Novel Fluorinated Antifolates. Enzyme Inhibition and Cytotoxicity Studies on 2′- and 3′-Fluoroaminopterin

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Two novel analogues of aminopterin with a single fluorine substitution in the 2′ (compound 8) or in the 3′ (compound 9) position of the *p*-aminobenzoyl group were synthesized and evaluated as inhibitors of dihydrofolate reductase from two bacterial species and from human HeLa cells. The 2′-fluoro compound was bound essentially the same as aminopterin itself, while the 3′-fluoro derivative bound two- to threefold more tightly in all cases. UV spectral shifts indicated normal binding of the pteridine. Cytotoxicity studies against mouse leukemia L1210 cells and the human stomach cancer line HuTu80 indicated equivalent toxicity of the parent drug with the 2′-fluoro analogue. 3′-Fluoroaminopterin was, however, twice as toxic as aminopterin to both cell lines.

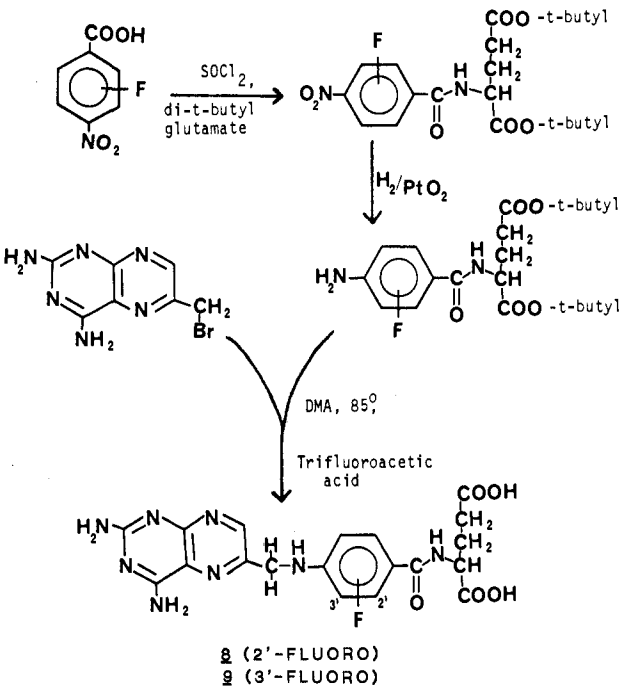
Aminopterin and its derivative methotrexate (MTX) are important chemotherapeutic agents by virtue of their powerful inhibition of dihydrofolate reductase (DHFR) and, thus, of folic acid mediated one-carbon metabolism.[1,2] These compounds and their analogues also interact to varying degrees with other folate-utilizing enzymes and folate transport systems, which, in turn, affect the specificity and toxicity of the drugs.[3,4] Analogues halogenated with iodine, bromine, and chlorine in the 3′ or 3′ and 5′ positions of the *p*-aminobenzoyl (PABA) moiety were among the first derivatives synthesized and showed binding to DHFR comparable to that of the parent compounds.[5] The chloro derivatives showed particular promise against leukemia L1210 in mice, being more potent than the parent compounds,[6] although results in clinical trials were less encouraging.[7]

Studies with correspondingly fluorinated antifolates have not previously been reported. If such compounds are found to interact with the target enzyme in a fashion similar to that of the parent drug, they would be expected to have several very useful properties, making them valuable both as enzyme probes and potential therapeutic agents. The low steric bulk and high electronegativity of fluorine and its ability to act as a hydrogen-bond acceptor[8] might lead to a more selective drug owing to differences in binding, transport, or metabolism. Fluorine has a strong environmentally sensitive NMR signal[9] which would allow the use of fluorinated antifolates as probes of the structure of folate-binding proteins, including DHFR, from a variety of species and as a means of titrating antifolate binding capacity in vivo (free vs. bound resonances). If the biological activity and disposition of fluoroaminopterins are similar to that of the parent drug and if an appropriate synthesis utilizing $H^{18}F$ at a late step can be achieved,[10] an ^{18}F positron-emitting radiopharmaceutical could potentially be utilized to image, at high resolution, the tissue distribution of the fluorinated antifolate in vivo by means of positron tomography.[11]

For the above reasons, we have synthesized 2′- and 3′-fluoroaminopterins and examined their binding to DHFR from two bacterial species and from human HeLa cells. We have also examined their cytotoxicity against two tumor cell lines.

