K. Patterson, Jr. (The Samuel Roberts Noble Foundation, Inc., Ardmore, OK 73401), and ASase was isolated as previously described.[31] The enzyme fraction used in the inhibition studies had a specific activity of 0.51 μmol of asparagine synthesized per milligram of protein in 30 min. L-Aspartic-^{14}C acid was incubated with L-glutamine, ASase, and other needed cofactors, and the L-asparagine-^{14}C synthesized was isolated as previously described.[31] The inhibitors were preincubated with ASase and necessary cofactors and then substrate aspartic acid was added, as outlined in paper 4 of this series.[1a]

Acknowledgment. This work was supported by grants from the American Cancer Society (CI-96) and the National Cancer Institute (CA-11714) for which the authors express their appreciation. The authors thank Dr. M.K. Patterson, Jr. (Samuel Roberts Noble Foundation), for supplying the frozen tissue from which the enzyme was isolated. The inhibition studies were performed by Mrs. Beth L. Trend, to whom the authors are grateful. Special thanks are accorded Timothy Stukus and James F. Bagaglio for preparation of starting materials.

Supplementary Material Available: Comparison infrared spectra of **2a** and **2b** (Figure 1) (1 page). Ordering information is given on any current masthead page.

Methotrexate Analogues. 13. Chemical and Pharmacological Studies on Amide, Hydrazide, and Hydroxamic Acid Derivatives of the Glutamate Side Chain

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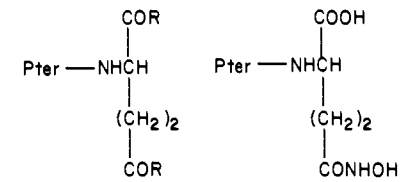
Carbodiimide-mediated condensation of 4-amino-4-deoxy-N^{10}-methylpteroic acid (APA) with several alkyl, aralkyl, and aryl amines, in the presence or absence of *N*-hydroxysuccinimide, was employed in order to prepare new lipid-soluble bis(amide) derivatives of methotrexate (MTX) as potential prodrugs. MTX dianilide was likewise prepared, in comparable yield, from APA and L-glutamic acid dianilide via the mixed carboxylic–carbonic anhydride method. Dihydrazide and bis(*N*-methylhydrazide) derivatives of MTX were formed readily from MTX diethyl ester. However, reaction with hydroxylamine led to MTX γ-monohydroxamic acid as the sole isolated product. The bis adduct appears to form, but is unstable during workup. The identity of the product was confirmed by independent mixed anhydride synthesis from APA and the γ-monohydroxamate of L-glutamic acid. Treatment of MTX dimethyl ester with *N,N*-dimethylhydrazine unexpectedly yielded MTX γ-monomethyl ester. MTX dianilide was active against L1210 leukemia in mice, with a +155% increase in life span at a dose of 160 mg/kg given ip in 10% Tween 80 on a q3d × 3 schedule. The bis(*p*-chlorobenzylamide), bis(*p*-methoxybenzylamide), and dihydrazide were also active against L1210 leukemia in vivo, but to a lesser extent than the dianilide. The γ-monohydroxamic acid derivative showed activity (+111% ILS at 40 mg/kg) similar to that of MTX and was found to bind to a partially purified dihydrofolate reductase preparation from L1210 cells with an ID_{50} of 0.005 μM as compared to 0.007 μM for MTX. In vivo experiments in mice indicated that the pharmacokinetic properties of this compound and of MTX are similar but failed to demonstrate any advantage over MTX in terms of selective uptake into tumor (sc implanted P388 leukemia) or improved penetration of the central nervous system. The activities of the dianilide, bis(benzylamide), and dihydrazide derivatives in vivo are of interest in view of their low toxicity relative to MTX against cells in culture, which suggests that these derivatives are probably acting as prodrugs in the intact animal.

Several previous reports from this laboratory have dealt with the chemical synthesis and biological evaluation of prodrug derivatives of methotrexate (4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamic acid, MTX). Classes of compounds which have been studied include diesters,[1a-e] bis(amides),[2,3] α- and γ-glutamyl conjugates,[4] and more recently a series of monoesters.[5a,b] In this paper we describe several additional examples of the amide type, whose structures (1–11) and methods of synthesis are shown in



1-11 12

Pter = 4-amino-4-deoxy-N^{10}-methylpteroyl

Table I. The monohydroxamic acid **12**, a heretofore unknown MTX analogue differing only in the replacement of the γ-COOH group by γ-CONHOH, was also prepared. Compound **12** was a good inhibitor of dihydrofolate reductase, was moderately toxic to human and mouse leukemic cells in culture, and showed in vivo antitumor activity comparable to that of MTX against L1210 leukemia in mice. Interest in this compound stemmed from the fact that, while the γ-CONHOH group is structurally very

(1) (a) A. Rosowsky, *J. Med. Chem.*, **16**, 1190 (1973); (b) G. A. Curt, J. S. Tobias, R. A. Kramer, A. Rosowsky, L. M. Parker, and M. H. N. Tattersall, *Biochem. Pharmacol.*, **25**, 1943 (1976); (c) A. Rosowsky and C.-S. Yu, in "Chemistry and Biology of Pteridines", R. L. Kisliuk and G. M. Brown, Eds., Elsevier/North Holland, New York, 1979, pp 377–381; (d) G. P. Beardsley, A. Rosowsky, R. P. McCaffrey, and H. T. Abelson, *Biochem. Pharmacol.*, **28**, 3069 (1979); (e) A. Rosowsky, H. Lazarus, G. C. Yuan, W. R. Beltz, L. Mangini, H. T. Abelson, E. J. Modest, and E. Frei III, *ibid.*, **29**, 648 (1980); (f) G. P. Beardsley and A. Rosowsky, *AACR Proc.*, **21**, 264 (1980).

