117654-80-5; **23** free base, 117654-94-1; **24**, 131275-34-8; **24** free base, 131275-35-9; **25**, 131275-36-0; **25** free base, 131275-37-1; **26**, 131275-38-2; **26** free base, 131275-39-3; **27**, 131275-40-6; **27** free base, 131296-22-5; **28**, 131275-41-7; **28** free base, 131275-42-8; **29**, 131275-43-9; **29** free base, 131275-44-0; **30**, 131275-45-1; **30** free base, 131275-46-2; **31**, 131275-47-3; **31** free base, 131275-48-4; **32**, 131275-49-5; **32** free base, 131275-50-8; **33**, 131275-51-9; **33** free base, 131275-52-0; **34**, 131275-53-1; **34** free base, 131275-54-2; **35**, 131275-55-3; **35** free base, 131275-56-4; **36**, 117654-75-8; **36** free base, 125763-86-2; **37**, 117654-76-9; **37** free base, 131275-57-5; **38**, 131275-58-6; **38** free base, 131275-59-7; **39**, 131275-60-0; **39** free base, 131275-61-1; **40**, 131275-62-2; **40** free base, 131275-63-3; **41**, 131275-64-4; **41** free base, 131275-65-5; **42**, 131296-23-6; **42** free base, 131275-66-6; **43**, 131275-67-7; **43** free base, 131275-68-8; **44a**, 1075-76-9; **44b**, 1488-20-6; **44c**, 131275-69-9; **46a**, 117654-95-2; **46b**, 131275-70-2; **46c**, 131275-71-3; **47a**, 117654-96-3; **47b**, 131275-72-4; **47c**, 131275-73-5; α,ω-bis[(cyanoethyl)amino]heptane, 131275-74-6; benzyl bromide, 100-39-0; spermine, 71-44-3; benzaldehyde, 100-52-7; 4-ethylbenzaldehyde, 4748-78-1; 1,7-dibromoheptane, 4549-31-9.

Synthesis and Biological Activity of Methotrexate Analogues with Two Acid Groups and a Hydrophobic Aromatic Ring in the Side Chain[1]

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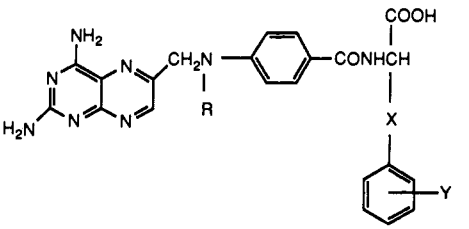
The heretofore unknown γ-(*m*-carboxyanilide) and γ-(*m*-boronoanilide) derivatives of methotrexate (MTX) and the γ-(*m*-carboxyanilide) derivatives of aminopterin (AMT) were prepared and tested as inhibitors of dihydrofolate reductase (DHFR) and as inhibitors of cell growth in culture with the aim of comparing their activity with that of N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine, a potent antifolate whose side chain likewise contains a hydrophobic aromatic ring with an acid group on the ring. All three anilides were potent DHFR inhibitors, with activity comparable to MTX and AMT. The γ-(*m*-boronoanilide) displayed growth inhibitory potency similar to that of the hemiphthaloylornithine analogue, with an IC_{50} of only 0.7 nM. This compound, which is the most potent of the γ-amides of MTX tested to date, is also the first reported example of an antifolate with a *$B(OH)_2$ group in the side chain* and is especially novel because of its potential to form a stable tetrahedral boronate complex by reaction with electron rich OH or NH_2 groups in the active site of DHFR or other folate enzymes. In antitumor assays against L1210 leukemia in mice, N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine gave a T/C of >263% at 20 mg/kg (qd×9) and 300% at 16 mg/kg (bid×10), whereas maximally tolerated doses of MTX of 8 mg/kg (qd×9) and 1 mg/kg (bid×10) gave T/C values of 213 and 188%, respectively. MTX γ-(*m*-boronoanilide) was also active, with a T/C of 175% at 32 mg/kg (qd×9), the highest dose tested.

This laboratory has been active for a number of years in the design and synthesis of analogues of the well-known anticancer drugs methotrexate (MTX) and aminopterin (AMT) for the purpose of systematically exploring the subtle relationships that exist between structure and biological activity in classical antifolates.[2] In two recent papers we reported the synthesis[3,4] and potent antifolate[3] activity of N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (**1**), an analogue of aminopterin (AMT) with three instead of two CH_2 groups in the side chain and the γ-COOH group replaced by a more liphophilic hemiphthaloyl group. Compound **1** had the unusual property of being not only more toxic than MTX to MTX sensitive cells but also more toxic to MTX resistant cells than MTX was to the parental cells from which the resistant sublines were derived.[3] With the aim of identifying the structural features that may contribute to this unusual level of activity, we wished to examine several possible factors: (a) whether the number of CH_2 groups in the side chain could be decreased from three to two, (b) whether the amide could be "reversed" from NHCO to CONH, (c) whether the COOH group on the phenyl ring could be moved from the ortho to the meta position, and (d) whether this group could be replaced by another acidic group. The present paper describes the synthesis of compounds **2**–**4** (Chart I) the first three analogues of **1** studied in this connection. Compounds **1**–**4** all inhibited DHFR from human leukemic cells with a potency similar to AMT and MTX and were also good inhibitors of cell growth in culture. Compound **4**, the first example of a folate analogue with a $B(OH)_2$ group in the side chain, was approximately ten times more potent than MTX as a cell

Chart I



	R	X	Y
1	H	$(CH_2)_3NHCO$	*o*-CO_2H
2	H	$(CH_2)_2CONH$	*m*-CO_2H
3	Me	$(CH_2)_2CONH$	*m*-CO_2H
4	Me	$(CH_2)_2CONH$	*m*-$B(OH)_2$

† Dana-Farber Cancer Institute.
‡ Medical College of Ohio.