The synthetic route for both target compounds was essentially the same and is outlined in Scheme I. Fluoronitrobenzoates (2 and 3) were coupled to the di-*tert*-butyl ester of *l*-glutamic acid and then reduced to the corresponding fluoro-PABA derivatives. These were condensed with 6-(bromomethyl)-2,4-pteridinediamine hydrobromide (1) to give the di-*tert*-butyl esters of the target compounds.

Scheme I



8 (2′-FLUORO)
9 (3′-FLUORO)

These could be much more cleanly purified by silica gel chromatography than could dimethyl or diethyl esters and led to much improved preparations of aminopterins in

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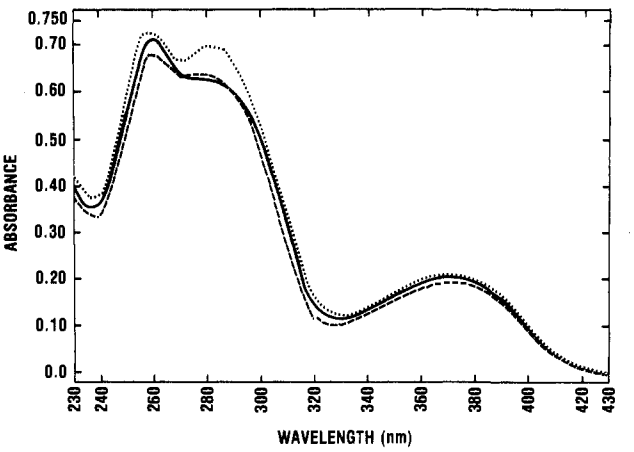

Figure 1. UV spectra of aminopterin derivatives. These were obtained in 0.05 M sodium phosphate buffer, pH 7.2. Each antifolate was present at a concentration of 25 μM: solid curve, aminopterin; dashed curve, 2′-fluoroaminopterin; dotted curve, 3′-fluoroaminopterin.

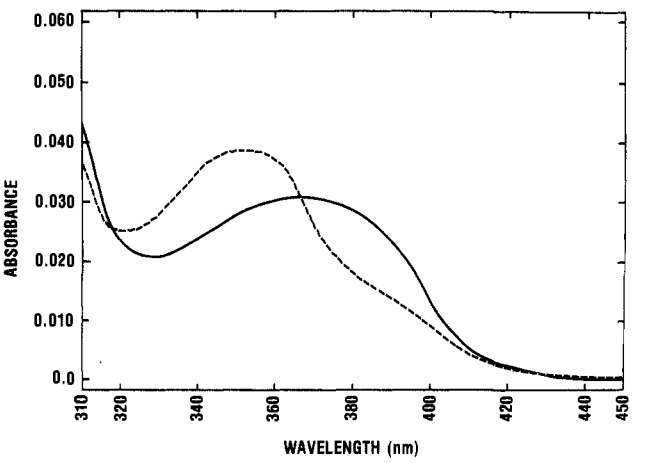

Figure 2. Pteridine spectral shift upon binding to *L. caseii* DHFR. The spectrum (solid curve) of 4 μM 2′-fluoroaminopterin was obtained in 0.05 M potassium phosphate buffer, pH 7.0, containing 0.5 M KCl. Addition of a small aliquot (25 μL) of enzyme solution to a final concentration of 5 μM in both the sample and reference cuvette gives the dased curve spectrum.

general. Finally, treatment with trifluoroacetic acid was used to remove the blocking groups. The ^{19}F NMR chemical shifts in the two compounds differed by more than 20 ppm as would be expected given the wide spectral ranges of ^{19}F chemical shifts.