(2) A. Rosowsky, W. D. Ensminger, H. Lazarus, and C.-S. Yu, *J. Med. Chem.*, **20**, 925 (1977).

(3) For related work on bis(amides) of MTX, see the following papers: (a) J. R. Piper and J. A. Montgomery, in "Chemistry and Biology of Pteridines", R. L. Kisliuk and G. M. Brown, Eds., Elsevier/North Holland, New York, 1979, pp 261–265; (b) F. M. Sirotnak, P. L. Chello, J. R. Piper, J. A. Montgomery, and J. I. DeGraw, *ibid.*, pp 597–602.

(4) A. Rosowsky and C.-S. Yu, *J. Med. Chem.*, **21**, 170 (1978).

(5) (a) A. Rosowsky, G. P. Beardsley, W. D. Ensminger, H. Lazarus, and C.-S. Yu, *J. Med. Chem.*, **21**, 380 (1978); (b) H. T. Abelson, G. P. Beardsley, W. D. Ensminger, E. J. Modest, and A. Rosowsky, *AACR Proc.*, **21**, 265 (1980).

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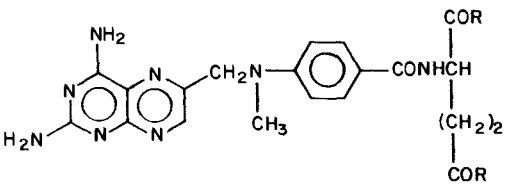
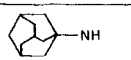
Table I. Synthesis and Purification of Methotrexate Bis(amides) and Dihydrazides



no.	R	method[a]	mol of amine; mol of MTX	col chromatogr and TLC data[b]	% yield[c]	mp, °C dec	formula[d]
1		A	0.0008; 0.0004	7 g; 100:0 (4 × 25 mL), 99:1 (4 × 25 mL), 98:2 (12 × 25 mL), 97:3 (4 × 25 mL) $CHCl_3$–MeOH; tubes 19–23; R_f 0.44 (10:1 $CHCl_3$–MeOH)	6.7	~160	$C_{40}H_{52}N_{10}O_3 \cdot 2H_2O$
2	c-$C_5H_{10}N$	A	0.0004; 0.0002	10 g; 98:2 (23 × 10 mL), 97:3 (20 × 10 mL), 96:4 (20 × 10 mL), 95:5 (20 × 20 mL), 94:6 (20 × 20 mL), CH_2Cl_2–MeOH; tubes 64–103; R_f 0.77 (1:1 CH_2Cl_2–MeOH)	35	157–185	$C_{30}H_{40}N_{10}O_3 \cdot CH_3OH$
3	4-$ClC_6H_4CH_2NH$	B	0.004; 0.002	40 g; 98:2 (50 × 20 mL), 96:4 (50 × 20 mL), 94:6 (50 × 20 mL), 92:8 (50 × 20 mL) $CHCl_3$–MeOH; tubes 61–110; R_f 0.75 (3:1 $CHCl_3$–MeOH)	62	143–151	$C_{34}H_{34}Cl_2N_{10}O_3$
4	4-$MeOC_6H_4CH_2NH$	B	0.0004; 0.0002	20 g; 99:1 (22 × 9 mL), 95:5 (77 × 9 mL) $CHCl_3$–MeOH; tubes 70–95; R_f 0.45 (6:1 $CHCl_3$–MeOH	66	130–143	$C_{36}H_{40}N_{10}O_5 \cdot H_2O$
5	$C_6H_5CH_2N(CH_3)$	A	0.004; 0.002	80 g; 100:0 (33 × 15 mL), 99:1 (33 × 15 mL), 98:2 (66 × 15 mL), 97:3 (33 × 15 mL), 96:4 (33 × 15 mL), 95:5 (99 × 15 mL);[e] tubes 237–280; R_f 0.59 (6:1 $CHCl_3$–MeOH)	67	130–135	$C_{36}H_{40}N_{10}O_3 \cdot 0.5H_2O$
6	$(C_6H_5CH_2)_2N$	A	0.0004; 0.0002	20 g; 100:0 (22 × 9 mL), 99:1 (22 × 9 mL), 98:2 (22 × 9 mL), 97:3 (22 × 9 mL), 96:4 (22 × 9 mL), 95:5 (22 × 9 mL), 94:6 (22 × 9 mL), 93:7 (22 × 9 mL), 92:8 (22 × 9 mL), 91:9 (22 × 9 mL) $CHCl_3$–MeOH; tubes 51–70; R_f 0.59 (6:1 $CHCl_3$–MeOH)	24	115–128	$C_{48}H_{48}N_{10}O_3 \cdot H_2O$
7	$C_6H_5CH(CH_3)NH$	A	0.0004; 0.0002	20 g; 100:0 (10 × 10 mL), 99:1 (10 × 10 mL), 98:2 (20 × 10 mL), 97:3 (20 × 10 mL), 96:4 (20 × 10 mL), 95:5 (20 × 10 mL), 94:6 (20 × 10 mL) $CHCl_3$–MeOH; tubes 76–87; R_f 0.33 (6:1 $CHCl_3$–MeOH)[f]	16	146–157	$C_{36}H_{40}N_{10}O_3 \cdot 0.5H_2O$