(1) Paper 43 in this series; for previous paper, see: Rosowsky, A.; Forsch, R. A.; Moran, R. G.; Freisheim, J. H. *J. Med. Chem.*, in press.
(2) For a recent comprehensive review of work in this and other laboratories on folate antagonists, see: Rosowsky, A. *Progr. Med. Chem.* **1989**, *26*, 1.
(3) Rosowsky, A.; Bader, H.; Cucchi, C. A.; Moran, R. G.; Kohler, W.; Freisheim, J. H. *J. Med. Chem.* **1988**, *31*, 1332.
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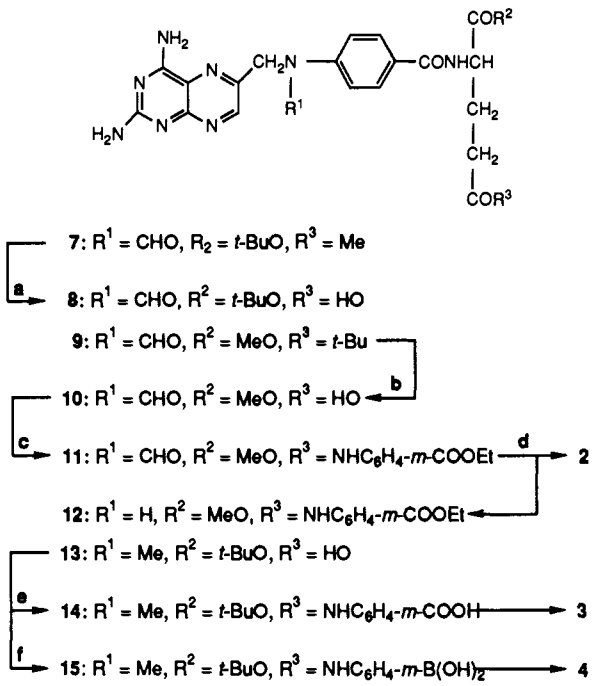
0022-2623/91/1834-0574$02.50/0

Scheme I[a]



[a] (a) NaOH/aq MeOH; (b) TFA; (c) (1) *i*-BuOCOCl/Et_3N, (2) $H_2NC_6H_4$-*m*-CO_2Et, (3) NH_4OH; (d) NaOH/DMSO; (e) (1) $H_2NC_6H_4$-*m*-CO_2Et/DPPA/Et_3N, (2) NH_4OH; (f) $H_2NC_6H_4$-*m*-$B(OH)_2$/DPPA/Et_3N.

growth inhibitor even though its anti-DHFR activity was similar. The presence of a $B(OH)_2$ group in **4** makes this an especially interesting DHFR ligand because of its potential for slow reaction with electron-rich groups in the active site to form a stable tetrahedral boronate complex.[5] Although this series of compounds is too limited to allow rigorous structure–activity correlations, the biological activity we have obtained to date points to the desirability of further work in this area.

Chemistry

A summary of the methods used to obtain the AMT and MTX γ-anilides **2–4** is given in Scheme I. Our initial intent, in the preparation of **2**, was to start with 4-amino-4-deoxy-N^{10}-formylpteroic (fAPA, **5**), which we had already used earlier as an intermediate in the synthesis of γ-ester[6] and γ-amide[7] derivatives as well as γ-sulfonate[8] and γ-phosphonate[9] analogues of AMT. Condensation of fAPA with α-*tert*-butyl γ-methyl L-glutamate hydrochloride (**6**·HCl)[10] by the mixed carboxylic–carbonic anhydride (MCA) method followed by gentle hydrolysis with NaOH in aqueous MeOH yielded **8** in 45% overall yield, without loss of the protecting group on N^{10}. However, attempts to condense **8** with ethyl m-aminobenzoate, either by the MCA method or with the aid of diphenylphosphoryl azide (DPPA), were unsuccessful. We therefore abandoned **8** and turned instead to N^{10}-formylaminopterin α-methyl γ-*tert*-butyl ester (**9**), which had used previously as an intermediate in the synthesis of the γ-*tert*-butyl ester of AMT.[6] Acidolysis of the *tert*-butyl group with trifluoroacetic acid afforded a nearly quantitative yield of the heretofore undescribed N^{10}-formyl monoester **10** as the trifluoroacetate salt. Condensation of **10** with ethyl *m*-aminobenzoate by the MCA method yielded a complex mixture of products from which it was possible to isolate the desired diester **11**, albeit in only 24% yield after preparative HPLC on C_{18} silica gel. Removal of both ester groups and the *N*-formyl group from **11** was performed in a single step with NaOH, and the product was purified by preparative HPLC and isolated as a hydrated ammonium salt. A minor product (2% yield) in the saponification reaction was tentatively identified as aminopterin α-methyl ester γ-(*m*-carbethoxyanilide) (**12**).

It may be noted that another approach to the preparation of **2**, patterned after our previous work on AMT γ-anilides,[7] would have been to condense the γ-(*m*-carbethoxyanilide) of α-benzyl-L-glutamate with fAPA. However, attempts to condense α-benzyl *N*-(*tert*-butyloxycarbonyl)-L-glutamate with ethyl *m*-aminobenzoate in the presence of DPPA consistently yielded complex mixtures from which little or none of the desired γ-anilide could be recovered. Since this was in marked contrast to our previous experience with other anilines,[7] we concluded that the NH_2 group in ethyl *m*-aminobenzoate is too weak a nucleophile to react efficiently with the intermediate azide in the DPPA reaction. This approach to **2** was therefore also abandoned.

