Folate Analogues. 22. Synthesis and Biological Evaluation of Two Analogues of Dihydrofolic Acid Possessing a 7,8-Dihydro-8-oxapterin Ring System[1]

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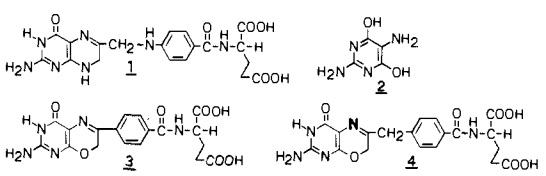
Two analogues of dihydrofolic acid possessing a 7,8-dihydro-8-oxapterin ring system have been synthesized and evaluated for their antifolate activities. These compounds, *N*-[(2-amino-4-hydroxy-7,8-dihydro-8-oxa-6-pteridinyl)benzoyl]-L-glutamic acid (**3**) and *N*-[[(2-amino-4-hydroxy-7,8-dihydro-8-oxa-6-pteridinyl)methyl]benzoyl]-L-glutamic acid (**4**), were synthesized by reacting the appropriately substituted α-halo ketones with 2,5-diamino-4,6-dihydroxypyrimidine (**2**). Elaboration of *p*-carbomethoxybenzaldehyde (**5**) to *p*-carbomethoxyphenacyl bromide (**7**) was accomplished by its oxidation with Jones reagent and the successive treatment of the oxidation product with $SOCl_2$, CH_2N_2, and HBr. Commercially available *p*-vinylbenzoic acid (**11**) was converted to its glutamate conjugate **12** and was further converted to the bromo ketone, diethyl *N*-[*p*-(1-bromo-2-oxopropyl)benzoyl]-L-glutamate (**17**), by a series of reactions involving epoxidation, oxirane ring opening with HBr, Jones oxidation, Zn/HOAc reduction, and successive treatment of the reduction product **16** with $SOCl_2$, CH_2N_2, and HBr. These bromo ketones, **7** and **17**, upon reaction with pyrimidine **2**, gave the diethyl esters of the target compounds, which were hydrolyzed to **3** and **4** with NaOH. Compound **4** underwent an interesting acid-catalyzed isomerization where the double bond of **4** was shifted from the 5,6-position to the 6,9-position to give the isomer **19**. Both compounds **3** and **4** were inactive against *Lactobacillus casei* (ATCC 7469) and did not serve as synthetic substrates of *L. casei* dihydrofolate reductase. Compound **4** showed activity against *Streptococcus faecium* (ATCC 8043), but **3** was inactive against this organism.

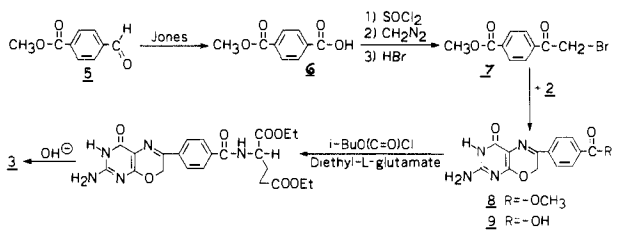
Analogues of dihydrofolic acid (**1**) capable of metabolic activation to antimetabolites in vivo are interesting candidates as potential chemotherapeutic agents against the treatment of those forms of cancers that have high levels of dihydrofolate reductase (EC 1.5.1.3).[2,3] If a synthetic substrate for dihydrofolate reductase can be enzymatically reduced to its tetrahydro derivative in vivo, and if the resulting product is capable of inhibiting a folate-dependent enzyme that is crucial to DNA biosynthesis, such as thymidylate synthase (EC 2.1.1.45), selective toxicity in the chemotherapy of those cancers that are resistant to methotrexate can be achieved with varying degrees of success.[2] This is due to the fact that many methotrexate-resistant tumors have elevated levels of dihydrofolate reductase, without the concomitant elevation of other folate-dependent enzymes in the mammalian system. As part of our continuing search for the development of such analogues, we were interested in the syntheses of a few stable analogues of dihydrofolic acid in which the amino group at the eighth position of **1** was replaced with either



an oxygen or sulfur.[4] This paper details the chemical syntheses and biological evaluation of two such analogues of dihydrofolic acid possessing a 7,8-dihydro-8-oxapterin ring system but also modified at the C^9,N^{10}-bridge region.

Chemistry. Reaction of an α-halo ketone with a 5-amino-6-hydroxypyrimidine was known to yield compounds with a pyrimido[4,5-*b*][1,4]oxazine ring system.[5] Therefore, it was decided to construct the appropriate α-halo ketones **7** and **17**, which on reaction with 2,5-diamino-4,6-dihydroxypyrimidine (**2**) were expected to yield pteroate analogues **8** and **22**. Elaboration of **8** and **22** to

Scheme I



target compounds **3** and **4** appeared straightforward. Commercially available *p*-carboxybenzaldehyde was methylated with diazomethane to **5**. Oxidation of **5** with Jones reagent[6] gave the monoester **6**, which upon successive treatment with thionyl chloride, diazomethane, and HBr gave the halo ketone **7** (Scheme I). Reaction of **7** with pyrimidine **2** gave the methyl pteroate analogue **8**, which was hydrolyzed with NaOH in acetonitrile to the pteroic acid **9**. This 6-aryl-substituted 7,8-dihydro-8-oxapterin was coupled with diethyl L-glutamate by the isobutyl chloroformate method,[7] and the glutamate conjugate **10** thus obtained was hydrolyzed to one of the target compounds, **3**.