Enzyme Studies. The UV spectra of the fluorinated aminopterins, along with that of aminopterin itself, are shown in Figure 1. The spectra are nearly identical, with the exception of a very slight difference in the 280–290-nm region for 3′-fluoroaminopterin, as expected, where the PABA ring makes the greatest contribution to absorption. Figure 2 shows expanded UV spectra of 2′-fluoroaminopterin in the long-wavelength pteridine absorption region in both the presence and absence of DHFR from *Lactobacillus caseii* (results with the *Escherichia coli* enzyme are identical). The spectral shift to shorter wavelength upon binding to enzyme is precisely the same as that observed with 3′-fluoroaminopterin, aminopterin itself, or MTX,[12] indicating that pteridine rings are similarly bound in all of these binary complexes. The shift has been attributed to protonation of the bound pteridine ring by an acidic side chain of the enzyme.[13] By observing the same

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Table I. Inhibition of Dihydrofolate Reductases by Aminopterin and Fluoroaminopterins

enzyme source	*E. coli*	*L. caseii*	HeLa cells[a]
folate, μM, in assay			114
dihydrofolate, μM, in assay	44	8.0	
	I_{50},[b] nM		
aminopterin	11	5.0	31
2′-fluoroaminopterin	10	4.7	27
3′-fluoroaminopterin	5.0	3.5	19
	K_i, nM		
aminopterin	0.09	0.11	
2′-fluoroaminopterin	0.08	0.095	
3′-fluoroaminopterin	0.03	0.06	

[a] K_i values were corrected for free inhibitor depletion due to enzyme binding, using the equation of Cha (Experimental Section). The concentration of pure bacterial enzymes ranged from 4 to 5 × 10^{-9} M in the assay. The enzyme concentration in HeLa cell extract was not determined. [b] All I_{50} values were accurate to within 10%.

Table II. Cytotoxicity of Fluoroaminopterins

antifolate	cell line:	EC_{50},[a] nM L1210	HuTu80
MTX		3.2	4.5
aminopterin		1.8	4.7
2′-fluoroaminopterin		2.3	4.8
3′-fluoroaminopterin		1.0	2.3

[a] EC_{50} values are an average of three determinations. Reproducibility was within 5% in all cases.

changes at the lowest detectable concentrations of E and I, an uppor limit for the dissociation constants K_d of the fluoroaminopterins in binary complex (E·I) can be set at 10^{-7} M and is probably much lower. The analogues and aminopterin itself are competitive inhibitors (against dihydrofolate) of DHFR from two bacterial species and from human cells. The kinetically determined I_{50} and K_i values of the fluoro compounds compared with aminopterin are shown in Table I. The values are very close to those of aminopterin, indicating little perturbation of binding due to the fluorine substitution. K_i more accurately compares binding affinity because it is corrected for inhibitor depletion due to enzyme binding, using the equation of Cha.[14] It is interesting that the 3′-fluoro derivative is actually more tightly bound to DHFR from all sources tested than is aminopterin.

Biological Data. Table II shows the inhibition of the growth of L1210 cells and HuTu80 cells by the fluoroaminopterins. Both analogues are more cytotoxic than MTX and are comparable in potency to aminopterin. It is striking that the 3′-fluoro compound is twice as toxic as the parent compound against both of the cell lines tested. The enhanced toxicity correlates with the increased affinity for DHFR, although additional factors may be involved. In all cases, cytotoxicity was reversed by the addition of 10 mM folinic acid. A combination of 10 μM thymidine and 500 μM hypoxanthine also substantially reversed the toxicity.

The above UV, enzyme kinetics, and cell growth inhibition studies indicate that 2′- and 3′-fluoroaminopterin interact with DHFR, the principal target enzyme of aminopterin and MTX, in essentially the same fashion as the

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latter drugs. It will be of considerable interest to determine how cellular uptake and efflux, as well as breakdown and excretion of fluorinated aminopterins in vivo, compare with that of the parent compound. The enhanced cytotoxicity of 3′-fluoroaminopterin makes it a promising candidate for animal studies similar to those described for chloroaminopterin.[6] While the syntheses of fluorinated aminopterins described here are not applicable to ^{18}F labeling, pilot studies in this laboratory indicate that it is possible to synthesize 2′-fluoroaminopterin by an alternative route using HF and dimethyl *N*-[(2-amino-4-fluorenylmethoxycarboxamido)benzoyl]-*l*-glutamate.[15] This suggests that H^{18}F can be used to label the compound for positron imaging studies.[10]