The preparation of the MTX γ-anilides **3** and **4** proved somewhat easier than that of **2**, but afforded at least one surprise: condensation of ethyl *m*-aminobenzoate with MTX α-*tert*-butyl ester (**13**) in the presence of DPPA and Et_3N followed by chromatography of the crude product on silica gel with 85:15:1 $CHCl_3$–MeOH–28% NH_4OH as the eluent unexpectedly gave the *ammonium salt* of **14** instead of a diester. This ease of hydrolysis of the aromatic ester group during chromatography spared us from having to cleave the ethyl ester group in a separate step, and allowed **3** to be obtained directly by cleavage of the *tert*-butyl ester with trifluoroacetic acid. The yield of **14** from **13** was 39%, and that of **3** from **14** was 65%. Condensation of **13** with (*m*-aminophenyl)boronic acid in the presence of DPPA and Et_3N afforded the γ-anilide **15** in 53% yield, and acidolysis of **15** in trifluoroacetic acid furnished **4** in 92% yield. Thus, coupling of the aromatic amine to the γ-carboxyl and acidolysis of the α-*tert*-butyl ester both appeared to proceed more cleanly with a $B(OH)_2$ than with a COOH group at the meta position.

From the success in obtaining the boronic acid derivative **4**, we were encouraged to attempt a coupling reaction between (*m*-aminophenyl)boronic acid and **10**. However, MCA condensation was found to give a complex mixture from which we were unable to isolate any of the desired product. Similarly unsuccessful were efforts to condense fAPA with the preformed γ-(*m*-boronoanilide) of α-benzyl

(5) For an example of the use of phenylboronic acid to chemically modify an active site residue in an enzyme (substilisin), see: Matthews, D. A.; Alden, R. A.; Birktoft, J. J.; Freer, S. T.; Kraut, J. *J. Biol. Chem.* **1975**, *250*, 7120. Other examples of work involving the use of boronopeptides to form tetrahedral boronate adducts of enzymes are reviewed in the following recent papers: Bone, R.; Shenvi, A. B.; Kettner, C. A.; Agard, D. A. *Biochemistry* **1987**, *26*, 7609. Kettner, C. A.; Bone, R.; Agard, D. A.; Bachovchin, W. W. *Biochemistry* **1988**, *27*, 7682. Bachovchin, W. W.; Wong, W. Y. L.; Farr-Jones, S.; Shenvi, A. B.; Kettner, C. A. *Biochemistry* **1988**, *27*, 7689.

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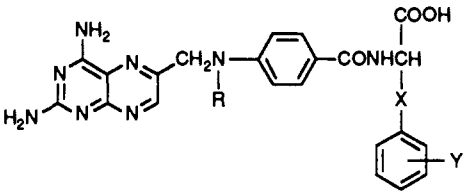
Table I. Biological Activity of Aminopterin and Methotrexate Analogues with Two Acid Groups and a Hydrophobic Phenyl Ring in the Side Chain



cmpd	R	X	Y	DHFR inhibition: IC_{50}, nM[a]	growth inhibition: IC_{50}, nM[b] L1210 cells	WI-L2 cells
1	H	$(CH_2)_3NHCO$	*o*-COOH	52[c]	0.75[c]	48
2	H	$(CH_2)_2CONH$	*m*-COOH	32	25	6.1
3	Me	$(CH_2)_2CONH$	*m*-COOH	35	1.6	2.2
4	Me	$(CH_2)_2CONH$	*m*-$B(OH)_2$	30	0.70	ND

[a] Purified DHFR from human cells (WI-L2/M4)[11] was used except with 1, which was assayed against purified enzyme from mouse cells (L1210/R71).[9] The IC_{50} values of MTX against the human and mouse enzyme were 24 and 25 nM, respectively; those of AMT were 25 and 35 nM, respectively. Assays were performed spectrophotometrically at 340 nm,[9,11] using 50 nM enzyme. [b] IC_{50} values obtained for MTX and AMT against L1210 cells were 4.6 and 2.0 nM, respectively. The IC_{50} of MTX against WI-L2 cells was 24 nM, and that of AMT was 7.1 nM. Cells were incubated with drug for 48 h and counted by the MTT method as described earlier.[3] ND = not determined. [c] Data for 1 are taken from ref 3.

L-glutamate, obtained from α-benzyl-*N*-(*tert*-butyloxycarbonyl)-L-glutamate by reaction with (*m*-aminophenyl)boronic acid and acidolysis of the product with *p*-toluenesulfonic acid. Neither the benzyl ester of the desired AMT derivative nor the free acid we attempted to obtain by saponification of the crude coupling product was formed in sufficient amount to warrant further work. Thus, **4** remains the first and only known folate analogue with a $B(OH)_2$ group in the side chain.

Biological Activity

Compounds **2–4** were tested as inhibitors of purified DHFR from human leukemic lymphoblasts (WI-L2/M4-cells).[11] As shown in Table I, the analogues all had IC_{50} values in the 30–35 nM range and were similar in activity to AMT and MTX, confirming previous findings with other γ-anilide derivatives of these antifolates.[7] Modifications of the amino acid side chain beyond the α-carbon are well known to have relatively little influence on binding to DHFR.[6-10,12] Since compounds **2–4** differ from compound **1** in that the latter contains an extra CH_2 group and a "reversed" amide group, the CONH group in **1** probably occupies a different position in space than the amide group in **2–4**. While it is conceivable that hydrogen bonding involving the CONH group next to the γ- or δ-carbon in these compounds can contribute to their interaction with DHFR, as has been proposed previously for the CONH group next to the α-carbon,[13] the present data suggest that this effect is not very significant.