For the synthesis of target compound **4**, bromo ketone **21** was required as an intermediate. The preparation of **21** from *p*-vinylbenzoic acid (**11**) via (*p*-carbomethoxy-

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(2) Misra, D. K.; Humphreys, S. R.; Friedkin, M.; Goldin, A.; Crawford, E. J. *Nature (London)* **1961**, *189*, 39.
(3) Goodman, L.; DeGraw, J. I.; Kisliuk, R. L.; Friedkin, M.; Pastore, E. J.; Crawford, E. J.; Plante, L. T.; Nahas, A.; Morningstar, Jr., J. F.; Kwok, G.; Wilson, L.; Donovan, E. F.; Ratzan, J. *J. Am. Chem. Soc.* **1964**, *86*, 308.
(4) Nair, M. G.; Berry, M. A.; Holman, L. *J. Org. Chem.* **1981**, *46*, 3354.
(5) Lin, S. C.; Holmes, G. P.; Dunn, D. L.; Skinner, C. G. *J. Med. Chem.* **1979**, *22*, 741.
(6) This reagent is a solution of chromic acid and sulfuric acid in water. A solution of the alcohol in acetone can be titrated with the reagent at 15–20 °C. For additional details, see Fieser, L. F.; Fieser, M. In "Reagents for Organic Synthesis"; Wiley: New York, 1967; p 142.
(7) Nair, M. G.; Campbell, P. T. *J. Med. Chem.* **1976**, *22*, 741.

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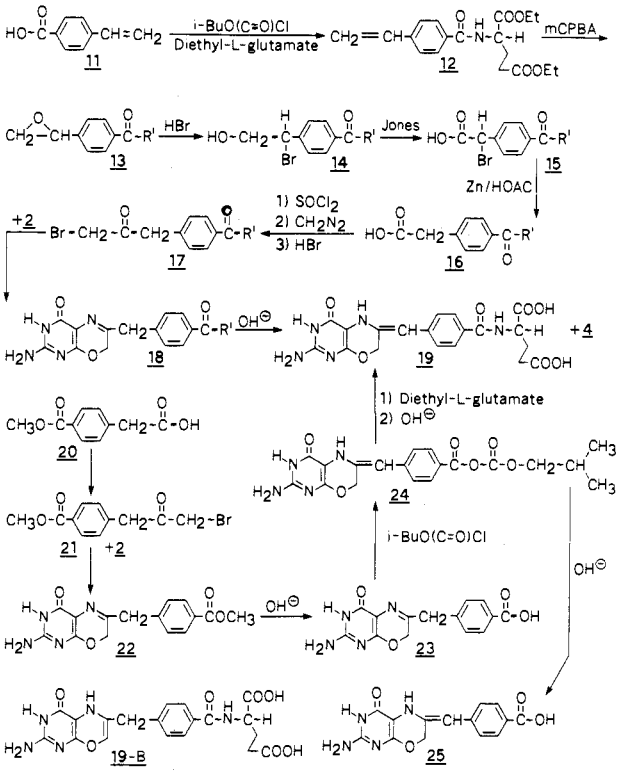
phenyl)acetic acid (**20**) has been described recently from this laboratory.[8] Reaction of **21** with **2** and subsequent hydrolysis of the product **22** gave the pteroic acid analogue **23** in good yield. No degradation of this 6-alkyl-substituted 7,8-dihydro-8-oxapterin was observed during the hydrolytic conditions using 0.1 N NaOH. Coupling of **23** with diethyl L-glutamate by the isobutyl chloroformate method gave a diethyl ester which was presumed to be **18**. Hydrolysis of this bright-yellow compound gave a folate analogue which, according to NMR and UV spectroscopy, did not clearly have the desired structure **4**.

The NMR spectrum of this moderately water soluble hydrolysate (**19**) displayed resonances of five protons in the aromatic region (δ 8.2–7.3) rather than the expected four protons of the benzene ring. By analogy, a characteristic two-proton resonance of **19** at δ 5.3 was assigned to the methylene protons of the oxazine ring. The UV spectrum of **19** in 0.1 N NaOH exhibited a λ_{max} at 400 nm, which was quite unexpected for a 7,8-dihydrofolate analogue but was indicative of extended conjugation of the double bond with the benzene ring or isomerization to the 6,7-position involving charge transfer. At any rate, the presence of an additional vinyl proton resonance in the aromatic region, coupled with the absence of a methylene resonance in the NMR spectrum of the hydrolysate **19**, was clearly indicative of the isomerization of the double bond from the 5,6-position to a new position involving the methylene protons either at the bridge region or the oxazine ring. Since base hydrolysis of 7,8-dihydro analogue **22** proceeded smoothly to **23**, it became apparent that the observed isomerization was induced by the acidic conditions employed for the preparation of the mixed anhydride **24**. To adduce independent evidence, the NMR and UV spectra of the recovered pteroic acid obtained by base hydrolysis of **24** was compared with the starting pteroic acid **23**. Not only was the high-field (δ 3.9) methylene resonance absent in the NMR spectrum of this compound, but also, as expected, an additional vinyl proton resonance appeared in the aromatic region. The chemical shift of the low-field methylene resonance was unaffected. Since the high-field methylene resonance of **22** and **23** can be assigned to the bridge protons, it follows that in the isomerization products the double bond is actually located between C6 and C9 of the molecule unless isomerization of the double bond to the 6,7-position, as in **19b**, is accompanied by considerable deshielding of the bridge protons so that these resonances exactly coincide with the original ring methylene resonances of **22** and **23**. Although the latter possibility cannot be ruled out, it appears that the most likely structures of the isomers are **19** and **25** (Scheme II). It should be noted that compounds **19** and **25** are thermodynamically more stable than **4**, **23**, or **19b**, and the reverse isomerization of the double bonds to the 5,6-position is unlikely. This has been verified experimentally. These observations, although interesting, suggested that alternate approaches precluding the use of acidic conditions are necessary for the synthesis of **4**.