8	C_6H_5NH	B	0.004; 0.002	60 g; 100:0 (25 × 20 mL), 99:1 (25 × 20 mL), 97:3 (25 × 20 mL), 95:5 (50 × 20 mL), 93:7 (50 × 20 mL), 91:9 (100 × 20 mL) $CHCl_3$–EtOH; tubes 174–205; R_f 0.49 (6:1 $CHCl_3$–MeOH)	19	163–176	$C_{32}H_{32}N_{10}O_3 \cdot 2H_2O$
		C	0.0053; 0.0028[g]	60 g; 98:2 (55 × 9 mL); 96:4 (55 × 9 mL), 92:8 (110 × 9 mL) $CHCl_3$–MeOH; tubes 110–182	21	162–171	
9	H_2NNH	D	*h*	recrystd from 50% aq MeOH; R_f 0.27 (alumina, 1:1 $CHCl_3$–MeOH)	95	196–204	$C_{20}H_{26}N_{12}O_3 \cdot 0.4H_2O$
10	CH_3NHNH	D	*h*	purified by TLC; R_f 0.26 (1:2 $CHCl_3$–MeOH)	28	192–228	$C_{22}H_{30}N_{12}O_3 \cdot 1.5CH_3OH \cdot 0.2CHCl_3$
11	c-$(CH_2CH_2)_2N$-NH	B	0.0004; 0.0004	20 g; 99:1 (22 × 9 mL), 90:10 (22 × 9 mL), 85:15 (22 × 9 mL) $CHCl_3$–MeOH; tubes 66–73; R_f 0.34 (6:1 $CHCl_3$–MeOH)	8	158–170	$C_{32}H_{46}N_{12}O_3 \cdot CH_3OH \cdot 0.6H_2O$

[a] Method A = DCC alone; method B = DCC + *N*-hydroxysuccinimide; method C = mixed anhydride coupling; method D = MTX diester + amine. [b] Weight of silica gel (Baker 5-3405, 60–200 mesh), composition of the eluent, number and volume of individual tubes, tube numbers from which the product was isolated, R_f values, and TLC developing solvent are given. Unless otherwise specified, TLC data refer to Anasil GF or OF plates (New England Nuclear Corp., Boston, MA). [c] Yields are for product that has been purified as indicated in the preceding column. [d] C, H, and N analyses were all within ±0.4% of theoretical values. [e] The analytical sample of this compound was obtained by rechromatography on a silica gel *dry column* (50 g, Woelm Activity Grade III/30 mm, ICN Pharmaceuticals, Inc., Cleveland, OH) which was eluted consecutively with 98:2 (20 × 20 mL), 96:4 (20 × 20 mL), and 94:6 (10 × 20 mL) $CHCl_3$–MeOH. [f] Evaporation of tubes 55–64 gave a bright yellow solid (0.064 g, 42% yield), mp 155–168 °C dec. The NMR spectrum of this compound in $CDCl_3$ solution clearly showed the presence of cyclohexyl moieties in the τ 7.0–9.2 region, and the IR spectrum exhibited, in addition to amide C=O bands at 1640 and 1615 cm^{-1}, a distinct shoulder at 1700 cm^{-1} consistent with the acylurea structures **13a** or **13b**. [g] Quantities refer to L-glutamic acid dianilide and the mixed anhydride from isobutyl chloroformate and 4-amino-4-deoxy-N^{10}-methylpteroic acid (cf. Experimental Section). [h] A large excess of hydrazine or *N*-methylhydrazine was used.

similar to the γ-COOH group, it should not be amenable to polyglutamate formation.

Chemistry. The reaction of MTX diethyl or dimethyl ester which was previously used to obtain bis(amides)[2] proceeded smoothly with ammonia, simple straight-chain *N*-alkylamides, or pyrrolidine but gave low yields (or failed entirely) with more hindered aliphatic amines such as 1-aminoadamantane, *N,N*-di-*n*-butylamine, or piperidine. When piperidine was used, the major products appeared to be γ-monoesters, whose formation was presumably due to the presence of residual moisture in the reactants or solvent.[5a] On the other hand, treatment of MTX with 2 molar equiv each of piperidine and *N,N*-dicyclohexylcarbodiimide (DCC) in dry DMF at room temperature for about 3 days resulted in a 35% yield of the desired bis(amide) **2**, after removal of a small amount of acylurea impurity (10% yield) by silica gel column chromatography. Acylurea side products[6] have been observed in this laboratory previously in DCC-mediated coupling reactions involving MTX[4] and appear to be general for this series. That compound **2** was a bis(amide) rather than mono(amide) derivative was apparent from its insolubility in aqueous base and its infrared spectrum, which showed no ester absorption in the 1740-cm^{-1} region.