In contrast to their similar IC_{50} values as DHFR inhibitors, compounds **2–4** exhibited a 10-fold range of potency as inhibitors of cell growth in culture. Interestingly, the AMT analogue **2** (IC_{50} = 25 nM) was 12.5-fold less potent against L1210 cells than AMT (IC_{50} = 2.0 nM), whereas the MTX analogue **3** (IC_{50} = 1.6 nM) was 3-fold *more potent* than MTX (IC_{50} = 4.6 nM). On the other hand, **2** (IC_{50} = 6.1 nM) was somewhat more potent against WI-L2 cells than either AMT (IC_{50} = 7.1 nM) or MTX (IC_{50} = 24 nM). Since **2** and **3** bind equally well to human DHFR, other factors must be considered to account for the results against WI-L2 cells. That small amounts of AMT and MTX were present in the test samples of **2** and **3** was ruled out by HPLC analysis. One possibility we cannot exclude at this time is that **3** accumulates more efficiently in the cells than MTX, resulting in a higher intracellular drug concentration in excess of the amount needed to just saturate DHFR, while **2** is less efficient in this regard than AMT. It is also conceivable that **2** and **3** undergo some hydrolysis to AMT and MTX inside the cell, with subsequent conversion to AMT and MTX polyglutamates, and that this process is more efficient with **2** than with **3**. Additional work using radiolabeled compounds would be required to address this problem. It would also be of interest to synthesize and test other analogues with a view to more fully exploring the subtle differences that appear to exist between pairs of compounds of the AMT (N^{10}–H) versus MTX (N^{10}–Me) type.

The in vivo activity of **1** and **4** against L1210 leukemia in mice was determined. As shown in Table II, qd×9 treatment with 20 mg/kg of **1** gave a T/C of >263%, whereas MTX at its maximally tolerated dose on this schedule (8 mg/kg) in the same experiment gave a T/C of 213%. In a separate experiment in which the drugs were given bid×10, 16 mg/kg of **1** gave a T/C of 300%, whereas 1 mg/kg of MTX, the maximally tolerated dose on this more frequent dosing schedule, gave a T/C of 188%. It thus appears that **1** is less toxic than MTX and produces a greater increase in survival in L1210 leukemic mice at optionally tolerated doses. Toxicity was not reached on the qd×9 schedule until the dose was 32 mg/kg. Compound **4** was less active than either **1** or MTX, giving a T/C of 175% at 32 mg/kg (qd×9). However **4** also appeared to be less toxic than **1** or MTX, causing <10% weight loss at 32 mg/kg. Lack of material prevented us from increasing the dose beyond 32 mg/kg on the qd×9 schedule or using the more frequent bid×10 schedule. Hence, the T/C value given in Table II may be suboptimal.

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(12) For additional examples showing that substitution on the γ-carboxyl (and even the γ-carbon) of the MTX side chain has little effect on DHFR binding, see: (a) Piper, J. R.; Montgomery, J. A.; Sirotnak, F. M.; Chello, P. L. *J. Med. Chem.* **1982**, *25*, 182. (b) Antonjuk, D. J.; Birdsall, B.; Cheung, H. T. A.; Clore, G. M.; Feeney, J.; Gronenborn, A.; Roberts, G. C. K.; Tran, T. Q. *Br. J. Pharmacol.* **1984**, *81*, 309. (c) Rosowsky, A.; Bader, H.; Kohler, W.; Freisheim, J. H.; Moran, R. G. *J. Med. Chem.* **1988**, *31*, 1338. (d) Tsushima, T.; Kawada, K.; Ishihara, S.; Uchida, N.; Shiratori, O.; Higaki, J.; Hirata, M. *Tetrahedron* **1988**, *44*, 5375. (e) McGuire, J. J.; Graber, M.; Licato, N.; Vincenz, C.; Coward, J. K.; Nimec, Z.; Galivan, J. *Cancer Res.* **1989**, *49*, 4517. See also earlier papers by this group.

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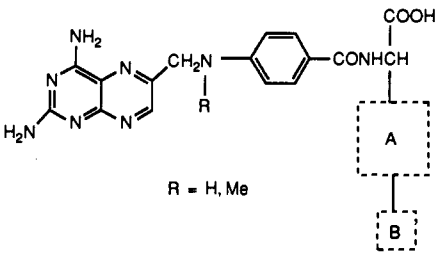
Table II. Activity of N^{α}-(4-Amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (1) and Aminopterin γ-(*m*-Boronoanilide) (4) against L1210 Leukemia in Mice

compd	dose, mg/kg[a]	no. of mice	schedule	7-day wt change, %	survival, days		T/C, %
					range	median	
1 expt 1[b]	2	9	qdx9	-4	12-13	13	163
	4	9		-3	13-15	14	175
	8	9		+4	14-16	15	188
	16	9		-5	13-19	18	225
	32	9		-11 (toxic)	9-19	11	138
1 expt 2[c]	12	9	qdx9	+1	13-18	15	188
	16	9		+1	14-21[f]	18	225
	20	9		-2	10-21[f]	>21	>263
	24	9		-3	15-21[f]	20	250
1 expt 3[d]	4	9	bidx10	+8	14-19	17	213
	8	9		+4	19-24	20	250
	12	9		+2	20-24	22	275
	16	9		-3	11-24	24	300
4[e]	8	9	qdx9	+8	9-12	11	138
	16	9		+4	11-14	12	150
	32	9		+5	12-15	14	175

