Plasmodium berghei. All the untreated infected animals, which serve as controls, die after 6–8 days and with a mean survival time of 6.2 days. Every compound is tested at several dose levels. At each level, the candidate drug is given subcutaneously in a single dose as a peanut oil suspension to five mice 72 h after they are infected. The compounds are judged to be "toxic" if the infected mice die before the 6th day, i.e., before the time when the untreated mice begin to die; "active" if the mean survival time of the mice is at least doubled; and "curative" if the mice survive 60 days postinfection. Details of the test procedure were given by Osdene, Russell, and Rane.[31]

Acknowledgment. We thank David H. Jun, Robert L. Runkle, and Dr. Thomas S. Woods for synthesizing several of the compounds reported here and Dr. Thomas R. Sweeney and Col. Craig J. Canfield for interest and encouragement throughout this investigation. We are also grateful to Col. David E. Davidson for useful discussions regarding the biological data.

Supplementary Material Available: Table V, infrared spectral correlation of 2-acetylpyridine 4-monosubstituted 3-thiosemicarbazones in KBr pellets, and Table VI, NMR spectral correlation of 2-acetylpyridine 4-monosubstituted 3-thiosemicarbazones and related compounds in $CDCl_3$ solution (2 pages). Ordering information is given on any current masthead page.

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Analogues of Methotrexate

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Analogues of methotrexate (MTX) were prepared by alkylation of side-chain precursors with 6-(bromomethyl)-2,4-pteridinediamine followed, where necessary, by saponification of the intermediate esters and, in two cases, by electrophilic substitution reactions in the pyridine ring portion of 3-deazamethotrexate. Effects of the various modifications on their ability to inhibit dihydrofolate reductase, cytotoxicity, and activity against L1210 leukemia in mice were examined in light of recent findings concerning active transport of MTX and related compounds and the binding features of the MTX–dihydrofolate reductase complex.

Methotrexate (MTX, 1) is perhaps the most useful *antimetabolite* presently employed in the treatment of cancer,[2] but attempts to improve the clinical activity of this agent by congener synthesis have not been successful.

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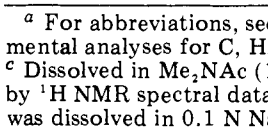
Table I. Reaction of 6-(Bromomethyl)-2,4-pteridinediamine Hydrobromide (3) with Side-Chain Precursors

precursor[a]	molar ratio of precursor/3	vol of Me_2NAc, mL/mmol of 3	react. time, days[b]	purif proced[c]	product % yield	product no.	product molec form.[d]
4c	1.2	5	3	A-1	14	**26**	$C_{21}H_{24}N_8O_5 \cdot H_2O$
5b	3	3.8	0.75	A-2	72	**29**	$C_{20}H_{22}N_8O_3 \cdot H_2O$
6b	1.1	5	4	A-1	37	**30**	$C_{19}H_{20}N_8O_5 \cdot 0.5H_2O$
7c	3	3.8	1	A-1	38	**31**	$C_{21}H_{24}N_8O_5 \cdot 2H_2O$
8c	2[e]	10.3	0.8	B-1	12	24	$C_{20}H_{30}N_8O_5 \cdot 0.5H_2O$[f]
9e	1.1	4	0.33[g]	A-3	66	**22**	$C_{25}H_{32}N_8O_5 \cdot 0.7EtOH \cdot 0.3H_2O$
10	1.1	5	0.75[g]	A-4	67	**20**	$C_{30}H_{34}N_8O_5$
11	1.1	5	0.75[g]	A-4	56	**21**	$C_{31}H_{36}N_8O_5$
12f	1.1[h]	3	5	A-5	68	19	$C_{25}H_{32}N_8O_5 \cdot 0.5H_2O$
HO-C_6H_4-COGluEt (**13**)[i]	2[j]	8.1	8	A-2	70	**18**	$C_{23}H_{27}N_7O_6 \cdot 0.5H_2O$
H_2N-C_5H_3N-COGluEt (**14**)[k]	1	4.2	7	B-2		23	$C_{22}H_{27}N_9O_5$[l]
H_2N-C_6H_4-SO_2Glu (**15**)[m]	1.1	6.4[n]	6	A-6	16	**32**	$C_{18}H_{18}MgN_8O_6S \cdot 7H_2O$[o]
H_2N-C_6H_4-COGly (**16**)[p]	1.1	3.3	4	C-1	55	**28**	$C_{16}H_{16}N_8O_3 \cdot 1.5H_2O$
H_2N-C_6H_4-$CONH(CH_2)_3$-CO_2H (**17**)[q,r]	3	6.7	2.5	C-2	63	**27**	$C_{18}H_{20}N_8O_3 \cdot HBr \cdot 0.25Me_2NAc \cdot 0.6H_2O$

[a] Abbreviations used are: Glu, $NHCH(CO_2H)(CH_2)_2CO_2H$; GluEt, $NHCH(CO_2Et)(CH_2)_2CO_2Et$; Gly, $NHCH_2CO_2H$. [b] At room temperature, except where noted. [c] See Experimental Section. [d] Elemental analyses for C, H, and N were obtained for each compound, except **23**. Results were within ±0.4% of calculated values, except for H on **31**: calcd, 5.59; found, 5.00. [e] Plus 4 equiv of KOC_4H_9-*t*. [f] Isolated as the dimethyl ester as a result of ester exchange. [g] 52 °C. [h] Et_3N (3 equiv) added to a solution of 12f·HBr before 3. [i] E. P. Fairburn, B. J. Magerlein, L. Stubberfield, E. Stapert, and D. I. Weisblat, *J. Am. Chem. Soc.*, **76**, 676 (1954). [j] Phenoxide ion generated with NaH equimolar with 13 prior to addition of 3. [k] Reference 35. [l] Not analyzed; used directly for conversion to 34 (see Table II). [m] Wagner-Jauregg and W.-H. Wagner, *Z. Naturforsch*, **1**, 229 (1946). [n] Hexamethylphosphoric triamide was used instead of Me_2NAc, apparently to no advantage. [o] Mg: calcd, 3.89; found, 3.64. [p] Aldrich Chemical Co., Inc. [q] B. R. Baker, D. V. Santi, P. I. Almaula, and W. C. Werkheiser, *J. Med. Chem.*, **7**, 24 (1964). [r] Nitro compound reduced with 5% Pd/C, and amine used without purification.