It appeared at this stage that if bromo ketone **17** with the glutamate moiety already attached to the side chain could be prepared, its reaction with **2** under neutral or basic conditions should yield the desired diethyl 7,8-dihydrofolate analogue **18**. Since the base hydrolysis of **22** to **23** did not involve double bond isomerization, it was reasonable to assume that hydrolysis of **18** should also proceed smoothly to give a good yield of **4**. With the emphasis placed on this assumption, procedures to synthesize **17** were undertaken.

Scheme II



Coupling of diethyl L-glutamate with *p*-vinylbenzoic acid by the isobutyl chloroformate method gave the glutamate conjugate **12**, which upon reaction with *m*-CPBA in the usual manner gave the epoxide **13**. Reaction of **13** with gaseous HBr in methylene chloride at 25 °C gave the bromohydrin **14**, which was oxidized with Jones reagent[6,8] to the α-bromo acid **15**. Reduction of **15** with Zn and HOAc gave the para-substituted phenylacetic acid **16**; successive reactions of **16** with thionyl chloride, diazomethane, and HBr gave the required bromohydrin **17** in acceptable yields after column chromatographic purification.

In order to avoid acidic conditions during the reaction of **17** with **2**, several acid scavengers were tried. The use of MgO in this reaction was shown to be best for this purpose. Thus, reaction of a methanolic solution of **2** and **17** under nitrogen in the presence of MgO as an acid scavenger at reflux conditions gave the expected diethyl ester **18** in good yield with no trace of the double bond isomer (NMR, UV). The only step that remained was the hydrolysis of **18** to **4**. The reaction conditions that were previously employed for the successful conversion of **22** to **23** were selected for this purpose. As expected, acidification of the hydrolysate of **18** gave the target compound **4**, albeit in low yield (30%). Unlike the pteroic acid analogue **23**, which precipitated immediately on acidification of the hydrolysate of **23** with HOAc, the precipitation of **4** was extremely slow and required overnight refrigeration. During this time, the acidified hydrolysate turned bright yellow. The cream-colored compound **4** was separated by filtration, washed with water, and dried. The bright-yellow filtrate showed a UV λ_{max} (0.1 N NaOH) at 400 nm and was identified as **19** by cochromatography with the original sample prepared from **23**. It is apparent that the enhanced solubility of the glutamate conjugate **4** in water compared to **23** resulted in its isomerization to **19** because of its prolonged contact in solution with organic

(8) Nair, M. G.; Rozmyslovicz, M. K.; Kisliuk, R. L.; Gaumont, Y.; Sirotnak, F. M. In "Pteridines and Folic Acid Derivatives"; Blair, J. A., Ed.; Walter de Gruyter: Berlin, NY, in press.

acid. A 30% yield of 4 was found to be acceptable, and no attempts were made to improve this yield.

Biological Evaluation and Discussion

The antifolate activities of 3 and 4 were evaluated by using two folate-requiring strains of microorganisms, *Lactobacillus casei* (ATCC 7469) and *Streptococcus faecium* (ATCC 8043). Both compounds were inactive against the growth of *L. casei* up to a concentration of 4 μg/mL. Compound 3 was also inactive against *S. faecium*, whereas 4 showed some activity (I_{50} = 1.4 μg/mL). Next the compounds were tested for their ability to function as synthetic substrates for *L. casei* dihydrofolate reductase; they were found to be devoid of any appreciable substrate activity even when the enzyme concentration was increased 10 times that of the concentration normally present in a standard assay. Both compounds were poor inhibitors of dihydrofolate reductase derived from MTX-resistant *L. casei*. None of these compounds inhibited *L. casei* thymidylate synthase at concentrations lower than 1×10^{-4} M. Compound 4 was at least 250 times as inhibitory methotrexate for methotrexate-resistant *L. casei*. It is also more inhibitory to resistant *L. casei* than to the sensitive strain (I_{50}, 2 vs. 4 μg/mL).