[a] Groups of BDF_1J mice were inoculated intraperitoneally with 10^5 L1210 cells on day 0, and drug treatment was started on day 1. Drugs were injected intraperitoneally in sterile solution at pH 7.5-7.8 on the indicated schedule. The bidx10 schedule refers to twice-daily injections on days 1-4, a double dose on days 5 and 6, and twice-daily injections on days 7-10. A 7-day weight loss of 10% or more is considered toxic. Untreated controls in each experiment (15 mice) had a median survival of 8 days (range, 7-8 days). [b] MTX (9 mice): T/C = 175% at 4 mg/kg. [c] MTX (9 mice): T/C = 188% at 4 mg/kg, 213% at 8 mg/kg. [d] MTX (9 mice): T/C = 175% at 0.75 mg/kg, 188% at 1 mg/kg. [e] MTX (9 mice): T/C = 200% at 4 mg/kg. [f] Mice moribund on day 21 were sacrificed: one at 16/mg/kg, three at 20 mg/kg, and two at 24 mg/kg. These animals all had ascites at the time of sacrifice.

Though the boronic acid derivative 4 appeared to be less effective than 1 against L1210 leukemia in vivo, its high potency (IC_{50} = 0.70 nM) against cultured L1210 cells is of interest because it is the highest among the many MTX γ-esters and γ-amides, or other analogues, we have examined to date. Our interest in this compound was kindled initially by the possibility that the $B(OH)_2$ function, which is known to form stable tetrahedral boronate complexes with electron-rich groups in proteins (e.g., the OH of active site serine in proteases),[5] might bind covalently to DHFR, as has been shown previously with MTX analogues in which L-glutamate was replaced by N^{ϵ}-(iodoacetyl)-L-lysine[14] or N^{δ}-(bromoacetyl)-L-ornithine.[15] Examination of stereo drawings of the structure for a crystalline complex of MTX and NADPH with murine[16] or human[17] DHFR does not clearly reveal how such covalent binding might occur, because the end of the MTX side chain appears to lie in a cavity where the γ-COOH group does not interact directly with the protein, though it may well be hydrogen bonded to water. However, bearing in mind that the side chain in 4 is somewhat longer than the side chain in MTX and that it might be possible for the DHFR to experience some conformational adaptation to the demands of the ligand, it is conceivable the $B(OH)_2$ group in 4 could come close enough to some active-site residue with an electron rich group to allow slow formation of a covalent tetrahedral boronate complex. A possible candidate in this regard might be the guanidino group of Arg-70 (chicken liver enzyme numbering).[18] Studies of such a covalent adduct by X-ray crystallography or nuclear magnetic resonance would be of interest.

Chart II



The results of this study suggest that it may be worthwhile to investigate a broader group of AMT and MTX analogues of the general type shown in Chart II, where A is a lipophilic moiety of appropriate size and B is a water-solubilizing acidic group. Domain A, consisting of a $(CH_2)_n$ spacer, an amide goup (NHCO or CONH), and an aromatic ring, would be expected to enhance binding to the "hydrophobic pocket" of DHFR, where the *p*-aminobenzoyl-L-glutamate moiety of folate and antifolate ligands normally lies. Group B, which could be on the ortho, meta, or para position of the aromatic ring in A, would have the function of improving uptake into cells via carrier-mediated transport. Evidence that hydrophobic interactions between certain residues on DHFR and the phenyl ring as well as side chain CH_2 groups of folates and antifolates play a major role in the binding of these molecules to the enzyme has come recently from site directed mutagenesis studies in several laboratories.[19-22] In view of the emerging recognition of the importance of these hydrophobic interactions, we believe it would be worth-

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while to examine folate analogues specifically designed to test this concept. Studies along this line are in progress in this laboratory.

Experimental Section

IR spectra (KBr disks) were obtained on a Perkin-Elmer Model 781 double-beam recording spectrophotometer, and UV spectra on a Varian Model 210 instrument. ^{1}H NMR spectra were obtained on a Varian EM360L spectrometer with Me_4Si as the reference. TLC analyses were on fluorescent Baker Si250F silica gel plates, Eastman 13181 silica gel sheets, or Eastman 13254 cellulose sheets. Spots were visualized under 254-nm UV illumination or with the aid of ninhydrin. TLC plates were developed with the following mixtures: 85:15:1 $CHCl_3$–MeOH–28% NH_4OH (system A); 28:12:1 $CHCl_3$–MeOH–28% NH_4OH (system B); 5:4:1 $CHCl_3$–MeOH–28% NH_4OH (system C); 9:1 $CHCl_3$–MeOH (system D); potassium phosphate, pH 7.4 (system E). Column chromatography was on Baker 3405 (60–200 mesh), Baker 7024-1 'Flash' silica gel, or Whatman DE-52 preswollen [(*N*,*N*-diethylamino)ethyl]cellulose (DEAE-cellulose). 4-Amino-4-deoxy-N^{10}-formylpteroic acid (fAPA, **5**),[6] α-*tert*-butyl γ-methyl L-glutamate hydrochloride (**6**·HCl),[7] N^{10}-formylaminopterin γ-*tert*-butyl α-methyl ester (**9**),[10] and MTX α-*tert*-butyl ester (**13**)[23] were prepared as reported earlier; other chemicals were purchased from Aldrich, Milwaukee, WI. Solvents for moisture sensitive reactions were dried by storage over Linde 4A molecular sieves (Fisher, Boston, MA). Analytical HPLC was on a Waters C_{18} radial compression cartridge column (5-μm particle size, 5 mm i.d. × 10 cm) connected to a Waters instrument equipped with a Model 490 multiwavelength detector and Model 660 programmable solvent gradient system. Preparative HPLC was on a Waters DeltraPrep 3000 instrument. Melting points were taken on a Fisher-Johns hot-stage apparatus (Fisher, Boston, MA) or in Pyrex capillary tubes in a Mel-Temp apparatus (Cambridge Laboratory Devices, Cambridge, MA) and are not corrected. Microanalyses were by Galbraith Laboratories, Knoxville, TN, MultiChem Laboratories, Lowell, MA, or Robertson Laboratory, Madison, NJ.