DCC coupling was also employed to obtain several bis(benzylamide) analogues from MTX. The best results (Table I) were obtained with *N*-benzyl-*N*-methylamine, which gave a 67% yield of the bis(amide) **5**. There appeared to be an inverse correlation between the yields of the bis(amides) in these reactions and the tendency to form acylureas. Thus, with 1-phenylethylamine the yield of the bis(amide) **7** was only 16%, whereas the acylurea product was obtained in 42% yield. On the basis of infrared and NMR spectral evidence, as well as microchemical data, the side product is probably a monoamide monoacylurea, though a definitive choice between structures **13a** and **13b**

13a

13b

cannot be made. That steric effects probably play a strong adverse role in the coupling reaction is indicated by the very low yield (6.7%) of the bis(amide) **1** from 1-aminoadamantane.

In some instances coupling was performed via the *N*-hydroxysuccinimide route (Table I, method B), which has been reported to be superior to DCC alone for the formation of peptide bonds.[6] When 4-chlorobenzylamine was condensed with MTX free acid in the presence of DCC, a 19% yield of the bis(amide) **3** was obtained; however, when the intermediate *N*-hydroxysuccinimide ester of MTX was prepared, the yield of compound **3** increased to 62%. Similar favorable results were observed when MTX

(6) D. H. Rich and J. Singh, in "The Peptides: Analysis, Synthesis, and Biology", Vol. 1, E. Gross and J. Meienhofer, Eds., Academic Press, New York, 1979, Chapter 5.

was condensed with 4-methoxybenzylamine in order to prepare the bis(amide) **4** (11 and 66% yields, respectively).

The dianilide derivative **8** was accessible directly from MTX via the DCC/*N*-hydroxysuccinimide approach (19% yield); however, in view of previous indications that some side-chain racemization might occur during MTX reactions involving DCC,[4] we chose to also prepare compound **8** via mixed anhydride condensation (Table I, method C). Reaction of *N*-carbobenzoxy-L-glutamic acid with aniline in the presence of DCC and *N*-hydroxysuccinimide, followed by removal of the Cbz group by catalytic hydrogenolysis and coupling of the dianilide with the mixed anhydride from isobutyl chloroformate and 4-amino-4-deoxy-N^{10}-methylpteroic acid, led to the desired product **8**. The yield of **8** in the final step was 21%, and a substantial amount of 4-amino-4-deoxy-N^{10}-methylpteroic acid was recovered unchanged, as is customary in this reaction.[4,5a]

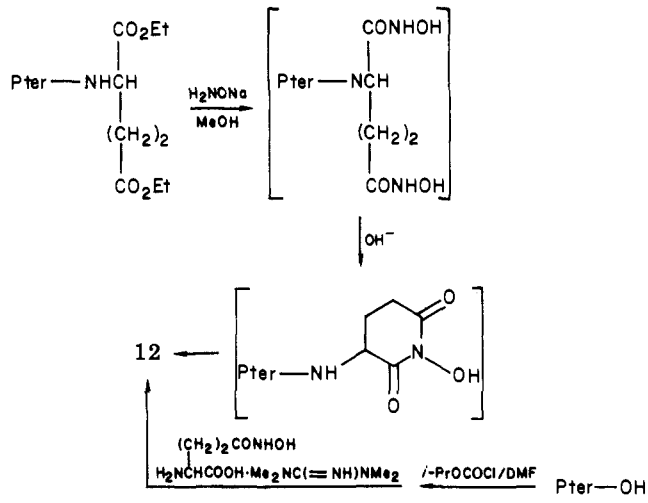
The dihydrazide derivatives **9** and **10** were obtained from MTX diethyl ester on reaction with hydrazine and *N*-methylhydrazine, respectively (Table I, method D). After only 30 min at room temperature, compound **9** was formed from hydrazine in almost quantitative yield. The reaction rate with *N*-methylhydrazine was slower; even after 48 h, the yield of compound **10** was only 28% and at least two other products were detected by TLC. The structure of **10** was assigned on the basis of a published study[7] reporting that acylation of *N*-methylhydrazine by esters occurs predominantly on the NH_2 group and is favored when the acyl group is large.

When MTX dimethyl ester was heated with *N,N*-dimethylhydrazine (48 h under reflux), none of the desired dihydrazide could be obtained; instead, a 57% yield of MTX γ-monomethyl ester was isolated. Similar cleavage of diesters to monoesters in the presence of *N,N*-dimethylhydrazine has been reported previously.[5a]