The 7,8-dihydro-8-oxapterin is isosteric with the 7,8-dihydropterin ring system. All model compounds with this ring system synthesized in this laboratory with a 6-aryl substitution were found to be stable toward both acidic and basic conditions. However, the 6-alkyl-substituted 7,8-dihydro-8-oxapterins were not very stable under acidic conditions but were found to be stable to 0.1 N NaOH, permitting hydrolysis of ethyl ester moieties present at the side chain without degrading the ring system. With regard to compound 4, the instability was traced to the shift of the double bond from the 5,6-position to the 6,9-position. This double bond isomer was also found to be stable in organic acids and base.

Analogues of dihydrofolate in which the amino group at the 8-position is replaced with a heteroatom, such as oxygen, may be expected to have some substrate activity toward DHFR provided the substituents at the 6-position are identical with those of folate or homofolate. Although compound 4 has a bridge methylene and the benzoyl-L-glutamate moiety, the lack of substrate activity of this analogue could be due to the shortening of the bridge region by one methylene unit. Recently, we have synthesized 10-deaza-9-norfolic acid,[8] which has only one methylene group at the bridge region. This compound was inactive against *L. casei* and *S. faecium*. It is hoped that the presence of a methylamino or an ethylamino group at the bridge region connecting the 6-position of the dihydro-8-oxapterin ring system and the benzene ring, as in folic acid and homofolic acid, will lead to synthetic substrates of DHFR. The total inactivity of compound 3 in all the systems examined can be explained in terms of the fact that the benzene ring and the pyrimidine ring are coplanar due to extended conjugation. This coplanarity may prevent the interaction of this compound with the active site of the enzyme.

Experimental Section

Melting points were determined on a Fisher Model 355 digital melting point analyzer. NMR spectra were run in $CDCl_3$ or CF_3COOH on a 90-MHz Perkin-Elmer R32 spectrometer with Me_4Si as internal lock signal. Field strengths of the various proton resonances are expressed in δ (parts per million), and coupling constants are expressed in hertz. Peak multiplicity is depicted as follows: s, singlet; d, doublet; t, triplet; q, quartet; br, broadened singlet or unresolved multiplet; c, complex set of signals, the center of which is given. UV spectra were determined on a Beckman Model 25 spectrophotometer. Ion-exchange chromatography was carried out on DEAE-cellulose in the chloride form with 2.5 × 20 cm packing unless otherwise specified. A linear NaCl gradient of 1 L each of 0–0.5 M NaCl in 0.005 M phosphate buffer, pH 7, was used to elute the column. Elemental analyses were by Galbraith Laboratories, Inc., Knoxville, TN. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements or functions were within ±0.4% of the theoretical values.

***p*-Carbomethoxyphenacyl Bromide (7).** In an oven-dried 500-mL round-bottomed flask, 4 g (22.2 mmol) of 6 was refluxed with a solution of 10 mL (60 mmol) of thionyl chloride in 250 mL of chloroform under strictly anhydrous conditions for 1 h. The reaction mixture was evaporated to remove $CHCl_3$ and $SOCl_2$ and evacuated for 1.5 h, and the residue was dissolved in 50 mL of $CHCl_3$. This solution of *p*-carbomethoxybenzoyl chloride was slowly added to a stirring 500-mL cold ethereal solution of 70 mmol of diazomethane. After the solution was stirred for 1 h at 25 °C, HBr was bubbled through the solution until it was distinctly acidic. The solvents were evaporated under reduced pressure at 30 °C, and the residue thus obtained was dissolved in minimum ether. On addition of hexane, crystals of 7 were formed, which were collected by filtration and dried: yield 3.83 g (67%); mp 109 °C; NMR ($CDCl_3$) δ 8.12 (br, 4 H, aromatic), 4.45 (s, 2 H, bromomethyl), 3.95 (s, 3 H, carbomethoxy). Anal. ($C_{10}H_9BrO_3$) C, H, Br.

2-Amino-4-hydroxy-6-(*p*-carboxyphenyl)-7,8-dihydro-8-oxapteridine (9). To 500 mL of absolute methanol in a 1-L round-bottomed flask fitted with a reflux condenser and nitrogen inlet, a mixture of 540 mg (3 mmol) of 2, 504 mg (6 mmol) of $NaHCO_3$, and 771 mg (3 mmol) of 7 was added and refluxed in N_2 for 2 h. The reaction mixture was filtered while hot, and the filtrate was evaporated to ~75 mL, whereupon yellow crystals of 8 separated. After chilling overnight in the refrigerator, the crystals were filtered, washed with methanol, and dried: yield 875 mg (94%); mp 289–290 °C; UV (0.1 N NaOH) λ_{max} 375 and 245 nm (equal intensity); NMR (TFA) δ 7.78 (c, 4 H, aromatic), 5.45 (s, 2 H, ring methylene), 3.6 (s, 3 H, carbomethoxy). Anal. ($C_{14}H_{12}N_4O_4 \cdot 0.5H_2O$) C, H, N.

The hydrolysis of the methyl ester 8 to the pteroic acid analogue 9 was carried out by stirring 600 mg of 8 with 100 mL of 0.1 N NaOH for 18 h and acidifying the hydrolysate to Ph 4.0 with glacial HOAc. After chilling overnight in the refrigerator, the bright-yellow solid was filtered, washed with water, and dried: yield 440 mg (76%); mp >300 °C; UV (0.1 N NaOH) λ_{max} 375 and 245 nm; NMR (Me_2SO-d_6) δ 8.1 (s, 4 H, aromatic), 5.4 (s, 2 H, ring methylene). Anal. ($C_{13}H_{10}N_4O_4 \cdot H_2O$) C, H, N.