N^{10}-Formylaminopterin α-*tert*-Butyl Ester (8). *i*-BuOCOCl (0.287 mg, 2.1 mmol) was added at room temperature to a stirred solution of **5**·1.5H_2O (0.772 mg, 2.1 mmol) and Et_3N (1.27 g, 12.6 mmol) in dry DMF (30 mL). After 15 min, **6**·HCl (0.535 g, 2.1 mmol) was added, followed after 10 min by a second portion of *i*-BuOCOCl (0.144 g, 1.05 mmol). After another 15 min, a second portion of amino ester (0.268 g, 1.05 mmol) was added, followed 15 min later by a third portion of *i*-BuOCOCl (0.772 g, 0.53 mmol) and 10 min later a third portion of amino ester (0.134 g, 0.53 mmol). The last cycle of addition of *i*-BuOCOCl and amino ester was repeated once more, thus bring the total of these reactants to 4.2 mmol. The solvent was evaporated under reduced pressure, and the residue was dissolved in TLC eluent system D (5 mL) and adsorbed onto a silica gel column (80 g, 3.0 × 38 cm) which was eluted with the same solvent mixture. Fractions showing a TLC spot with R_f 0.40 (silica gel, system D) were pooled and evaporated, the residue was taken up in MeOH, and the MeOH was added to Et_2O to obtain a solid. Filtration and drying in vacuo at 60 °C over P_2O_5 afforded the N^{10}-formyl diester **7** as a yellow powder (1.06 g): mp 148–150 °C; IR ν 3325 (NH_2), 1740 (ester C=O), 1645, 1610 (amide C=O) cm^{-1}.

A portion (0.246 g) of the crude diester **7** was dissolved in a mixture of MeOH (15 mL) and H_2O (10 mL), and 0.15 N NaOH (1.8 mL) was added dropwise over 10 min until a pH of 9.0 was reached. The reaction was monitored for the appearance of a blue-fluorescent spot at R_f 0.58 by TLC (silica gel, system A). When hydrolysis was complete, the solution was acidified with AcOH until a pH of 4.3 was reached. The MeOH was partially evaporated on the rotary evaporator, and the remaining mixture was kept at 0 °C for 18 h. Filtration followed by drying, first on a lyophilizer and then in vacuo at 80 °C over P_2O_5, afforded **8** as a yellow powder (65 mg), mp 154–157 °C.

The mother liquor was evaporated to dryness, and the residue was redissolved in TLC eluent system A and applied onto a silica gel column (7 g, 1.0 × 23 cm), which was prepared and eluted with the same solvent mixture. Pooling of fractions with R_f 0.58 gave, after evaporation and drying, another 59 mg of product; total 124 mg (45% overall yield from **5**); IR ν 3340 (NH_2), 3200 (NH^+), 1730 (ester C=O), 1660 (acid C=O), 1640 (amide C=O) 1607 (aromatic) cm^{-1}; NMR (pyridine-d_5) δ 1.43 (s, 9 H, *t*-Bu), 2.0–2.7 (m, 4 H, CH_2CH_2), 3.5 (s, ca. 3 H, CH_3OH), 5.0 (m, 1 H, α-CH), 5.3 (s, 2 H, CH_2N), 7.13 (d, 2 H, J = 5 Hz, $C_{2'}$- and $C_{6'}$-H), 8.23 (d, 2 H, J = 5 Hz, $C_{3'}$- and $C_{5'}$-H), 8.87 (s, 1 H, CHO), 8.96 (s, 1 H, C_7-H). Anal. ($C_{24}H_{28}N_8O_6$·1.3CH_3OH) C, H, N.

N^{10}-Formylaminopterin α-Methyl Ester (10). A. A mixture of **9**·H_2O (0.20 g, 0.359 mmol) and precooled (−20 °C) trifluoroacetic acid was sonicated for 5 min to dissolve all the solid and then kept at 0 °C for 1 h. After addition of the solution to excess Et_2O with stirring, the precipitate was filtered and dried at room temperature under reduced pressure. The solid was redissolved in 10% NH_4OH, and the solution was acidified to pH 4.0 with 10% AcOH and cooled. The precipitated solid was filtered and dried, first on a lyophilizer and then in vacuo at 80 °C over P_2O_5, to obtain a yellow powder (190 mg, ca. 100% yield): mp 162–164 °C; IR ν 3325, 3220, 2960 (NH_2, NH^+), 1745 (ester C=O), 1690–1640 (acid and amide C=O), 1610 (amide C=O) cm^{-1}; TLC R_f 0.47, blue-fluorescent (cellulose, system E); R_f 0.70, blue-fluorescent (silica gel, system C); NMR (pyridine-d_5) δ 2.0-3.0 (m, 4 H CH_2CH_2), 2.56 (s, ca. 1 H, CH_3CO_2H), 3.60 (s, 3 H, CH_3O), 5.15 (m, 1 H, α-CH), 5.26 (s, 2 H, CH_2NH), 7.4 (d, 2 H, J = 5 Hz, $C_{2'}$- and $C_{6'}$-H), 8.16 (d, J = 5 Hz, $C_{3'}$- and $C_{5'}$-H), 8.83 (s, 1 H, CHO), 8.90 (s, 1 H, C_7-H). Anal. ($C_{21}H_{22}N_8O_6$·0.5CH_3CO_2H·H_2O) C, H, N.