The monohydroxamic acid analogue **12** was obtained directly from MTX diethyl ester and hydroxylamine in the presence of base. When a methanol solution containing MTX diethyl ester and an excess of hydroxylamine sodium salt was stirred at room temperature for 2 days and the reaction was terminated by adding ether, a TLC-homogeneous solid (R_f 0.43, cellulose, pH 7.4) was isolated which gave a positive hydroxamate test (violet color with $FeCl_3$) and whose IR spectrum showed no ester C=O absorption in the 1740-cm^{-1} region. On passage through a DEAE-cellulose column, the initial product underwent transformation to a *new* TLC-homogeneous compound (R_f 0.61, cellulose, pH 7.4) which still gave a positive $FeCl_3$ test for the hydroxamic acid function. Though definitive evidence is lacking, it appears that the initial product (R_f 0.41) may be a bis(adduct) which loses a molecule of hydroxylamine rapidly during ion-exchange chromatography in alkaline solution. That the final product (R_f 0.61) is the γ-monosubstituted derivative **12** is clear, however, from the fact that this compound was also obtained from 4-amino-4-deoxy-N^{10}-methylpteroic acid and L-glutamic γ-monohydroxamic acid via the mixed anhydride route (Scheme I). Mixed anhydride coupling of APA to *unprotected* amino acids in the presence of 1,1,3,3-tetramethylguanidine has been used previously in this laboratory to prepare MTX γ-monoethyl ester[5a] and has other precedents in the peptide literature.[8]

Bioassay. Most of the compounds synthesized during this investigation were tested as inhibitors of the growth

Scheme I[a]



[a] Pter = 4-amino-4-deoxy-N^{10}-methylpteroyl.

Table II. Activity of MTX Amides against Leukemic Cells in Culture

	ID_{50},[a] μM		
compd	human lymphoblastic leukemia (CEM)	murine leukemia (L1210)	rat basophilic leukemia (RBL)
MTX	0.003	0.01	0.003
1	>10 (38%)	3.4	1.6
2		>10	
3	>10 (29%)	6.6	
4	>10 (44%)	8.2	
5	6.4	9.4	
6	7.6	0.69	
7	>10 (25%)	>10	
8	3.3	0.41	
9	7.5	0.95	0.96
12	0.25	0.062	

[a] See ref 16 for details of the assay procedure. Values given in parentheses are percent inhibitions of cell growth (48 h) at a drug concentration of 10 μM, which was the highest dose tested. Results are averages of triplicate experiments and have a standard deviation of ±10%. [b] See ref 2.

of CEM human lymphoblastic leukemia cells and L1210 mouse leukemia cells in vitro (Table II). Against CEM cells, slight activity was shown by the dianilide **8** (ID_{50} = 3.3 μM). A low level of activity was likewise observed with the bis(*N*-benzyl-*N*-methylamide) and bis(*N,N*-dibenzylamide) derivatives (compounds **5** and **6**), but the addition of a methyl group on the benzylic carbon (compound **7**) reversed this effect. The bis(*p*-chlorobenzylamide) and bis(*p*-methoxybenzylamide) derivatives (compounds **3** and **4**) were less active than the previously reported bis(benzyl) analogue.[2] Against L1210 cells, the most active bis(amide) was once again the dianilide **8**, which had an ID_{50} value of 0.41 μM. Also active below 1 μM were the bis(*N,N*-dibenzylamide) **6** and the dihydrazide **9**, but not the bis(*N*-benzyl-*N*-methylamide) **5**. The other bis(amides) were less active, though it may be noted that, for the most part, their ID_{50} values were lower against L1210 cells than against CEM cells. In several instances (compounds **6**, **8**, and **9**), this difference in activity was approximately 10-fold. Two compounds were also tested against cultured rat basophilic leukemia cells (RBL)[2] and were found to have greater activity than against CEM cells. The dihydrazide **9** had an ID_{50} value of 7.5 μM against CEM cells but only 0.96 μM against RBL cells. This 8-fold increase in activity against the RBL line was similar to our earlier

(7) R. L. Hinman and D. Fulton, *J. Am. Chem. Soc.*, **80**, 1895 (1958).
(8) D. S. Kemp, S.-W. Wang, J. Rebek, Jr., R. C. Mollan, C. Banquer, and G. Subramanyan, *Tetrahedron*, **30**, 3955 (1974).

Table III. Binding of MTX Amides to Dihydrofolate Reductase

compd	ID_{50}, μM: L1210 enzyme (ligand-binding assay)	ID_{50}, μM: *L. casei* (kinetic assay)
MTX	0.0068 ± 0.0022[a]	0.021 ± 0.008[a]
1	3.1	6.3
2	0.012	0.25
3	0.18	2.3
4	0.13	0.40
5	0.026	0.44
6	0.53	0.86
7	0.011	0.31
8	0.074	1.1
9	0.020	0.36
12	0.0048	0.030

[a] Mean ± SD from three separate experiments are reported for MTX.

observations with other bis(amide) derivatives of MTX, for which several possible explanations were advanced including the possibility that RBL cells, which are of myeloid rather than lymphoid origin, contain elevated levels of amidases. The present finding that the ID_{50} values for compound **9** against the lymphoid L1210 line and myeloid RBL line are essentially the same appears to rule out such an interpretation, however, and suggests that the data are a reflection primarily of species differences (i.e., human vs. rodent).

Of all the compounds tested in this work, the most cytotoxic proved to be the γ-monohydroxamic acid ester **12**, which had an ID_{50} value of 0.25 μM against CEM cells. Against L1210 cells, compound **12** had an ID_{50} value of only 0.062 μM and was thus only 6-fold less active than MTX itself, whose ID_{50} against this cell line was 0.01 μM.