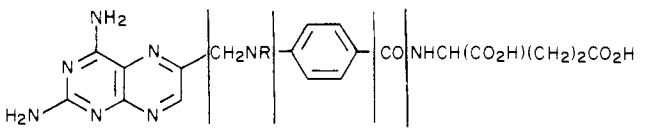
Table II. Ester Saponification

ester[a]	amt, g (mmol)	EtOH, mL	1 N NaOH, mL	time, h[j]	product % yield	product no.	product molec form.[b]
DaptCH_2O-C_6H_4-COGluEt (**18**)	1 (2)	c	c	19	72	40	$C_{19}H_{19}N_7O_6 \cdot H_2O$
DaptCH_2N(Me)CH_2-C_6H_4-COGluEt (19)	1.8 (3.4)	120	12	22	74	39	$C_{21}H_{22}Na_2N_8O_5 \cdot 0.2EtOH \cdot 3H_2O$[d]
DaptCH_2N(Bn)-C_6H_4-COGluEt (**20**)	0.59 (1)	100	2.4	20	94	37	$C_{26}H_{26}N_8O_5 \cdot H_2O$
DaptCH_2N(Phe)-C_6H_4-COGluEt (21)	0.60 (1)	100	2.4	20	95	38	$C_{27}H_{26}N_8O_5 \cdot 1.3H_2O$
DaptCH_2N(Et)-C_6H_4-COGluEt (**22**)	0.50 (0.9)	90	2.2	20	91	36	$C_{21}H_{24}N_8O_5 \cdot H_2O$
DaptCH_2NH-C_5H_3N-COGluMe (**23**)	c	200[f]	48	6	11[g]	34	$C_{18}H_{19}N_9O_5 \cdot H_2O$
DaptCH_2N(Me)$(CH_2)_4$COGluMe (24)	0.54 (1.2)	h	h	4	89	35	$C_{18}H_{26}N_8O_5 \cdot 2HCl \cdot 1.5H_2O$[i]
	0.17 (0.3)	30	0.7	20	93	33	$C_{21}H_{22}Cl_2N_8O_5 \cdot 0.9H_2O$

[a] For abbreviations, see Table I, footnote *a*; GluMe, $NHCH(CO_2Me)(CH_2)_2CO_2Me$; Dapt, 2,4-diamino-6-pteridinyl. [b] Elemental analyses for C, H, and N were within ±0.4% of calculated values, except for H on 39: calcd, 5.11; found, 4.48. [c] Dissolved in Me_2NAc (15 mL) and the solution was treated with 0.1 N NaOH (40 mL). [d] Solvation by EtOH confirmed by ^1H NMR spectral data. [e] Crude **23** (see Table I) was used directly. [f] Plus 230 mL of H_2O. [g] Yield from 3. [h] The ester was dissolved in 0.1 N NaOH (92 mL). [i] Cl: calcd, 13.27; found, 13.54. [j] 25–30 °C.

3′,5′-Dichloromethotrexate is superior to MTX against leukemia L1210 in mice,[3] but this advantage may be too small to detect in the treatment of human disease.[4] Aminopterin (**2**), differing only by lack of a methyl group at N^{10}, appeared to be more toxic than MTX[5] and, consequently, was dropped from clinical use. Recent studies with neoplasms in rodents have shown that **2** is better transported, by an energy-dependent mechanism, into gut cells of the mouse than MTX but not into tumor cells.[6] Such a difference could explain the greater clinical toxicity of **2** and the recent failure of citrovorum factor to reverse its toxicity as effectively as it does the toxicity of MTX.[7] The importance of these differences in active transport—both influx and efflux—of analogues of MTX has only recently been established[6,8,9] and provides a new approach to maximize the selective toxicity of dihydrofolate reductase inhibitors. Thus, the purpose of this study was to prepare effective inhibitors of dihydrofolate reductase with altered transport characteristics aimed at selective transport into tumor cells.[8,9]

Since much less is known about the binding of analogues to the active-transport mechanism than about binding to the enzyme, the only approach open was to systematically modify the structure of MTX and assess the results of these structural modifications. For this purpose, alterations were made in five segments (see structures **1** and **2**)

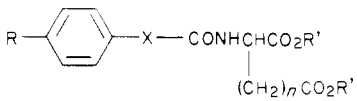


1, R = Me
2, R = H

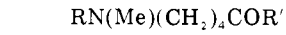
of the parent structure.

Analogues bearing the (2,4-diamino-6-pteridinyl)methyl

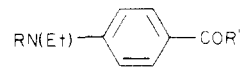
Chart I



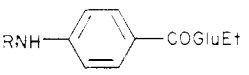
	R	X	n	R'
4a	NO_2		4	Me
b	NO_2		4	H
c	NH_2		4	H
5a	NO_2	CH_2	2	H
b	NH_2	CH_2	2	H
6a	NO_2	CH_2	1	H
b	NH_2	CH_2	1	H
7a	NO_2	CH=CH	2	Et
b	NO_2	CH=CH	2	H
c	NH_2	CH_2CH_2	2	H