Experimental Section

IR spectra were recorded on a Perkin-Elmer 710B spectrophotometer. UV spectra were obtained on a Hewlett-Packard 8450A microprocessor controlled parallel-detection spectrophotometer. Enzyme rates were obtained on the same instrument but at fixed wavelength with OD read and stored every 2 s over the assay interval. ^1H NMR spectra were taken on a 60-MHz Varian 360A spectrometer. ^{19}F NMR was obtained at 245 MHz by pulsed Fourier transform methods with a Bruker 270-MHz HXS 270 spectrometer. Microanalyses were carried out by Galbraith Laboratories, Knoxville, TN, or by Atlantic Microlabs, Atlanta, GA. Analytical results obtained for elements were within ±0.4% of theoretical values except where otherwise indicated.

NADPH, aminopterin, MTX, and di-*tert*-butyl *l*-glutamate were from Sigma Chemical Co. [^3H]Folic acid was from Amersham Corp. and was repurified just prior to use by HPLC. 7,8-Dihydrofolic acid was freshly prepared by dithionite reduction of folic acid according to Blakley.[16]

6-(Bromomethyl)-2,4-pteridinediamine Hydrobromide (1). This compound was prepared according to the method of Piper and Montgomery[17] as modified by Boyle and Pfleiderer.[18]

2-Fluoro- and 3-Fluoro-4-nitrobenzoic Acid (2 and 3). 2-Fluoro- or 3-fluoro-4-nitrotoluene (3.1 g, 20 mmol) was oxidized in refluxing aqueous $KMnO_4$ essentially by the method of Schmelkes and Rubin.[19] **2**: NMR (acetone-d_6 + 10% D_2O) aromatic H only (δ 7.9–8.3, largest peak at δ 8.15). **3**: NMR (acetone-d_6 + 10% D_2O) aromatic H only (δ 7.8–8.5, largest peak at δ 8.10).

Di-*tert*-butyl *N*-(2-Fluoro-4-nitrobenzoyl)-*l*-glutamate (4). 2-Fluoro-4-nitrobenzoic acid (**2**; 0.185 g, 1.0 mmol) was stirred at 70–75 °C in 10 mL of dry benzene containing 0.24 g (2 mmol) of thionyl chloride and 5 mg of dimethylformamide. Periodic dilution of a reaction aliquot into methanol, followed by TLC (silica gel, ether–hexane, 1:1) showed that the conversion to acid chloride (indicated as methyl ester) was nearly complete in 4 h. The solvent and $SOCl_2$ was then removed under vacuum with careful exclusion of moisture. The residue was then stirred for 2 h with a freshly prepared solution containing 0.4 g (1.25 mmol) of di-*tert*-butyl *l*-glutamate hydrochloride and 0.25 g (2.5 mmol) of triethylamine in 10 mL of dichloromethane. Solvent was evaporated, and the residue was taken up in 25 mL of dry ether. Salts were removed by filtration, and the volume of solvent was concentrated to 5 mL. This solution (including some suspended salts) was applied to a 1.5 × 20 cm column of dry silica gel (32–63 mesh) and eluted with ether–hexane (1:1). A single intensely UV absorbing peak was obtained which gave 0.19 g (48 yield) of the amide (**4**) as a pale yellow amorphous solid upon removal of solvent: NMR ($CDCl_3$) δ 1.45 and 1.52 (2 s *t*-Bu, 18 H), 2.20–2.45 (m, glutamate CH_2CH_2, 4 H), 4.77 (dd α-H, 1 H), 7.50 (broad, NH, 1 H), 7.9–8.3 (aromatics, 3 H); IR ($CHCl_3$) 3010 (arom CH), 1720, (ester C=O) 1665 (amide C=O), 1530 and 1350 (NO_2), 930 (butyl CH), 1215 (intense CF), 2430 (sharp band, probably CF overtone) cm^{-1}. Anal. ($C_{20}H_{27}N_2O_7F$) H, N, F; C: calcd, 56.34; found, 56.87.