***N*-[(2-Amino-4-hydroxy-7,8-dihydro-8-oxa-6-pteridinyl)benzoyl]-L-glutamic Acid (3).** A solution of 306 mg (1 mmol) of 9 in 100 mL of dry Me_2SO was stirred with 0.28 mL (2.5 mmol) of *N*-methylmorpholine and 0.262 mL (2 mmol) of isobutyl chloroformate for 15 min. During this period a solution of 480 mg (2 mmol) of diethyl L-glutamate hydrochloride and 2 mmol of *N*-methylmorpholine was prepared in 15 mL of Me_2SO. This solution was added to the mixed anhydride solution and stirred overnight at room temperature. The solvent Me_2SO was removed under vacuum at 80 °C, and the bright-yellow residue thus obtained was triturated with water and filtered. This yellow solid was stirred at 25 °C with 100 mL of 0.1 N NaOH for 6 h, the pH was adjusted to 7.3, and the solution was chromatographed on a DEAE-cellulose column. Two bright-yellow compounds were eluted from the column. The column effluents corresponding to each of these products were pooled, concentrated to a small volume, and acidified to pH 4 with glacial HOAc. The precipitated products were filtered, and the filtrates were washed and dried. The less polar material was identified as the starting pteroic acid 9, and the more polar one was the desired target compound 3; yield 265 mg (60%); mp >300 °C; UV (0.1 N NaOH) λ_{max} 375 and 247 nm; NMR (TFA, Me_4Si external standard) δ 7.7 (br, 4 H, aromatic), 5.5 (s, 2 H, ring methylene). Anal. ($C_{18}H_{17}N_5O_7 \cdot 1.5H_2O$) C, H, N, O.

Preparation of Diethyl *p*-Vinylbenzoyl-L-glutamate (12). A solution of 4.4 g (30 mmol) of *p*-vinylbenzoic acid (11) and 4.24 mL (37.5 mmol) of *N*-methylmorpholine in 75 mL of dry DMF was placed in a 500-mL round-bottomed flask, to which 3.93 mL (30 mmol) of freshly distilled isobutyl chloroformate was added.

This was stirred for 30 min. In a separate flask, a solution of 14.4 g (60 mmol) of diethyl L-glutamate hydrochloride in 75 mL of DMF was neutralized by the addition of 6.72 mL (60 mmol) of *N*-methylmorpholine. These two solutions were then combined and stirred for 18 h at 25 °C. After evaporation of DMF under vacuum, the viscous residue was triturated with 100 mL of distilled water, followed by the addition of 200 mL of EtOAc, and the contents were transferred to a separatory funnel. The EtOAc layer was washed several times with a solution of saturated sodium bicarbonate to remove unreacted 11 from the product; after washing twice with distilled water, it was dried over Na_2SO_4 and evaporated. This crude product was judged 95% pure by TLC and was repurified by column chromatography over silica gel by using CH_2Cl_2 as the eluting solvent. The pure product, which was a colorless gum, weighed 8.3 g (66%): NMR ($CDCl_3$) δ 7.8 and 7.45 (2 d, 4 H, aromatic), 6.75 (q, 1 H, vinyl), 5.8 and 5.3 (2 d, 2 H, vinyl), 4.8 (c, 1 H, α proton of glutamate), 4.15 (c, 4 H, ethoxy), 2.5–2 (c, 4 H, glutamate), 1.35 (2 t, 6 H, ethoxys). Anal. ($C_{18}H_{23}NO_5$) C, H, N.

Preparation of Diethyl *p*-(1,2-Epoxyethyl)benzoyl-L-glutamate (13). In an Erlenmeyer flask, 7.7 g (23 mmol) of 12 was dissolved in 500 mL of chloroform, and 6.2 g (36 mmol) of *m*-chloroperbenzoic acid was added. This solution was allowed to remain at 25 °C for 72 h and then extracted repeatedly with100-mL portions of saturated $NaHCO_3$ until there was no more effervescence. The chloroform layer was washed twice with 50-mL portions of distilled water, followed by vigorous shaking with 100 mL of a 5% solution of KI. After 30 s, enough sodium thiosulfate solution (10% in water) was added while shaking to decolorize the solution. The chloroform layer was separated, washed again with two 100-mL portions of distilled water, dried over anhydrous Na_2SO_4, and evaporated to obtain crude 13 in almost quantitative yield. This noncrystalline product showed only a single spot on TLC (silica gel GF, 3% MeOH in $CHCl_3$). Further purification of this product was unnecessary; therefore, it was used directly for the next reaction: NMR ($CDCl_3$) δ 7.8 and 7.3 (2 d, 4 H, aromatic), 7.1 (d, amide NH), 4.75 (c, 1 H, α proton of glutamate), 4.15 (c, 4 H, ethoxy), 3.85 (q, 1 H, epoxide), 3.15 (2 d, 1 H, epoxide), 2.72 (2 d, 1 H, epoxide), 2.5–2 (c, 4 H, glutamate), 1.25 (2 t, 6H, ethoxys). Anal. ($C_{18}H_{23}NO_6$) C, H, N.