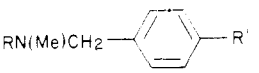
	R	R'
8a	Ts	OH
b	Ts	GluEt
c	H	GluEt



	R	R'
9a	Ts	OEt
b	Ts	OH
c	Ts	Cl
d	Ts	GluEt
e	H	GluEt



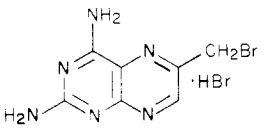
	R
10	$C_6H_5CH_2$
11	$C_6H_5(CH_2)_2$



	R	R'
12a	Ts	CN
b	Ts	CO_2Me
c	Ts	CO_2H
d	Ts	COCl
e	Ts	COGluEt
f	H	COGluEt

Ts = 4-$MeC_6H_4SO_2$-

grouping attached to varied side chains (see Table I) were prepared by alkylation of the corresponding precursors with 6-(bromomethyl)-2,4-pteridinediamine hydrobromide (**3**) under conditions similar to those used in improved

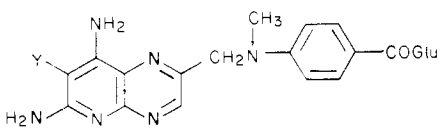


3

syntheses of **1** and **2** based on **3**.[10] Most of the side-chain precursors were synthesized by standard routes that involved coupling appropriate carboxylic acids with amino acids or esters of amino acids, followed by deprotection of tosylated alkylamino groups or reduction of nitro groups. Exceptions were compounds **10** and **11** (see Chart I), which were prepared by reductive amination[11] of the appropriate aldehydes with diethyl *N*-(4-aminobenzoyl)-L-glutamate. The remaining precursors were obtained from a commercial source or prepared by reported procedures, as indicated in the footnotes to Table I.

Related compounds bearing side chains devoid of a terminal amino acid grouping have been prepared in similar fashion from **3**.[12] Other investigators using different synthetic approaches have prepared MTX analogues with varied side chains.[13] A lengthy synthesis of 10-oxaaminopterin (**40**) was described[13c] soon after a preliminary report of its synthesis from **3**.[14]

Bromination and nitration of 3-deazamethotrexate[15] (**41**)



41, Y = H
42, Y = Br
43, Y = NO_2

gave the respective products **42** and **43** whose structural assignments are supported by two features in their ^1H NMR spectra: (1) disappearance of the singlet assigned to the pyridine ring proton of **41**[15] and (2) retention of the 1,4-disubstituted phenylene spin pattern.

Biologic Data. It is generally accepted that the anticancer activity of MTX and its analogues is due primarily to its tight binding to dihydrofolate reductase.[16] For this reason, all of the analogues described herein were assayed for their ability to inhibit this enzyme. Although the source of the enzyme used in these studies was pigeon liver,[17] there is generally good agreement between results obtained with the enzyme from this source and from leukemia L1210 cells with a number of other analogues of MTX.[18,19]

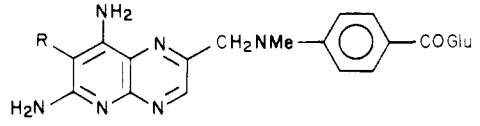
The cytotoxicity of these compounds to KB cells in culture was also determined. The results obtained in this test will depend not only on the ability of a compound to inhibit the enzyme but also on its ability to pass the cell membrane by either active transport or passive diffusion. Compounds highly ionized at physiologic pH that are not substrates for the active-transport mechanism seem likely to enter cells poorly by passive diffusion, judging from results with nucleotides[20] and certain carboxylic acids[21] for which there are no active-transport mechanisms. Thus, a good enzyme inhibitor may show little cytotoxicity.[22]

Finally, the compounds were evaluated for their ability to prolong the life of mice injected intraperitoneally with 10^5 L1210 leukemia cells. Activity in this test system may depend on several factors: (1) relative ability to inhibit dihydrofolate reductase of L1210 cells and normal mouse cells; (2) relative ability to enter L1210 cells and normal mouse cells—particularly bone marrow stem cells and crypt cells of the gut; (3) drug distribution in the mouse; and (4) drug metabolism. Selective toxicity may depend on all these factors, although work to date would indicate little difference in the active site of the reductase isolated from leukemic and normal cells.[23] On the other hand, there are favorable differences in transport of MTX and some of its analogues into L1210 cells compared to mouse gut epithelial cells.[6]

The data in Table III show, in this series of compounds, a good correlation between the I_{50} for the inhibition of dihydrofolate reductase, cell culture cytotoxicity, and activity against leukemia L1210 in the mouse. All of the compounds within one order of magnitude of MTX with respect to their I_{50} for enzyme inhibition were also within one order of magnitude with respect to their cytotoxicity and gave >50% increase in life span of leukemic mice. With one exception (**39**), compounds with an I_{50} for enzyme inhibition more than one order of magnitude larger than that of MTX showed no cytotoxicity at the highest level tested (100 μg/mL) or had a MTX ratio of around 1000 or greater. Of these latter compounds, only four showed any activity against leukemia L1210 and these were all marginal. Thus, ability to inhibit the enzyme is a good predictor of biologic activity, although transport, or lack of it, may also be a factor in the cytotoxicity of some of these analogues (e.g., **28**, **29**, **31**, and **38**).

All of the analogues that are highly inhibitory (**26**, **33**, **34**, **36**, and **41**) are very closely related structurally to MTX and aminopterin. A number of changes at the 10 position are well tolerated, although the steric consequences of the benzyl group at N-10 (**37**) are apparent. Removal of the phenyl group by an additional methylene group (**38**) restores enzyme inhibition to a large extent and results in some cytotoxicity but not L1210 activity. Minor changes in the benzene ring, such as chlorination (**33**) or substitution of the pyridine ring for it (**34**), are well tolerated,