Di-*tert*-butyl *N*-(3-Fluoro-4-nitrobenzoyl)-*l*-glutamate (5). Essentially the same procedure as above for converting **2** to **4** was used to convert **3** to **5**. The product(s) (60% yield), purified by silica gel chromatography, was, however, a yellow oil rather than a solid: NMR ($CDCl_3$) δ 1.75 and 1.52 (2 s, *t*-Bu, 18 H), 2.20–2.45 (m, 4 H), 4.60 (dd, α-H, 1 H), 7.65 (broad, NH, 1 H), 7.65–8.27 (complex aromatic, 3 H); IR ($CHCl_3$) 3020 (arom CH), 1720 (ester C=O), 1665 (amide C=O), 1600 (amide II), 1525 and 1350 (NO_2), 930 (butyl CH), 1210 (intense CF), 2420 (sharp band, probably CF overtone) cm^{-1}. Anal. ($C_{20}H_{27}N_2O_7F$) C, H, N, F.

Di-*tert*-butyl *N*-(2-Fluoro-4-aminobenzoyl)-*l*-glutamate (6). The nitro compound **4** (0.142 g, 0.33 mmol) was dissolved in 10 mL of ethyl acetate and hydrogenated for 16 h under 1 atm of H_2 introduced via a gas buret using 10 mg of Adam's catalyst. Solvent was thoroughly removed under reduced pressure, and the residue was dissolved in 20 mL of ether and applied to a 2 × 15 cm column of dry silica gel (32–63 mesh) and eluted first with 1:1 ether–hexane and then with ether to give a single ninhydrin-positive peak. When taken to dryness, this gave 0.117 g of **6**, a white solid (91% yield): NMR ($CDCl_3$) δ 1.43 and 1.51 (2 s, Bu, 18 H), 2.2–2.4 (m, 4 H), 4.36 (broad, NH_2, 2 H), 4.71 (broad, α-H, 1 H), 6.1–6.6 (complex aromatic, 2 H), 7.75–8.05 (arom, t, 1 H), 6.95–7.4 (m, broad NH); IR ($CHCl_3$) 3650 and 3590 (amine NH), 3020 (aromatic CH), 1720 (ester C=O), 1625 (amide C=O), 1210 (intense, CF), 2420 (sharp band, probably CF overtone) cm^{-1}. Anal. ($C_{20}H_{29}N_2O_5F$) H, N, F; C: calcd, 60.59; found, 61.49.

Di-*tert*-butyl *N*-(3-Fluoro-4-aminobenzoyl)-*l*-glutamate (7). The same hydrogenation and purification procedures as described above for converting **4** to **6** were applied in obtaining (90% yield) the *o*-fluoroaniline (**7**) from the *o*-fluoronitro compound (**5**). The product was, however, a colorless oil rather than a white solid. Like **6**, the product (**7**) also gave a positive ninhydrin reaction: NMR ($CDCl_3$) δ 1.43 and 1.51 (2 s Bu, 18 H), 2.2–2.5 (m, 4 H), 4.27 (broad s, NH_2, 2 H), 4.68 (m, α-H, 1 H), 6.73 (t, arom, 1 H), 7.02 (d, amide, NH, 1 H), 7.35–7.66 (arom, 2 H); IR ($CHCl_3$) 3640 and 3580 (amine NH), 3020 (aromatic CH), 1720 (ester C=O), 1635 (amide C=O), 1210 (intense, CF), 2420 (sharp band, probably CF overtone) cm^{-1}. Anal. ($C_{20}H_{29}N_2O_5F$) C, H, N, F.

