenase inhibitor currently under clinical investigation for the treatment of psoriasis, has been prepared and evaluated for topical inhibitory activity against arachidonic acid induced mouse ear edema. The results of these studies demonstrate that introduction of the fused 2,3-alkylidenedioxy ring, in place of the acyclic 2,3-dialkoxy substituent pattern characteristic of the previous series, caused a modest dimunition in overall potency within the series. These results suggest a potential steric intolerance for these extended planar analogues, in comparison with their 2,3-dialkoxy predecessors.

The regulation of abnormal arachidonic acid metabolism for the relief of chronic inflammatory or autoimmune conditons has been a prime target for pharmacological intervention in these disease states.[2-4] In particular, the development of 5-lipoxygenase (5-LO) inhibitors as therapeutic agents for diseases potentially caused or sustained by leukotriene B_4 (LTB_4) and other chemotactic lipoxygenase products has received increasing attention.[5] The viability of this approach has been most widely recognized in the treatment of psoriasis,[6] where elevated levels of arachidonic acid metabolites, especially the leukotrienes, have been detected.[7-10] A variety of compounds now identified as lipoxygenase (LO) or mixed cyclooxygenase–lipoxygenase (CO-LO) inhibitors are targeted for the topical or systemic treatment of psoriasis.[6]

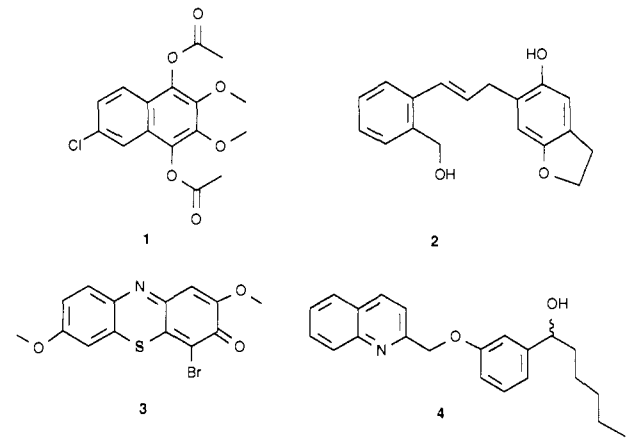
Among 5-LO inhibitors with demonstrated topical activity (**2–4**,[11] Chart I), our interest has focused on lonapalene (**1**),[12] already shown to have clinical efficacy comparable to steroid therapy[13] and currently undergoing advanced clinical evaluation as a topical treatment for psoriasis. Previous structure–activity correlations related the activity of 1 and a series of close analogues in the arachidonic acid induced mouse ear edema assay to net lipophilicity and ease of ester hydrolysis.[12] In this study, a series of 2,3-(alkylidenedioxy)naphthalene lonapalene analogues (**5a–p**), distinguished by the incorporation of the



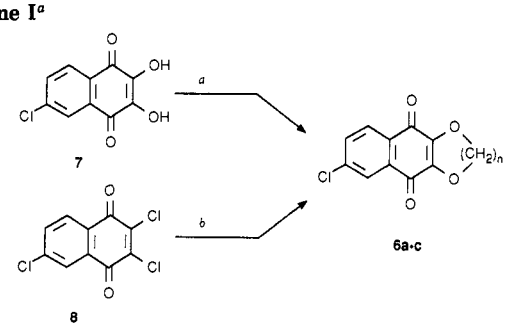
5a-p

2,3-dialkoxy substituents into 5- to 7-membered rings, were prepared to investigate the effects of the introduction of

Chart I



Scheme I[a]



[a] Reagents: a, $Br(CH_2)_nBr$/NaH/DMF; b, $HO(CH_2)_nOH$/NaH/DMF or no solvent.

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(1) Contribution No. 286 from the Institute of Bio-Organic Chemistry. For part 1, see ref 12. Address inquiries to Dr. John M. Young.
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