Table III. Biologic Data

no.	DaptCH$_2$NH—C$_6$H$_4$—CONHR[a] R	inhibn of DHFR[b] I_{50}, μM	inhibn of DHFR[b] MTX ratio[c]	cytotox to KB cells[d] ED_{50}, μg/mL	cytotox to KB cells[d] MTX ratio	inhibn of leukemia L1210[e] daily dose, mg/kg	inhibn of leukemia L1210[e] % ILS
2	$CH(CO_2H)(CH_2)_2CO_2H$	0.026	1	0.003	0.75	0.67	74
26	$CH(CO_2H)(CH_2)_4CO_2H$	0.013	0.5	0.025	6.3	20	57
27	$(CH_2)_3CO_2H$	0.38	15	>100		25[f]	I
28	CH_2CO_2H	1.1	42	88	22 000	80	I
	DaptCH$_2$NH—C$_6$H$_4$—R R						
	R	0.38	15	41	10 000	500	35
29	CH_2COGlu	1.0	38	>100		200	29
30	CH_2COAsp	0.46	33	37	9 300	500	I
31	$(CH_2)_2COGlu$	1.1	39	45	11 000	100	27
32	SO_2Glu						
	DaptCH$_2$NR$_1$R$_2$ R$_1$ R$_2$						
33	Et	0.050	1.1	0.022	5.5	100	70
34	H	0.030	1.2	0.006	1.5	0.44	69
35	Me -$(CH_2)_4COGlu$	20	770	>100		80	I
	DaptCH$_2$X—C$_6$H$_4$—COGlu[a] X						
2	-NH-	0.026	1	0.003	0.75	0.67	74
1	-NMe-	0.026	1	0.004	1	1.5	72
36	-NEt-	0.045	1	0.005	1.3	10	117
37	-NBn-	14	310	>100		80	I
38	-NPhe-	0.28	11	26	6 500	80	I
39	$-NMeCH_2-$	0.75	29	0.043	11	50	36
40	-O-	0.51	20	3.5	875	1.3	32
	 R						
41	H	0.063	4.8	0.05[g]	13	100	64
42	Br	2.5	190	76	19 000	200[h]	I
43	NO_2	38	1400	>100		200[h]	I

[a] For abbreviations, see footnote *a* of both Tables I and II. [b] Dihydrofolate reductase from pigeon liver assayed by the procedure of Baker [*J. Heterocycl. Chem.*, 1, 88 (1964)]. [c] The I_{50} for MTX varied from 0.014 to 0.045 μM. [d] Procedure described by R. I. Geran, N. H. Greenberg, M. M. Macdonald, A. M. Shumacher, and B. J. Abbott, *Cancer Chemother. Rep., Part 3*, 3 (2), 59 (1972). [e] 10^5 cells, ip. Treatment ip, qd 1-9. The dose given is the optimal dose based on maximum percent increase in lifespan (ILS), except for inactive (I) compounds in which case the maximum nontoxic dose tested is given. [f] Highest nontoxic dose. [g] H. Ep.-2 cells. [h] Days 2 and 6 only.

but substitution of an aliphatic group of about the same length (in the extended or staggered conformation) (**35**) results in a great loss of activity (against the enzyme). Since X-ray data[24] show that the aromatic ring of the *p*-aminobenzoyl portion of MTX resides in a hydrophobic pocket, this loss may be in part due to the somewhat greater hydrophobic binding of the planar benzene moiety,[25] although folding or balling of the side chain[26] may be a more important factor. Extension of the length of the molecule by inclusion of one or two methylene groups on either side of the benzene ring (**29**, **31**, and **39**) decreases binding by somewhat more than one order of magnitude, as does substitution of monocarboxylic acids for glutamic acid (**27** and **28**). Extension of the chain between the two carboxyls of the glutamate moiety (**26**), however, apparently results in a slight increase in binding but somewhat lower cytotoxicity, perhaps a reflection of poorer transport. The antileukemic activity is not significantly affected.

It has been proposed, on the basis of X-ray crystallographic studies of the methotrexate–dihydrofolate reductase complex from two bacterial sources,[24,27] that a hydrogen bond between an aspartic acid carboxyl and N-1 of the inhibitor contributes greatly to the inhibitor's tight binding. Support for this position is provided by the binding and biologic activity of 3-deazamethotrexate (**41**) relative to 1-deazamethotrexate, since the 3-deazamethotrexate (**41**) closely resembles MTX itself, whereas the 1-deaza analogue is a poor inhibitor of the enzyme with little biologic activity.[28] Further support is provided herein by the incremental changes in binding, and cytotoxicity, caused by bromination (**42**) and nitration (**43**) of 3-deazamethotrexate. Although the steric factor cannot be evaluated, the relative effect of these two groups on basicity (pK_a values for **42**, **43**, and MTX are respectively

5.1, 4.3, and 5.5[29]) is reflected in the decreased binding and cytotoxicity of the analogues.

None of these analogues appears to be a significantly better inhibitor of dihydrofolate reductase (from pigeon liver) than MTX or therapeutically superior to it in the L1210 system, although several appear to be equivalent (**26**, **33**, **34**, **36**, and **41**).

Experimental Section

^{1}H NMR (determined in Me_2SO-d_6 with a Varian XL-100-15 spectrometer) and UV spectra (determined in 0.1 N HCl, pH 7, buffer, and 0.1 N NaOH with a Cary 17 spectrophotometer) obtained for all of the compounds listed in Tables I and II were consistent with assigned structures. Analytical results indicated by element symbols were within ±0.4% of the theoretical values. Spectral determinations and some of the elemental analyses were performed in the Molecular Spectroscopy Section of Southern Research Institute under the direction of Dr. W. C. Coburn, Jr. Elemental analyses were also performed by Galbraith Laboratories, Knoxville, Tenn. Examinations of intermediates (including esters of final products) by TLC were performed on Analtech precoated (250 μm) silica gel G(F) plates. Examinations of final products listed in Tables I and II by TLC were performed on DEAE-cellulose sheets (Bakerflex) using 0.5 M NaCl, 0.2 M mercaptoethanol, 0.005 M KH_2PO_4 buffer solution at pH 7.0. Unless other conditions are specified, evaporations were performed with a rotary evaporator and a water aspirator, and products were dried in vacuo (<1 mm) at room temperature.