B. Under the same conditions as in the preceding experiment, a solution obtained from **9**·H_2O (0.854 g, 1.53 mmol) in trifluoroacetic acid (4 mL) was kept at 0 °C for 1 h and poured with stirring into Et_2O (150 mL). The solid was filtered and dried in vacuo at 80 °C over P_2O_5 to obtain the *trifluoroacetate salt* as a yellow powder (0.87 g, 95% yield); mp >300 °C with decomposition: TLC R_f 0.72, blue-fluorescent (silica gel, system C); HPLC 3.9 min, flow rate 1.0 mL/min. Anal. ($C_{21}H_{22}N_8O_6$·CF_3CO_2H·0.3H_2O) C, H, F, N.

N^{10}-Formylaminopterin α-Methyl Ester γ-(*m*-Carbethoxyanilide) (11). *i*-BuOCOCl (124 mg, 0.91 mmol) was added at room temperature to a stirred solution of **10**–CF_3CO_2H (548 mg, 0.91 mmol) and *N*-methylmorpholine (556 mg, 5.5 mmol) in dry DMF (35 mL). After 20 min, 4-(*N*,*N*-dimethylamino)pyridine (45 mg) was added, followed by ethyl *m*-aminobenzoate (150 mg, 0.91 mmol). Monitoring of the reaction by HPLC (C_{18} silica gel, 25% MeCN in 0.1 M NH_4OAc, pH 7.0; flow rate 1.0 mL/min) showed a constant ratio of product (retention time 32 min) and ethyl *m*-aminobenzoate (retention time 27 min) after 3 h. After being stirred at room temperature for 4 days, the reaction mixture was evaporated to dryness under reduced pressure. The residue was washed with H_2O and redissolved in 95:5 $CHCl_3$–MeOH, and the solution was applied onto a silica gel column (40–140 mesh, 100 g, 27.5 × 3 cm) which was eluted successively with 98:2, 95:5, and 9:1 $CHCl_3$–MeOH. Fractions showing a major blue-fluorescent TLC spot with R_f 0.51 (silica gel, system A), along with several minor spots indicating the presence of impurities, were pooled and evaporated to obtain a yellow soild (222 mg, 38% yield). This material was further purified by preparative HPLC (silica gel C_{18} silica gel, 25% MeCN in 0.01 M NH_4OAc, pH 7.5; flow rate 15 mL/min). A major peak with a retention time of 15 min (product A) and minor peak with a retention of 17.9 min (product B) were collected separately, evaporated to dryness, and repeatedly lyophilized with additions of H_2O for several days to constant weight. Product A, the desired diester (11), was a yellow powder (139 mg, 24% yield): mp 128–130 °C after drying in vacuo over P_2O_5 at 40 °C; TLC R_f 0.48, blue-fluorescent (silica gel, system A); IR ν 3400 (NH_2), 1720, 1700 (ester C=O), 1660–1600 (amide C=O, aromatic) cm^{-1}. Anal. ($C_{30}H_{31}N_9O_7$·H_2O) C, H, N.

Product B, formulated as aminopterin α-methyl ester γ-(*m*-carbethoxyanilide) (**12**) from its microchemical analysis, was a yellow powder (11 mg, 2%): mp 97–99 °C; TLC R_f 0.56, blue-fluorescent (silica gel, system A); IR ν 3400 (NH_2), 1730 (ester C=O), 1675–1600 (amide C=O, aromatic) cm^{-1}. Anal. ($C_{29}H_{29}N_9O_7$·3H_2O) C, H, N.

Aminopterin γ-(*m*-Carboxyanilide) (2). A suspension of **11** (113 mg 0.174 mmol) in 0.5 N NaOH (5.2 mL) was sonicated

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until almost all the solid dissolved (ca. 7 min). After 3 h at room temperature, a trace of solid was removed by filtration and the filtrate was acidified to pH 4.2 with 10% AcOH. The mixture was stirred at room temperature for 30 min, then cooled at -20 °C for 30 min and centrifuged. The solid was washed with H_2O, centrifuged again, and freeze dried overnight to obtain a dark-yellow powder (105 mg). Analytical HPLC on C_{18} silica gel (5% MeCN in 0.1 M NH_4OAc, pH 7.0; flow rate 1 mL/min) showed a major peak (10.8 min, 71%) along with five other peaks (2.9 min, 4.5%; 5.2 min, 6.5%; 6.2 min, 2%; 7.0 min, 2%; 8.0 min, 13%). The crude hydrolysis product was subjected to preparative HPLC (C_{18} silica gel, 7% MeCN in 0.1 M NH_4OAc, pH 7.0; flow rate 15 mL/min), and the main peak (16.5 min) was collected in several fractions. Two fractions found to be >99% pure were combined and evaporated to dryness. The residue was taken up in dilute ammonia and lyophilized repeatedly with additions of H_2O for several days to constant weight to obtain the hydrated *ammonium salt* as a yellow solid (73 mg, 67%): mp 210 °C dec (softening at 135 °C); TLC R_f 0.14 (silica gel, system C); IR (KBr) ν 3430, 1645 (acid C=O), 1555 (amide C=O) cm^{-1}. Anal. ($C_{26}H_{25}N_9O_6 \cdot NH_3 \cdot 3H_2O$) C, H, N.