Diethyl *p*-(2-Bromo-1-hydroxyethyl)benzoyl-L-glutamate (14). In a round-bottomed flask, 7.5 g (21.5 mmol) of epoxide 13 was dissolved in 200 mL of CH_2Cl_2, and gaseous HBr was bubbled through the solution for 20 min. By this time the solution became saturated with HBr. The solvent and HBr were removed by rotary evaporation under reduced pressure at 25 °C. The viscous residue of the crude bromohydrin was triturated with ice-cold water (200 mL) and extracted with EtOAc. The EtOAc layer was washed several times with water, dried with anhydrous Na_2SO_4, and evaporated to obtain a golden yellow viscous product, which was judged ~95% pure by TLC. This compound was freed of minor impurities by column chromatography over silica gel by using benzene–CH_2Cl_2 as eluting solvent: yield 6.8 g (75%); NMR ($CDCl_3$) δ 7.9 and 7.6 (2 d, 4 H, aromatic), 7.32 (d, 1 H, amide), 5.15 (t, 1 H, benzylic), 4.35 (d, 2 H, hydroxymethyl), 4.2 (c, 4 H, ethoxys), 2.8–2.2 (c, 4 H, glutamate), 1.35 (2 t, 6 H, ethoxys). Anal. ($C_{18}H_{24}BrNO_6$) C, H, Br.

Diethyl *N*-[*p*-(Carboxybromomethyl)benzyl]-L-glutamate (15). In an Erlenmeyer flask, 5 g (11.6 mmol) of 14 was dissolved in 300 mL of acetone, and 15 mL of Jones reagent[6] was slowly added with vigorous stirring at room temperature over a period of 20 min. This mixture was transferred to a round-bottomed flask and evaporated to ~20 mL at 30 °C. After dilution with 200 mL of water, the oxidation product was extracted in 100 mL of EtOAc. The EtOAc layer was washed several times with water, dried over anhydrous Na_2SO_4, and evaporated to a gum, which was almost pure by both TLC and NMR criteria. This compound was used as such without further purification for the next step. The oxidation of the primary alcohol to the acid, and not to an aldehyde, under these conditions was established by the collapse of the one-proton triplet at δ 5.15 of 14 to a sharp singlet which resonated at 5.5 ppm. Attempts to crystallize this material were unsuccessful: NMR ($CDCl_3$) δ 9.1 (s, acid proton), 7.9 and 7.7 (2 d, 4 H, aromatic), 5.5 (s, 1 H, benzylic), 4.9 (t, 1 H, α proton of glutamate), 4.25 (c, 4 H, ethoxys), 2.7–2.2 (c, 4 H, glutamate), 1.35 (t, t, 6 H, ethoxys): yield 4.12 g (92.6%).

Diethyl *N*-[*p*-(Carboxymethyl)benzoyl]-L-glutamate (16). The reductive dehalogenation of 15 to 16 was carried out by dissolving 4 g (9 mmol) of 15 in 150 mL of glacial HOAc and adding portionwise 20 g of zinc dust with vigorous stirring over a 4-h period. The reduction was allowed to proceed for another hour, after which the solution was filtered. On evaporation of the filtrate under vacuum at 60 °C, the desired phenylacetic acid 16 was obtained as a gum, which was triturated with 100 mL of ice-cold distilled water. The slightly yellow viscous product 16 was extracted with 100 mL of EtOAc, washed three times with distilled water, dried over Na_2SO_4, and evaporated. The NMR ($CDCl_3$) spectrum of this compound displayed no resonance due to the benzylic proton at 5.5 ppm, indicating complete reduction; other NMR resonances were at δ 8.0 (d, 1 H, amide), 7.8 and 7.35 (2 d, 4 H, aromatic), 4.85 (t, 1 H, α proton of glutamate), 4.2 (c, 4 H, ethoxys), 3.7 (s, 2 H, benzylic), 2.6–2.2 (c, 4 H, glutamate), 1.3 (2 t, 6 H, ethoxys). This product was dried over P_2O_5 under vacuum for 48 h before it was used in the next step: yield 3.15 g (96%). Anal. ($C_{18}H_{23}NO_7$) C, H, O.

Diethyl *N*-[*p*-(1-Bromo-2-oxopropyl)benzoyl]-L-glutamate (17). A mixture of 3 g (8.2 mmol) of 16, 10 mL of thionyl chloride, and 100 mL of benzene was refluxed with stirring for 1.5 h and then evaporated. More benzene (50 mL) was added to the residue, and the solution was reevaporated to remove traces of thionyl chloride, evacuated in a vacuum oven over KOH for 2 h, and then dissolved in 100 mL of dry CH_2Cl_2. This solution of the acid chloride was slowly added, while stirring, to a cold solution of 40 mmol of diazomethane in 300 mL of ether. A vigorous reaction, which subsided after 5 min, occurred. After the solution was stirred for 1 h, gaseous HBr was bubbled through the solution (~15 min) until the solution was distinctly acidic. External cooling of the solution with an ice bath was necessary to maintain the temperature of the contents of the flask at 25 °C during HBr treatment. The solvents were removed at reduced pressure at 25 °C, and the residue was treated with 200 mL of ice-cold water. The water-insoluble oily product was extracted in EtOAc, washed three times with water, dried over Na_2SO_4, and evaporated at 25–30 °C.