Dimethyl 2-(4-Nitrobenzamido)heptanedioate (4a). A solution of DL-2-aminoheptanedioic acid (2.55 g, 14.5 mmol) in MeOH saturated with dry HCl was kept 16 h at 25 °C, refluxed for 6 h, and then evaporated. Solutions of the residue in $CHCl_3$ were repeatedly evaporated to dryness, and the oily ester hydrochloride that remained was dissolved in pyridine (50 mL) along with 4-nitrobenzoyl chloride (5.38 g, 29.0 mmol). The mixture was stirred for 64 h at 25 °C and then for 2 h at 70–75 °C, cooled, and treated with MeOH (10 mL). After 20 h at 25 °C, the mixture was evaporated. A suspension of the solid residue in EtOAc was washed with 1 N HCl, saturated $NaHCO_3$ solution, and H_2O. The dried (Na_2SO_4) EtOAc solution was evaporated to give a gum which was dissolved in $CHCl_3$ and applied to a silica gel column (175 g, Merck silica gel H). Elution with $CHCl_3$–MeOH (199:1) led to pure **4a**, mp 69 °C, in 74% yield (3.79 g). Anal. ($C_{16}H_{20}N_2O_7$) C, H, H.

2-(4-Aminobenzamido)heptanedioic Acid (4c). A solution of **4a** (3.37 g, 9.56 mmol) in MeOH (40 mL) was combined with 0.5 N NaOH (153 mL). After 4 h at 25 °C, the solution was concentrated by evaporation to 100 mL. Acidification to pH 3.5 followed by refrigeration gave a first crop (1.56 g) of 2-(4-nitrobenzamido)heptanedioic acid (**4b**), mp 232–234 °C dec. Concentration of the filtrate led to two additional crops (0.89 and 0.31 g), each with mp 232–234 °C dec.

Reduction of **4b** (2.70 g) was carried out in H_2O (200 mL) at ambient conditions using 5% Pd on C (300 mg) as catalyst. After H_2 consumption (610 mL) had ceased, the mixture was filtered, and the filtrate was evaporated to dryness to give **4c** (2.34 g, 85% overall yield) as a colorless syrup homogeneous by TLC (*n*-BuOH–AcOH–H_2O, 5:2:3) and with ^{1}H NMR spectrum consistent with that expected. This material was used directly for conversion to **26**.

***N*-[(4-Nitrophenyl)acetyl]-L-glutamic acid (5a)**, mp 141–142 °C, was prepared from 4-nitrophenylacetyl chloride[30] and L-glutamic acid by an adaptation of the method used to prepare its 4-nitrobenzoyl analogue.[31] Anal. ($C_{13}H_{14}N_2O_7 \cdot 0.5H_2O$) C, H, N.

***N*-[(4-Aminophenyl)acetyl]-L-glutamic Acid (5b).** Catalytic reduction (at 40 psi) of **5a** (20 g) suspended in H_2O (200 mL) with 5% Pd on C (4 g) gave pure **5b**, mp 190–191 °C, in 74% yield (13.4 g) after filtration and concentration. Anal. ($C_{13}H_{16}N_2O_5$) C, H, N.

***N*-[(4-Aminophenyl)acetyl]-L-aspartic Acid (6b).** Pulverized 4-nitrophenylacetyl chloride[30] (20 g, 0.10 mol) was added to a solution of L-aspartic acid (12.0 g, 0.090 mol) in 1.15 N NaOH (235 mL) also containing Na_2CO_3 (19.1 g) at 10 °C. The stirred mixture was warmed to 50 °C, kept at 40–50 °C for 2 h, then cooled, and extracted with Et_2O. The aqueous phase was filtered and acidified to pH 4 to give **6a** (7.24 g, 24% yield). Part (5.65 g) of this material was reduced catalytically (Raney Ni) in H_2O (200 mL) at ambient conditions. After H_2 uptake had ceased (1.22 L), the catalyst was filtered off, and the solution was lyophilized to give 4.27 g of solid whose ^{1}H NMR spectrum was as expected for **6b**. This material was used without further purification for conversion to **30**.

Diethyl *N*-(4-Nitrocinnamoyl)-L-glutamate (7a). A mixture of 142 mmol each of 4-nitrocinnamoyl chloride[32] (30.0 g) and diethyl L-glutamate hydrochloride (34.2 g) in dry pyridine (400 mL) was stirred for 1 h at 25–30 °C and then for 2 h at 80–90 °C. The solution was evaporated, and the residue was stirred with warm C_6H_6 (750 mL). Pyridine hydrochloride was removed by filtration, and the C_6H_6 solution was washed successively with H_2O, saturated $NaHCO_3$ solution, and H_2O, then dried (Na_2SO_4), clarified (Norit, Celite), and evaporated to give **7a**, mp 117–118 °C, in 88% yield (47.2 g).

***N*-(4-Nitrocinnamoyl)-L-glutamic Acid (7b).** A mixture of **7a** (25.0 g, 66.1 mmol), EtOH (100 mL), and 1 N NaOH (300 mL) was stirred for 48 h at 25 °C. The filtered (Norit, Celite) solution was neutralized by the addition of HCl (to pH 7.5) and evaporated to dryness in vacuo (bath to 40 °C). The residue was dissolved in H_2O (150 mL), and addition of HCl to pH 3.5 gave a granular solid. The collected solid was suspended in H_2O (100 mL), redissolved by the addition of 50% NaOH to pH 7.5, and reprecipitated by the addition of HCl to pH 3.5 to give pure **7b**, mp 194–195 °C, in 80% yield (17.4 g, dried in vacuo at 65 °C over P_2O_5). Anal. ($C_{14}H_{14}N_2O_7 \cdot 0.5H_2O$) C, H, N.

***N*-(4-Aminohydrocinnamoyl)-L-glutamic Acid (7c).** Hydrogenation of **7b** (14.9 g) in H_2O (300 mL) in the presence of 5% Pd on C (1.0 g) at approximately 40 psi (Parr shaker) was complete within 1.5 h. Evaporation of the filtered solution gave **7c** as a solid, which was pulverized under C_6H_6 before being collected and dried in vacuo (65 °C, P_2O_5): yield 97% (13.2 g); mp 168–169 °C. Anal. ($C_{14}H_{18}N_2O_5 \cdot 0.5H_2O$) C, N; H: calcd, 6.31; found, 5.76.