The affinities of the MTX bis(amides) **1–9** and γ-monohydroxamic acid **12** for partially purified bacterial (*Lactobacillus casei*) and mammalian (L1210 mouse leukemia) dihydrofolate reductase was likewise investigated. A competitive ligand-binding assay[9,10] was employed with the L1210 enzyme, whereas studies with the *L. casei* enzyme involved a standard spectrophotometric assay.[11] The competitive ligand-binding assay, which depends on the ability of a test compound to compete with [^{3}H]MTX for binding to the enzyme, is convenient when limited amounts of enzyme are available and is known to agree well with kinetic assays based on spectrophometric measurement of the rate of conversion of NADPH to NADP. The ID_{50} values obtained for MTX and the amide derivatives in these two assays are listed in Table III. Under our assay conditions, MTX showed ID_{50} values of approximately 0.02 μM against the *L. casei* enzyme (kinetic assay) and 0.007 μM against the L1210 enzyme (ligand-binding assay). The γ-monohydroxamic acid **12** was effective against both enzymes and, in fact, appeared to have a slightly higher affinity for the L1210 enzyme than MTX itself. The bis(4-chlorobenzylamide) (**2**), bis(*N*-benzyl-*N*-methylamide) (**5**), bis(1-phenylethylamide) (**7**), and dianilide (**8**) derivatives were likewise fairly active against L1210 dihydrofolate reductase, with ID_{50} values of 0.01–0.1 μM. Activities against the *L. casei* enzyme followed a qualitatively similar trend, with **12** being the most active and **1** being the least active in the series.

Table IV. Activity of MTX Derivatives against L1210 Leukemia in Mice[a]

compd		dose, mg/kg q3d 1, 4, 7	MTX equiv, mg/kg	no. of animals	median T/C, days	% ILS
3		80	65	5	11.0/9.0	+22
		160	130	5	13.0/9.0	+44
4		80	64	5	11.0/9.0	+22
		160	128	5	15.0/9.0	+66
8		40	30	5	13.0/9.0	+44
		80	60	5	19.0/9.0	+111
		120	90	5	23.0/9.0	+155[c]
9	expt 1	7.5	7.1	5	14.0/10.0	+40
		15	14.2	5	14.0/10.0	+40
	expt 2	20	18.8	5	16.0/9.0	+77
		40	37.6	5	12.0/9.0	+33
12	expt 1	7.5	7.3	5	15.0/10.0	+50
		15	14.6	5	16.0/10.0	+60
	expt 2	20	19.4	5	17.0/9.0	+89
		40	38.8	5	19.0/9.0	+111
MTX[b]		15		20	15.5/9.0	+72
		30		20	16.5/9.0	+83
		60		20	18.0/9.0	+100

[a] Groups of five B6D2F$_1$ mice were injected ip with 10^5 L1210 cells on day 0. Test compounds were administered ip in 10% Tween 80 or 1:1:8 emulphor–ethanol–water suspension, and MTX was administered ip as the disodium salt in water. [b] Data given for MTX represent median survivals from several experiments. [c] The ILS was >250% in two out of five animals at this dose.

Examination of the data in Table III revealed that the γ-monohydroxamic acid **12**, which was the most active compound against the L1210 enzyme in the ligand-binding system, was also the most toxic to intact L1210 cells. Similarly, the dianilide **8** and dihydrazide **9**, which had ID_{50} values of <0.1 μM against intact cells, were among the most active members of the series in the enzyme assay.

It is of interest that the most cytotoxic compound in this group was the γ-monohydroxamic acid **12**, which had an ID_{50} value of 0.062 μM against L1210 cells and showed virtually the same affinity for L1210 dihydrofolate reductase as MTX itself. This compound is structurally very close to MTX γ-monoamide, which was described recently by other investigators.[3a,b] The ID_{50} value for the γ-monoamide against KB cells (human epithelial carcinoma) was reported to be 0.32 μg/mL (0.71 μM),[3a] and the K_i against purified L1210 dihydrofolate reductase was given as 0.0027 nM as compared with 0.0047 nM for MTX.[3b] The lower than expected cytotoxicity of the γ-monoamide was ascribed to decreased uptake into cells, rather than to a lack of enzyme binding.[3b] A reasonable explanation for decreased net uptake is that γ-monosubstituted MTX derivatives such as **12** and the γ-monoamide, since they lack a free γ-COOH group, cannot accumulate intracellularly in the form of polyglutamates. Another possibility which cannot be ruled out is that these compounds owe their low toxicity to human cells in culture to a low affinity for human dihydrofolate reductase. Binding studies employing enzyme derived from CEM or other human cells should provide an answer to this question.

Five of the compounds which were available in sufficient amount for in vivo antitumor evaluation were tested against L1210 leukemia in mice (Table IV). The bis(*p*-chlorobenzylamide) and bis(*p*-methoxybenzylamide) derivatives **3** and **4** were active at doses of 160 mg/kg, given on days 1, 4, and 7 following tumor implantation. The increases in life span (44 and 66%, respectively) were approximately the same as was observed earlier with the

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unsubstituted bis(benzyl) analogue. It was found also that the dianilide 8, which had only modest activity in vitro as compared to MTX, was quite active in vivo (+155% ILS at 160 mg/kg, with two out of five animals surviving 30 days). Thus, it appears that bis(amide) derivatives of MTX are active in vivo when the *N*-substituent on the amide function is aromatic (i.e., phenyl or benzyl). In view of our finding that the liver of mice treated with MTX bis(benzylamide) contains free MTX, there seems to be a requirement for aromatic substitution on nitrogen in order for enzymatic bioactivation of MTX prodrugs of the amide type to occur. The dihydrazide derivative 9 was likewise more active in vivo (+77% ILS at 20 mg/kg) than would be predicted on the basis of the cell culture data. Examination by TLC of the plasma of a mouse 0.5 h after intraperitoneal administration of a 250 mg/kg dose of 9 revealed no detectable free MTX, but did show the formation of a metabolite which may be the as yet unknown γ-monohydrazide. Work is in progress to prepare an authentic specimen of this compound in order to confirm its identity and conduct further studies of its biological properties.