Examination of the semisolid product by TLC ($CHCl_3$–MeOH, 99:1) on silica gel plates revealed that the desired product was contaminated with two less polar impurities. Purification was accomplished by column chromatography over silica gel CC7. A 1:1 mixture of benzene and CH_2Cl_2 eluted the less polar impurities, which were not characterized. After the complete elution of the impurities, the column was eluted with EtOAc, whereupon the bromo ketone 17 was recovered from the column. Evaporation of the EtOAc effluents from the column gave 2.56 g of the pure bromo ketone (73%): NMR ($CDCl_3$) δ 7.85 and 7.35 (2 d, 4 H, aromatic), 7.15 (d, 1 H, amide), 4.75 (t, 1 H, α proton of glutamate), 4.15 (d, 4 H, ethoxys), 4.0 and 3.9 (2 s, 4 H, bromomethyl and benzyl), 2.6–2.1 (c, 4 H, glutamate), 1.25 (2 t, 6 H, ethoxys). Anal. ($C_{19}H_{24}BrNO_6$) C, H, N.

Diethyl *N*-[[(2-Amino-4-hydroxy-7,8-dihydro-8-oxa-6-pteridinyl)methyl]benzoyl]-L-glutamate (18). A 500-mL three-necked round-bottomed flask was fitted with a reflux condenser and a nitrogen inlet, and 200 mL of absolute MeOH was deaerated by bubbling nitrogen for 15 min. To this solvent was added 134 mg (0.75 mmol) of 2,5-diamino-4,6-dihydroxypyrimidine hydrochloride, and the mixture was vigorously stirred with heating. This was followed by the simultaneous addition of 332 mg (0.73 mmol) of bromo ketone 17 and 60 mg (1.5 mmol) of magnesium oxide. The reaction mixture was refluxed under nitrogen for 3 h and then filtered while hot. The filtrate was evaporated to ~25 mL and chilled in the refrigerator overnight. The diethyl ester of the target compound 18 separated as brownish crystals, which were collected by filtration and washed with ether: yield 110 mg (30%); mp >300 °C; NMR (TFA) δ 8.0 and 7.6 (2 d, 4 H, aromatic), 5.52 (s, 2 H, ring methylene), 5.0 (α proton of glutamate), 4.45 (s, 2 H, bridge methylene), 4.40 (c, 4 H, ethoxy), 2.9–2.2 (c, 4 H, glutamate), 1.4 (2 t 6 H, ethoxys). Anal. ($C_{23}H_{27}N_5O_7$) C, H, N.

***N*-[[(2-Amino-4-hydroxy-7,8-dihydro-8-oxa-6-pteridinyl)methyl]benzoyl]-L-glutamate (4).** The hydrolysis was carried out by stirring 430 mg (1 mmol) of 18 in a deaerated (bubbling N_2, 5 min) mixture of 100 mL of 0.1 N NaOH and 35 mL of MeCN for 6 h at 25 °C. The MeCN was evaporated off in vacuum, and

the clear hydrolysate was acidified with citric acid to pH 4.0. Upon chilling in the refrigerator for 4 h, cream-colored 4 separated and was collected by filtration, washed with water, and dried: yield 120 mg; UV (0.1 N NaOH) λ_{max} 325, 275, and 245 nm; NMR (TFA) δ 7.98 and 7.6 (2 d, 4 H, aromatic), 5.5 (s, 2 H, ring methylene), 4.41 (s, 2 H, bridge methylene), 3.9–2.2 (c, 4 H, glutamate). Anal. ($C_{19}H_{19}N_5O_7$) C, H, O.

The filtrate from which 4 was separated was deep yellow in color. This solution was adjusted to pH 7.0 with Na_2CO_3 and evaporated to ~20 mL; the pH was readjusted to 3.5 with citric acid, and the solution was refrigerated for 3 days. A bright yellow solid separated, which was washed quickly (moderately water soluble) with ice-cold water and dried: UV (0.1 N NaOH) λ_{max} 400, 250 nm; NMR (TFA) δ 7.9 and 7.75 (c, 5 H, 4 aromatic and 1 vinyl), 5.5 (s, 2 H, ring methylene), 4.9 (t, α proton of glutamate), 2.8–2.2 (c, 4 H, glutamate). This compound was found to be identical in all respects with the product **19** obtained by the glutamate coupling of **24**. Anal. ($C_{19}H_{19}N_5O_7$) C, H, N, O.

2-Amino-4-hydroxy-6-(*p*-carbomethoxybenzyl)-7,8-dihydro-8-oxapteridine (22). The synthesis of bromomethyl ketone **21** has been described previously from this laboratory.[8] In a three-necked round-bottomed flask, a mixture of 1.78 g (10 mmol) of **2**, 2.7 g (10 mmol) of bromo ketone **21**, and 400 mg of MgO was refluxed with 500 mL of MeOH in N_2 for 4 h. The reaction mixture was filtered hot, evaporated to ~100 mL, and chilled. Brown crystals of **22** were formed overnight; these were filtered, washed with water followed by minimum MeOH, and dried: yield 1.5 g (50%); NMR (TFA) δ 7.71 and 7.0 (2 d, 4 H, aromatic), 5.0 (s, 2 H, ring methylene), 4.25 (s, 3 H, carbomethoxy), 3.98 (s, 2 H, bridge methylene). Me_4Si was used as an external standard. Anal. ($C_{15}H_{14}N_4O_4 \cdot 0.5H_2O$) C, H, N.