5-[[(4-Methylphenyl)sulfonyl]methylamino]pentanoic Acid (8a). Me_2SO_4 (54.0 g, 0.428 mol) was added dropwise to a stirred solution of 5-[[(4-methylphenyl)sulfonyl]amino]pentanoic acid[33] (58.0 g, 0.214 mol) in 3 N NaOH (428 mL) at 65–75 °C. The stirred mixture was kept at 65–75 °C for 2 h, then at 25 °C for 16 h. Excess Me_2SO_4 was removed by extraction with Et_2O, and the clear aqueous phase was acidified to give **8a** as an oily precipitate which was extracted with Et_2O. Evaporation of the H_2O-washed and dried (Na_2SO_4) Et_2O solution gave **8a** as an oil that eventually solidified, mp 68–72 °C; yield 95% (57.8 g). Anal. ($C_{13}H_{19}NO_4S$) H, N; C: calcd, 54.72; found, 54.25.

Diethyl *N*-[5-[[(4-Methylphenyl)sulfonyl]methylamino]pentanoyl]-L-glutamate (8b). A stirred mixture of **8a** (9.4 g, 33 mmol) and diethyl L-glutamate hydrochloride (7.9 g, 33 mmol) in CH_2Cl_2 (60 mL) was treated with *N*,*N*′-dicyclohexylcarbodiimide (DCC; 3.4 g, 16 mmol) and Et_3N (3.3 g, 33 mmol). Addition of another 3.4-g portion of DCC caused an exothermic reaction, and the mixture was cooled externally to 25 °C and then left overnight. Precipitated *N*,*N*′-dicyclohexylurea was removed by filtration, and the filtrate was washed successively with 1 N HCl, saturated $NaHCO_3$ solution, and H_2O, then dried (Na_2SO_4), and evaporated. The residue was dissolved in $CHCl_3$, and a small amount of *N*,*N*′-dicyclohexylurea was removed by filtration. Evaporation of the filtrate gave an oil which was subjected to column chromatography (500 g, Merck silica gel H) using $CHCl_3$ as the initial eluant followed by $CHCl_3$–MeOH (99:1). Fractions homogeneous by TLC were combined and evaporated to give **8b** as an oil (^{1}H NMR spectrum as expected) in 49% yield (7.53 g). Anal. ($C_{22}H_{34}N_2O_7S \cdot 0.1CHCl_3$) C, H, N.

Diethyl *N*-[5-(Methylamino)pentanoyl]-L-glutamate (8c) Hydrobromide. A solution of **8b** (14.5 g, 31.0 mmol) in 30% dry HBr–AcOH solution (150 mL) containing phenol (11.7 g, 124 mmol) was kept at 25 °C for 65 h. Addition of Et_2O (1.5 L) caused precipitation of crude **8c**·HBr as a viscous oil. After several hours, the clear ethereal solution was removed by decantation. After two reprecipitations from EtOH (50–75 mL) solution by addition of Et_2O (1.5 L), the gummy product was again dissolved in EtOH, and the solution was evaporated in vacuo (final conditions, <1 mm at 25 °C) to give **8c**·HBr as an oil containing Et_2O (molar

Table IV

no.	yield, %	mp, °C	molec formula	anal.
9a	94	126	$C_{18}H_{21}NO_4S$	C, H, N
9b	97	181	$C_{16}H_{17}NO_4S$	C, H, N
9c		oil[a]	$C_{16}H_{16}ClNO_3S$	
9d		oil[a]	$C_{25}H_{32}N_2O_7S$	
9e	40[b]	73	$C_{18}H_{26}N_2O_5$	C, H, N

[a] Used directly in succeeding step. [b] Based on starting amount of 9b.

ratio 8c·HBr to Et_2O about 4:1 according to ^1H NMR spectral data). This material was used without further purification for conversion to **35**.

Diethyl *N*-[4-(Ethylamino)benzoyl]-L-glutamate (9e). The five-step sequence described by Santi[34] for the conversion of ethyl 4-[[(4-methylphenyl)sulfonyl]amino]benzoate to the Me homologue of **9e** proved readily adaptable. Results and data on intermediates are listed in Table IV.

Diethyl *N*-[4-[(Phenylmethyl)amino]benzoyl]-L-glutamate (10). A solution of benzaldehyde (1.97 g, 18.6 mmol) and diethyl *N*-(4-aminobenzoyl)-L-glutamate (5.73 g, 17.7 mmol) in MeOH (75 mL) containing molecular sieve (12 g, Linde 3 Å) was treated in portions during 30 min with sodium cyanoborohydride (702 mg, 11.2 mmol) while the mixture was kept near pH 6 by addition of 0.8 N dry HCl in MeOH as required. The mixture was then stirred at 25 °C for 22 h before it was filtered and diluted with Et_2O (750 mL). This solution was washed with saturated $NaHCO_3$ solution (2 × 100 mL), H_2O (100 mL), 1 N HCl (2 × 40 mL), and H_2O (2 × 50 mL). Evaporation (final conditions, <1 mm at 70 °C) gave crude **10** as an oil (6.25 g). A portion (3.25 g) of this material was purified by preparative liquid chromatography using a Waters Associates Prep LC/System 500 equipped with a 30 × 5.7 cm PrepPak 500 silica gel column and a refractive index detector. $CHCl_3$ stabilized with 1% EtOH (Burdick and Jackson Laboratories) was used as developing solvent at a flow rate of 200 mL/min. Fractions with a retention time 9–17 min were combined and evaporated to give 1.64 g of pure **10**, mp 113 °C. The amount obtained corresponds to a 43% yield. Anal. ($C_{23}H_{28}N_2O_5$) C, H, N.