The last compound tested was the γ-monohydroxamic acid 12. This molecule was of particular interest because, apart from its obvious structural similarity to MTX, it would not be expected to undergo polyglutamate formation and therefore ought to be cleared more rapidly from the tissues than MTX. An encouraging level of activity was observed with compound 12 (+111% ILS at 40 mg/kg) which was closely comparable to that of MTX. Of the compounds tested, this was the only one for which there was a correlation between in vitro and in vivo test results.

Since compound 12 appeared to possess the most promising combination of enzyme-binding ability, in vitro cytoxicity, and in vivo antitumor effect among the derivatives prepared in this work, it was of interest to obtain some information about its pharmacological behavior in the mouse. In particular, we desired to examine the possibility that the uptake of this compound into tumors might be superior to that of MTX or that its ability to penetrate the central nervous system might exceed that of MTX, which is known for its low penetration of the blood–brain barrier.[12] Plasma, brain, and tumor levels of these two agents were measured 0.5, 2, and 4 h after intraperitoneal injection into mice bearing 11-day-old subcutaneously implanted P388 tumors. Concentrations were determined by means of a radioimmunoassay employing a commercially obtained goat anti-MTX antibody which had approximately the same reactivity toward compound 12 as it did toward MTX. The radioimmunoassay procedure was adapted from the published method of Loeffler and co-workers,[13] with the following minor changes: (a) binding was conducted at pH 6.2; (b) the [^{3}H]MTX and unlabeled ligands (MTX or 12) were combined before the addition of the antibody; and (c) the antibody–ligand mixture was incubated at 37 °C. The ID_{50} values, i.e., the concentrations of nonradioactive ligands required to decrease [^{3}H]MTX binding to the antibody by 50%, were found to be 10 nM for compound 12 and 7.6 nM for MTX. Since the antibody thus appeared to be almost completely cross-reactive toward these molecules, we assumed that the method would allow us to obtain a close approximation of the concentration of compound 12 in tissue specimens and that even if 12 were partially biotransformed into MTX the results would still provide a useful estimate of the *total concentration* of the two species in tissues.

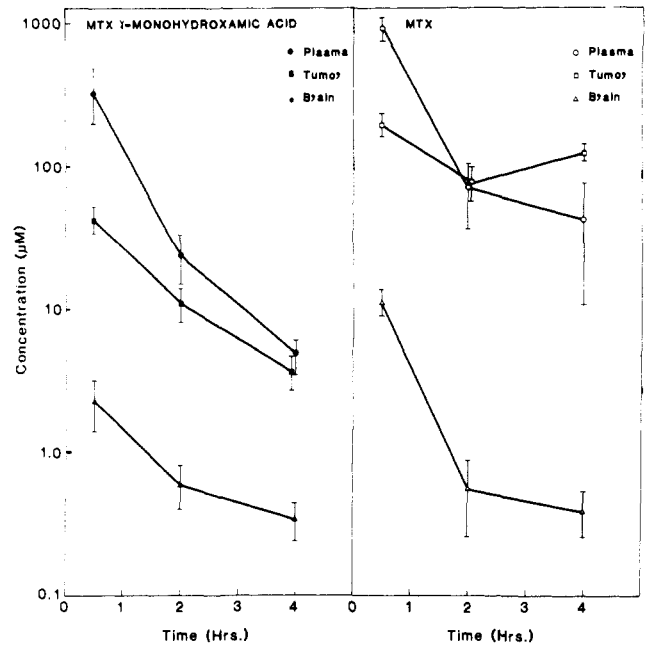

Figure 1. Pharmacokinetic comparison of MTX γ-monohydroxamic acid (12) and MTX in mice bearing subcutaneously implanted P388 leukemia.

As shown in Figure 1, the plasma level of total antibody-reactive material 0.5 h after a 250 mg/kg ip dose of 12 was 3×10^{-4} M and there was approximately a tenfold drop in concentration after 2 h, with another 10-fold decrease after 4 h. Corresponding levels in the tumor and brain extracts at 0.5 h were 4×10^{-5} and 2×10^{-6} M, respectively, and there was a qualitatively similar decrease in concentration over time. In animals treated with the same doses of MTX, the levels of MTX in plasma ranged from 1×10^{-3} M at 0.5 h to 5×10^{-5} M at 4 h whereas the levels in brain extract ranged from 1×10^{-5} M at 0.5 h to 4×10^{-7} M at 4 h. All the initial drug levels were higher following MTX treatment, suggesting that compound 12 may not be absorbed as well as MTX from the peritoneal cavity or that it is cleared more rapidly from the circulation. Compound 12 is less water soluble than MTX, which could cause some precipitation to occur following injection. Apart from this, and an apparently greater persistence of MTX in the tumor which is probably not statistically significant, the overall pharmacokinetic characteristics of the two drugs were very comparable. Since the brain/plasma ratio was approximately 0.01 for both compounds, there seems to be no improved penetration of the blood–brain barrier on converting the γ-COOH group of MTX to γ-CONHOH. Furthermore, since the plasma/tumor ratios for the two compounds were likewise very similar, there was no evidence for more selective tumor uptake by the γ-substituted derivative. In this experimental system, therefore, MTX γ-monohydroxamic acid (12) cannot be said to possess a pharmacological advantage over the parent molecule. Whether this would be true in other animal species, or in mice with tumors other than subcutaneously implanted P388 leukemia, remains to be determined.