Methotrexate α-*tert*-Butyl Ester γ-(*m*-Carboxyanilide) (14). A solution of **13** (511 mg, 1 mmol) in dry DMF (20 mL) was cooled to -10 °C, and to the solution was added ethyl *m*-aminobenzoate (205 mg, 1 mmol) followed by DPPA (605 mg, 2.2 mmol) and Et_3N (445 mg, 4.4 mmol). The solution was kept at 0 °C for 6 h and then at room temperature for 74 h. The solvent was removed under reduced pressure, and the residue was dissolved in a small volume of TLC eluent system A and adsorbed onto a silica gel column (27 g, 2.0 × 27 cm) which was eluted with the same solvent mixture. Fractions showing a TLC spot at R_f 0.51 (silica gel, system A) were pooled, evaporated to dryness, redissolved in 97.5-2.5 $CHCl_3$-MeOH, and applied onto a second identical column, which was eluted first with the same solvent mixture and then with 95:5 $CHCl_3$-MeOH. Fractions giving a TLC spot with R_f 0.30 (silica gel, system D) were pooled, evaporated, and taken up in $CHCl_3$. The $CHCl_3$ solution was added in a fine stream to excess Et_2O, and the precipitated solid was collected and dried in vacuo over P_2O_5 at 80 °C to obtain the *ammonium salt* as a yellow powder (256 mg, 39% yield): mp 150-151 °C; IR ν 3330 (NH_2), 1710 (ester C=O), 1650-1600 (acid and amide C=O) cm^{-1}. Anal. ($C_{31}H_{35}N_9O_6 \cdot NH_3 \cdot 0.5H_2O$) C, H, N.

Methotrexate γ-(*m*-Carboxyanilide) (3). A solution of **14** (240 mg, 0.366 mmol) in trifluoroacetic acid (1.5 mL) was obtained by brief sonication, kept at 0 °C for 1.5 h, and then added dropwise with stirring to Et_2O (75 mL). The solid was filtered, washed with Et_2O, and dried in vacuo at room temperature. The resultant yellow powder was suspended in H_2O, and enough concentrated NH_4OH and MeOH were added to obtain a clear solution. The solution was filtered, most of the MeOH was removed by rotary evaporation, and 10% AcOH was added until a pH of 4.1 was reached. After storage at 0 °C, the solid was collected, washed with H_2O, and dried on a lyophilizer and then in vacuo at 80 °C over P_2O_5 to obtain a yellow powder (140 mg, 65% yield): mp 197-200 °C; TLC R_f 0.23 (silica gel, system B); IR ν 3400 (NH_2), 1705, 1640, 1610 (acid and amide C=O) cm^{-1}. Anal. ($C_{27}H_{27}N_9O_6 \cdot 0.8H_2O$) C, H, N.

Methotrexate α-*tert*-Butyl Ester γ-(*m*-Boronoanilide) (15). *m*-Aminophenylboronic acid monohydrate (74 mg, 0.48 mmol) was added to a solution of **13** (204 mg, 0.4 mmol) in dry DMF (8 mL) under nitrogen. The solution was cooled to 0 °C and Et_3N (0.18 μL, 1.8 mmol) was added, followed by DPPA (242 mg, 0.88 mmol). The solution was kept at 0 °C for 6 h, and then left at room temperature for 65 h. After solvent evaporation under reduced pressure, the residue was dissolved in 10:6:1 $CHCl_3$-MeOH-28% NH_4OH (5 mL) and the solution applied onto a silica gel column (13 g, 1.5 × 22 cm), which was eluted with the same solvent mixture. Fractions giving a TLC spot with R_f 0.42 (silica gel, system C) were combined and evaporated to dryness. The residue was taken up in MeOH and Et_2O was added until a solid precipitated. Filtration and drying in vacuo at 60 °C over P_2O_5 gave a yellow powder (143 mg, 53%): mp > 300 °C; IR ν 3410, 1730 (ester C=O), 1640-1610 cm^{-1}. Anal. ($C_{30}H_{36}BN_9O_6 \cdot 2.4H_2O$) C, H, B, N.

Methotrexate γ-(*m*-Bromoanilide) (4). A mixture of **15** (50 mg, 0.074 mmol) and TFA (1 mL) was stirred at 0 °C for 5 h. A solution formed within 10 min. The reaction was monitored by TLC (silica gel, system C) for the disappearance of **15** (R_f 0.35) and formation of **4** (R_f 0.06). The solution was added with stirring to Et_2O (50 mL), and the precipitated solid was filtered and dried in vacuo overnight at 80 °C over P_2O_5 to obtain a yellow powder (50 mg, 92%): mp ca. 270 °C (charring); IR ν 3430, 2940, 1645, 1610 cm^{-1}. Anal. ($C_{26}H_{28}BN_9O_6 \cdot 1.2CF_3CO_2H \cdot 1.25H_2O$) C, H, B, F, N.

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Registry No. 1, 113857-87-7; **2** (free base), 130799-21-2; **2**·NH_3, 130829-30-0; **3**, 130799-22-3; **4** (free base), 130799-23-4; **4**·TFA, 130799-32-5; **5**, 89043-75-4; **6**·HCl, 34582-33-7; **7**, 130799-24-5; **8**, 130799-25-6; **9**, 95484-99-4; **10**·$^1/_2$HOAC, 130799-27-8; **10**·TFA, 130799-33-6; **11**, 130799-28-9; **12**, 130799-29-0; **13**, 79640-70-3; **14**, 130799-30-3; **15**, 130799-31-4; DHFR, 9002-03-3; 3-$H_2NC_6H_4COOEt$, 582-33-2; 3-$H_2NC_6H_4B(OH)_2$, 30418-59-8.