Diethyl *N*-[4-[(2-Phenylethyl)amino]benzoyl]-L-glutamate (11). Reductive alkylation of diethyl *N*-(4-aminobenzoyl)-L-glutamate (5.00 g, 15.5 mmol) with phenylacetaldehyde (2.05 g, 17.1 mmol) and sodium cyanoborohydride (646 mg, 10.3 mmol) as described above for the preparation of **10** led to crude **11** as an oil (6.0 g), which was purified by column chromatography on silica gel (563 g, Mallinckrodt SilicAR TLC-7) using cyclohexane–EtOAc (1:1) as eluant: yield 44% (2.93 g) of waxy solid; mp 55–60° C. Anal. ($C_{24}H_{30}N_2O_5$) C, H, N.

4-[[[(4-Methylphenyl)sulfonyl]methylamino]methyl]benzonitrile (12a). A solution of 0.100 mol each of 4-(bromomethyl)benzonitrile (19.6 g) and *N*-methyl-4-toluenesulfonamide (18.5 g) in DMF (100 mL) was added during 1.5 h to a stirred suspension of NaH (5.00 g of 50% dispersion in oil, 0.104 mol) in DMF (25 mL) kept below 40 °C. After 40 h at 25 °C, the solution was combined with H_2O (500 mL) to give an oily precipitate that solidified while being stirred. The collected solid was washed with cyclohexane and recrystallized from MeOH to give pure **12a**, mp 108–110 °C, in 50% yield (14.9 g). Anal. ($C_{16}H_{16}N_2O_2S$) C, H, N.

Methyl 4-[[[(4-methylphenyl)sulfonyl]methylamino]methyl]benzoate (12b) was prepared from methyl 4-(bromomethyl)benzoate in a manner similar to that described above for **12a**. The yield of **12b**, mp 125–128 °C, of purity suitable for conversion to **12c** was 33%. An analytical sample, mp 129–131 °C, was obtained by recrystallization from MeOH. Anal. ($C_{17}H_{19}NO_4S$) C, H, N.

4-[[[(4-Methylphenyl)sulfonyl]methylamino]methyl]benzoic Acid (12c). A. From 12a. A solution of **12a** (1.00 g) in EtOH (15 mL) and H_2O (5 mL) containing KOH (0.86 g) was refluxed for 40 h before more H_2O (15 mL) was added, and refluxing was continued for 8 h longer. The mixture was filtered (Norit, Celite), and most of the EtOH was removed by boiling in an open flask. The solution was then diluted with hot H_2O (to 30 mL) and treated with glacial AcOH (2 mL) to give **12c**, mp 181–185 °C, in 90% yield (0.95 g). A sample recrystallized from MeOH had mp 182–184 °C, identical with that of analytically pure **12c** described below.

B. From 12b. A solution of **12b** (2.00 g) in EtOH (15 mL) and 2 N NaOH (15 mL) was refluxed for 2 h and then distilled until most of the EtOH had been removed. The remaining mixture, which contained an oily phase, was refluxed until a nearly clear solution resulted. The cooled solution was clarified (extraction with Et_2O followed by filtration) and acidified to give pure **12c**, mp 182–184 °C, in 80% yield (1.53 g). Anal. ($C_{16}H_{17}NO_4S$) C, H, N.

Diethyl *N*-[4-[(methylamino)methyl]benzoyl]-L-glutamate (12f) hydrobromide was prepared from **12c** in three steps as follows.

A. Aroyl Chloride 12d. A solution of **12c** (7.20 g, 22.6 mmol) in C_6H_6 (100 mL) containing $SOCl_2$ (8 mL) was refluxed for 2.5 h, cooled, and evaporated. Solutions of the residue in C_6H_6 were repeatedly evaporated to dryness to give **12d**, mp 115–118 °C.

B. Diethyl *N*-Aroyl-L-glutamate 12e. The sample of **12d** was dissolved along with diethyl L-glutamate hydrochloride (5.90 g, 24.6 mmol) in 1,2-dichloroethane (125 mL). This solution was stirred at about 5 °C while a solution of triethylamine (5.00 g, 49.5 mmol) in 1,2-dichloroethane (25 mL) was added during 20 min. The mixture was then stirred at 25 °C for 1.5 h before it was washed with 1 N HCl (2 × 125 mL) and H_2O (125 mL), then dried (Na_2SO_4), and evaporated to give the crude coupled product **12e** (11.5 g) as a viscous oil.

C. 12f·HBr. The oil was warmed to 35–40 °C (bath temperature) before crystalline phenol (8 g) was added. The mixture was stirred until the phenol had dissolved to give a solution which dissolved readily in 30% dry HBr–AcOH solution (60 mL). After 6 days at 25 °C, the solution was combined with Et_2O (200 mL) to give a gummy precipitate. The supernatant was removed by decantation, and the precipitate was washed once with Et_2O and then dissolved in 5:1 (v/v) EtOH–C_6H_6 (180 mL). The solution was kept at 25 °C for 24 h, refluxed for 2 h, and then distilled during 2 h until the vapor-line temperature reached 77 °C. The remaining solution was evaporated to give a solid residue (7.6 g). Recrystallization from EtOH–Et_2O gave pure **12f**·HBr, mp 124–127 °C, in 54% overall yield (5.26 g). Anal. ($C_{18}H_{26}N_2O_5$·HBr) C, H, N.

Diethyl *N*-[4-[[(2,4-Diamino-6-pteridinyl)methyl]ethylamino]-3,5-dichlorobenzoyl]-L-glutamate (25). A solution of **22** (562 mg, 1.00 mmol; see Table I) in formamide (6 mL) was treated with Cl_2 (500 mg, 7.0 mmol) during 30 min at 0–5 °C, kept for 2 h at 0 °C, diluted with H_2O (25 mL), neutralized with 1 N NaOH, and kept overnight in a refrigerator. Crude **25** (432 mg) was collected by centrifugation and purified by preparative TLC (Merck silica gel 60 F-254 PLC plates, developed with 9:1 $CHCl_3$–EtOH), followed by recrystallization from EtOH, yield 37% (219 mg). Anal. ($C_{25}H_{30}Cl_2N_8O_5$) C, H, Cl, N.