2-Amino-4-hydroxy-6-(*p*-carboxybenzyl)-7,8-dihydro-8-oxapteridine (23). One gram of **22** was stirred with 300 mL of 0.1 N NaOH and 75 mL of MeCN in a 500-mL round-bottomed flask under nitrogen for 6 h. The clear solution was concentrated on a rotary evaporator to ~200 mL and filtered. The clear filtrate was acidified to pH 3.4 with glacial HOAc. A thick cream-colored precipitate that formed was filtered, washed with water, and dried: yield 750 mg; mp >300 °C; UV (0.1 N NaOH) λ_{max} 320, 270 nm; NMR (TFA) δ 7.76 and 7.70 (2 d, 4 H, aromatic), 5.02 (s, 2 H, ring methylene), 3.95 (s, 2 H, bridge methylene). Anal. ($C_{14}H_{12}N_4O_4 \cdot 0.5H_2O$) C, H, N.

***N*-[[(2-Amino-4-hydroxy-7,8-dihydro-8-oxa-6-pteridinyl)methenyl]benzoyl]-L-glutamate (19).** This reaction was done by dissolving 600 mg (2 mmol) of **23** in 50 mL of dry Me_2SO and activating the carboxyl group by the addition of 0.28 mL (2.5 mmol) of *N*-methylmorpholine, followed by 0.226 mL of freshly distilled isobutyl chloroformate. After 15 min, this solution was mixed with a solution of 960 mg (4 mmol) of diethyl glutamate hydrochloride in 20 mL of Me_2SO containing 0.45 mL (4 mmol) of *N*-methylmorpholine and stirred overnight. The solvent Me_2SO was distilled off in a vacuum at 80 °C and the bright-yellow residue was triturated with water and filtered. This crude product was hydrolyzed with 0.1 N NaOH (250 mL) and MeCN (30 mL) as described for **22**. Upon acidification of the hydrolysate, no precipitate was formed. The solution was adjusted to pH 7.5, diluted to 1 L, and chromatographed on DEAE-cellulose. Two bright-yellow compounds were eluted; the less polar was the isomer **25** of starting pteroic acid **23**, and the more polar glutamate conjugate was identified as the product **19**.

Biological Evaluation. The antimicrobial activities of these compounds were evaluated by procedures that have been published previously.[9,10] All assays with thymidylate synthase were performed according to the procedure of Friedkin.[11]

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Registry No. **2**, 40769-69-5; **2**·HCl, 56830-58-1; **3**, 85828-43-9; **4**, 85828-44-0; **6**, 1679-64-7; **7**, 56893-25-5; **8**, 85828-45-1; **9**, 85828-46-2; **10**, 85828-47-3; **11**, 1075-49-6; **12**, 85828-48-4; **13**, 85828-49-5; **14**, 85828-50-8; **15**, 85828-51-9; **16**, 85828-52-0; **17**, 85828-53-1; **18**, 85828-54-2; **19**, 85828-55-3; **21**, 85828-56-4; **22**, 85828-57-5; **23**, 85828-58-6; **25**, 85828-59-7; diethyl L-glutamate, 16450-41-2; dihydrofolate reductase, 9002-03-3.

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2-Haloethylating Agents for Cancer Chemotherapy. 2-Haloethyl Sulfonates

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Because certain (2-chloroethyl)triazenes and (2-haloethyl)nitrosoureas have high antineoplastic activity, 2-chloroethyl and 2-fluoroethyl sulfonates were prepared to try to develop additional types of 2-haloethylating agents. In this initial study, it was demonstrated that antineoplastic activity much superior to that of the prototype, 2-chloroethyl methanesulfonate, could be found among 2-chloroethyl sulfonates. Among a variety of 2-chloroethyl alkane- and arenesulfonates, several substituted methanesulfonates displayed significant activity against P388 leukemia in mice; the chloromethanesulfonate showed high activity (T/C = 218%). None of the arenesulfonates were active in this test.

In tests against transplanted leukemia L1210 in mice, 5-[3,3-bis(2-chloroethyl)-1-triazenyl]-1*H*-imidazole-4-carboxamide (BIC), the chloroethyl analogue of the clinical anticancer drug DTIC[1-3] (dacarbazine), effects many cures[4,5] and is very much superior to DTIC and to other imidazole and benzenoid triazenes lacking a chloroethyl group.[6,7] By analogy to the metabolism[8-11] of DTIC,

(1) DTIC = 5-(3,3-dimethyl-1-triazenyl)-1*H*-imidazole-4-carboxamide; MCIC = 5-[3-(2-chloroethyl)-1-triazenyl]-1*H*-imidazole-4-carboxamide; BFIC = 5-[3,3-bis(2-fluoroethyl)-1-triazenyl]-1*H*-imidazole-4-carboxamide; MFIC = 5-[3-(2-fluoroethyl)-1-triazenyl]-1*H*-imidazole-4-carboxamide.
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