2′-Fluoroaminopterin (8). The amine **6** (0.079 g, 0.2 mmol) was stirred with 0.060 g (0.15 mmol) of the (bromomethyl)diaminopteridine **1** in 0.5 mL of distilled dimethylacetamide for 1 h at 85 °C. "Proton Sponge" (Aldrich Chemical Co., 0.15 mmol) was then added, and the reaction was continued for an additional hour. Most of the solvent was removed under vacuum at 50 °C. The dark residue was taken up in 10 mL of chloroform containing 0.1 g of triethylamine, and the resulting solution was applied to a 1.5 × 20 cm column of dry silica gel (32–63 mesh). Washing of the column with 30 mL of $CHCl_3$ was carried out and was followed by elution with 95% ethanol–$CHCl_3$ (1:4, v/v). The major bright yellow band was collected. This material displayed a UV spectrum in ethanol very similar to that of authentic aminopterin with peaks near 260 and 280 nm and a smaller pteridine absorption near 370 nm. The partially pure product was concentrated, dissolved in 5 mL of $CHCl_3$, reapplied to a 1.5 × 30 cm silica gel column, and eluted with a stepwise gradient of 95% ethanol in $CHCl_3$ from 0 to 25%, v/v. The desired fluoroaminopterin di-*tert*-butyl ester was thus isolated and shown to be pure by TLC on silica gel and by HPLC (reverse phase). The product was concentrated to a yellow solid (40 mg), which was then treated for 2 h at room temperature with trifluoroacetic acid. The reagent was thoroughly removed under vacuum, giving a residue which was no longer soluble in $CHCl_3$ but was dissolved readily in 15 mL of 0.01 M aqueous NaOH. The pH was then adjusted to 4.2–4.4 by addition of 1 N HCl, giving precipitate of 2′-fluoroaminopterin, which was collected by centrifugation. The pellet was resuspended in 10 mL of H_2O, washed, repelleted three additional times, and finally dried under vacuum to give 28 mg (40% yield) of **8**: NMR of disodium salt, pH ~11 (D_2O, DHO at δ 4.8), δ 2.24 (m, 4 H, glutamate CH_2CH_2), 4.04 (broad, 2 H C_9 CH_2), 4.34 (broad, α-H, 1 H), 5.91–6.30 (2 H, aromatic), 7.32 (t, aromatic), 8.35 (s 1 H, pteridine C_7); UV (0.1 M phosphate, pH 7.2) λ_{max} 260 nm (ϵ_M 28 000) 279 (26 000), 371 (7950); ^{19}F NMR (0.05 M phosphate, pH 7.2, 25% D_2O for lock) sharp multiplet at 35.86 ppm upfield of trifluoroacetate. Anal. ($C_{18}H_{19}N_8O_5$-

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F·2.5H_2O) C, H, F (dried at 50 °C under vacuum); N: calcd, 22.79; found, 21.24.

3′-Fluoroaminopterin (9). This isomer was obtained in similar yield to 8 by condensing 1 and 7 in an identical reaction and purification scheme as described above for obtaining 8 from 6. The product, a pale yellow fluffy solid, was identified as follows: NMR of disodium salt, pH ~11 (D_2O, DHO at δ 4.8), δ 2.22 (m, 4 H), 4.32 (broad, 2 H), 4.38 (broad, α-H, 1H), 6.60 (t, arom, 1 H), 7.32–7.52 (arom, 2 H), 8.54 (s, 1 H); UV (0.1 M phosphate, pH 7.2) λ_{max} 260 nm (ϵ_M 28400), 279 (27800), 371 (7950); ^{19}F NMR (0.05 M phosphate, pH 7.2, 25% D_2O) sharp multiplet centered at 58.57 ppm upfield of trifluoroacetate. Anal. ($C_{18}H_{19}N_8O_5$F·2.5H_2O) C, H, F (after drying for 16 h at 50 °C); C: calcd, 44.15; found, 45.43; N: calcd, 22.70; found, 21.40.