***N*-[4-[[(6,8-Diamino-7-bromopyrido[2,3-*b*]pyrazin-2-yl)methyl]methylamino]benzoyl]-L-glutamic Acid (42).** A solution of 3-deazamethotrexate (**41**;[15] 47.5 mg, 0.100 mmol) in 6 N HCl (1 mL) at 0 °C was treated dropwise with a solution of Br_2 (16.0 mg, 0.100 mmol) in glacial AcOH (0.5 mL), kept at 0 °C for 1.5 h, treated with 50% NaOH to pH 3, and kept overnight in a refrigerator. The yellow solid was collected; washed with H_2O, Me_2CO, and Et_2O; and then dried (100 °C) to give pure **42** in 84% yield (47 mg). Spectral data: UV λ_{max} ($\epsilon \times 10^{-3}$) at pH 1 223 nm (ϵ 40.4), 313 (19.2), 332 (18.7), at pH 7 221 nm (ϵ 38.1), 263 (21.2), 304 (21.8), 352 (12.5), in 0.1 N NaOH 263 nm (ϵ 22.2), 304 (21.9), 354 (12.7). Anal. ($C_{21}H_{22}BrN_7O_5$·0.7HCl) C, H, N.

***N*-[4-[[(6,8-Diamino-7-nitropyrido[2,3-*b*]pyrazin-2-yl)methyl]methylamino]benzoyl]-L-glutamic Acid (43).** A solution of 3-deazamethotrexate (**41**;[15] 95.0 mg, 0.200 mmol) in 96% H_2SO_4 (1 mL) was treated at 0 °C under N_2 with a solution of 70% HNO_3 (13.3 μL, 0.210 mmol) in 96% H_2SO_4 (1 mL), kept at 0 °C for 10 min and then at 25 °C for 30 min, and finally at 35 °C for 30 min before it was poured onto ice (about 30 g). The resulting mixture was treated with 50% NaOH to pH 3 and then refrigerated overnight. The collected solid was washed with H_2O and dried in vacuo (100 °C), yield 90% (94 mg). Spectral data: UV λ_{max} ($\epsilon \times 10^{-3}$) in 0.1 N HCl 211 nm (ϵ 38.8), 272 (20.8), 299 (26.6), at pH 7 240 nm (ϵ 23.3), 312 (30.2), 410 (sh) (3.51), in 0.1 N NaOH unstable. Anal. ($C_{21}H_{22}N_8O_7$·0.22H_2SO_4) C, H, N.

General Procedure for Reaction of 3 with Side-Chain Precursors. Quantities given in Table I of **3**, the side-chain precursor, and Me_2NAc (and in some instances an auxiliary base) were stirred for the time indicated. Products were isolated by one of the following procedures and indicated in Table I.

Purification Procedures. A. Products that did not precipitate from the reaction mixtures were caused to precipitate by the addition of H_2O, but in some instances indicated below Et_3N was added before H_2O. One of the following actions was then taken: (1) The collected precipitate was dissolved in dilute NaOH (final pH about 8), and the solution was filtered (Norit, Celite), treated with 1 N HCl to pH 6, filtered again, and then acidified to pH 4. The precipitate was then collected, washed with H_2O, and dried. (2) The precipitate was collected, washed with H_2O followed by Et_2O, and dried. (3) Et_3N (2 equiv) was added before H_2O; the collected precipitate was recrystallized from EtOH. (4) Et_3N (2 equiv) was added before H_2O; the precipitate was extracted with $CHCl_3$, and the residue from evaporation of the $CHCl_3$ was recrystallized from EtOH. (5) The collected and washed precipitate was recrystallized from EtOH–H_2O. (6) The acid was reprecipitated once from a solution of its Na salt and was then treated in hot H_2O with MgO to give the Mg salt.[35]

B. Me_2NAc was removed by evaporation (final conditions, <1 mm at 30 °C) before one of the following actions: (1) The pure dimethyl ester **24** was obtained by column chromatography on silica gel (Brinkmann H, Type 60) using $CHCl_3$–MeOH (4:1) as eluant. The precursor to **24** was a diethyl ester, but transesterification occurred during the workup. (2) The crude ester was used directly in the saponification step without further purification.

C. The product precipitated from the reaction mixture and was collected with the aid of Me_2NAc and then treated as follows: (1) Reprecipitation as in A-1 afforded the pure product. (2) The solid was washed with Et_2O and dried in vacuo (78 °C, P_2O_5).

Ester Saponification and Product Isolation. The ester in the amount given in Table II was dissolved by warming in the medium indicated, and the solution was cooled and treated with the listed volume of 1 N NaOH (exceptions were esters **18** and **24**; details are given in the footnotes to Table II). The mixture was stirred for the time given, and the product was isolated as follows.

40. The solution was filtered (Norit, Celite), and the filtrate (pH 8.2) was treated with 1 N HCl to pH 3.0. The yellow solid was collected, suspended in H_2O (5 mL), and dissolved by the addition of 0.3 N NaOH (11 mL). This solution was filtered (Norit, Celite), and pure **40** was precipitated by treatment with 5 N HCl to pH 3.0.

33 and 36–38. Adaptations of the procedure described earlier for obtaining MTX following saponification of its diethyl ester under these conditions[10] readily sufficed.

39. The precipitated disodium salt was collected, washed with EtOH and Et_2O, and dried (100 °C for 30 min, P_2O_5).

34. An isolation procedure like that indicated for **33** led to impure **34**, which was purified by column chromatography on DEAE-cellulose as described for the corresponding folic acid analogue.[36]

35. The solution was treated with 1 N HCl to pH 6, and the product was isolated by column chromatography on DEAE-cellulose using 0.02 N HCl as eluant followed by lyophilization.

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References and Notes

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