Enzyme Assays. *L. casei* dihydrofolate reductase (DHFR) was purified to homogeneity from an MTX-resistant overproducer strain by the procedure of Dann et al.[20] Enzyme was assayed spectrophotometrically at pH 7.2, 400 mM KCl in the presence of 22 μM NADPH. For dihydrofolate, K_m = 0.36 μM under these conditions.[20] *E. coli* (MB 1428) MTX-resistant overproducer DHFR was purified to homogeneity according to the method of Poe[21] as modified by Williams[22] using MTX affinity chromatography. The enzyme was assayed spectrophotometrically at pH 7.2 in the presence of 40 μM NADPH, under conditions described by Poe et al., where the K_m for dihydrofolate is equal to 0.44 μM.[21] The concentrations of the above enzymes were established by MTX titration.[12] Rates (OD/min) were taken in linear regions from 0.5 to 2 min where less than 10% of the dihydrofolate was consumed. I_{50} is the inhibitor concentration that is found to reduce the observed rate to one-half its control value. K_i was calculated by the equation of Cha:[14]

$$I_{50} = K_i(1 + S/K_m) + 0.5E_t$$

HeLa cell DHFR was obtained from an overproducer (500-fold) strain of HeLa S-3 cells which are resistant to MTX and are grown in the presence of the drug.[23] These cells (HeLa S-3-500) were then grown for 2 generations in MTX-free, Dulbecco's modified Eagle's medium. Harvested cells were sonicated at 0 °C in phosphate buffer, and cell debris was removed by centrifugation at 40000*g*. The supernatant was assayed utilizing tritiated folic acid as substrate according to the method of Rothenberg[24] as modified by Alt et al.[25]

Biological Studies. Cytotoxicity studies were carried out on L1210 cells obtained from EG and G Mason Research Institute and on human stomach cancer line HuTu 80. Testing was carried out by standard methods:[26] Cells (Ca. 1 × 10^5) were suspended in 1 mL of their original media containing specified inhibitors and incubated at 37 °C for 72 h. Cell number was determined on a Coulter counter ZB_1 and maximally increased 10- to 20-fold over that inoculated. EC_{50} values refer to the concentration of inhibitor necessary to inhibit cell growth by 50% compared to controls grown in the absence of inhibitor.

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Registry No. 1, 52853-40-4; **2**, 403-24-7; **3**, 403-21-4; 4, 85803-25-4; **5**, 85803-26-5; **6**, 85803-27-6; **7**, 85803-28-7; **8**, 85803-29-8; 8 di-*tert*-butyl ester, 85803-30-1; 8·2Na, 85803-32-3; **9**, 85803-34-5; **9** di-*tert*-butyl ester, 85803-31-2; **9**·2Na, 85803-33-4; 2-fluoro-4-nitrotoluene, 1427-07-2; 3-fluoro-4-nitrotoluene, 446-34-4; di-*tert*-butyl L-glutamate hydrochloride, 32677-01-3; dihydrofolate reductase, 9002-03-3.

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Inhibitors of Glycolic Acid Oxidase. 4-Substituted 2,4-Dioxobutanoic Acid Derivatives

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Fourteen new 4-substituted 2,4-dioxobutanoic acids have been synthesized. These compounds, all of which contain lipophilic 4-substituents, are potent inhibitors in vitro of porcine liver glycolic acid oxidase. The I_{50} value of the two most potent representatives, 4-(4′-bromo[1,1′-biphenyl]-4-yl)-2,4-dioxobutanoic acid (8) and 4-[4′-[[(3,4-dihydro-3-hydroxy-2*H*-1,5-benzodioxepin-3-yl)methyl]thio][1,1′-biphenyl]-4-yl]-2,4-dioxobutanoic acid (13) is 6 × 10^{-8} M.

Recently we described[1] a series of 4-substituted 3-hydroxy-1*H*-pyrrole-2,5-dione derivatives which were shown to be potent inhibitors of the enzyme glycolic acid oxidase (glycolate:O_2 oxidoreductase, EC 1.1.3.1) (GAO). This enzyme, which catalyzes the oxidation of glycolate to glyoxylate along with the conversion of the latter to oxalate, is considered to play an important role in oxalate production in plants and animals.[2] Earlier studies by Schuman and Massey[2a,3] had suggested that the active site of porcine liver GAO contained two cationic groups in close proximity along with a hydrophobic bonding region. On

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