In summary, we have shown that it is possible to prepare prodrug derivatives of MTX of the dianilide, bis(benzylamide), and dihydrazide type and that the in vivo antitumor effect of these compounds is comparable, in some instances, to that of MTX itself. The absence of significant cytotoxicity against tumor cells in culture suggests in vivo

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transformation to free MTX or to the α- or γ-monoamides. The increased activity of the dianilide and bis(benzylamide) derivatives relative to the previously studied aliphatic bis(amides)[2] suggests that aromatic substitution may favor enzymatic cleavage of the amide bonds. The heretofore undescribed γ-monohydroxamic acid derivative of MTX was also synthesized and shown to possess in vitro and in vivo biological activity similar, but not superior, to that of MTX itself. Studies on side-chain modification of MTX are continuing in our laboratory.

Experimental Section

Infrared spectra were obtained on a Perkin-Elmer Model 137B double-beam recording spectrophotometer. NMR spectra were determined by means of a Varian T60A instrument, with tetramethylsilane as the reference. TLC was performed on 250 μm Analtech silica gel GF plates, Eastman 13181 silica gel sheets, or Eastman 13254 cellulose sheets. Spots were visualized under ordinary fluorescent light or 254-nm ultraviolet light or by iodine staining. Column chromatography was carried out on Baker 3405 silica gel (60–200 mesh) as described in Table I, unless otherwise specified. Melting points were measured in Pyrex capillary tubes in a Mel-Temp apparatus (Laboratory Devices, Inc., Cambridge, MA) and are not corrected. Microchemical analyses were performed by Galbraith Laboratories, Knoxville, TN, and were within ±0.4% of the calculated C, H, N values. Radioactivity measurements were made by means of a Beckman LS7000 scintillation counter, and spectrophotometric assays of enzyme inhibition were performed with the aid of a Gilford Model 240 spectrophotometer equipped with a thermostatted programmable cell-changer assembly.

Preparation of MTX Bis(amides) by DCC Coupling (Method A). MTX Bis(*N*-benzyl-*N*-methylamide) (5). To a solution of MTX free acid (1.0 g, 0.002 mol) in dry DMF (30 mL) was added *N*-benzyl-*N*-methylamine (0.49 g, 0.004 mol), followed by a solution of DCC (1.0 g, 0.0048 mol) in dry DMF (10 mL). After being stirred at room temperature for 2 h, the reaction mixture was filtered and evaporated under reduced pressure. Addition of a small volume of fresh DMF to the residue caused precipitation of *N,N*-dicyclohexylurea (0.85 g, 78% yield). Repeated rotary evaporation of the DMF and trituration of the residue with ether gave a yellow-orange solid (1.6 g).

MTX Bis(1-phenylethylamide) (7). To a solution of MTX free acid (0.1 g, 0.002 mol) in dry DMF (3 mL) was added 1-phenylethylamine (0.049 g, 0.0004 mol), followed by a solution of DCC (0.1 g, 0.0004 mol) in dry DMF (1 mL). After 20 h of stirring at room temperature, the precipitated *N,N*-dicyclohexylurea was filtered off (0.042 g, 39% yield) and the DMF was removed by rotary evaporation. Trituration of the residue with ether gave a yellow solid: IR (KCl) ν 1640, 1615 (amide C=O), 695 (monosubstituted phenyl) cm^{-1}.

Preparation of MTX Bis(amides) via *N*-Hydroxysuccinimide Esters (Method B). MTX Bis(4-chlorobenzylamide) (2). Solid DCC (1 g, 0.0048 mol) was added to an ice-cold solution of MTX (1 g, 0.002 mol) and *N*-hydroxysuccinimide (0.46 g, 0.004 mol) in dry DMF (10 mL), and the mixture was allowed to stand in the refrigerator for 23 h. At the end of this period a solution of 4-chlorobenzylamine (0.58 g, 0.004 mol) in DMF (4 mL) was added, the mixture was stirred at room temperature for 2 h, the precipitated *N,N*-dicyclohexylurea was filtered off (0.85 g, 78% yield), and the DMF was removed by rotary evaporation. Trituration of the amber-colored semisolid with pH 7.4 phosphate buffer (100 mL), suction filtration, washing with water, and drying in vacuo at 70 °C gave a yellow-orange solid (1.7 g): IR (KCl) ν 1615, 1650 (amide C=O) cm^{-1}; NMR (Me_2SO-d_6) τ 7.8 (br m, 4 H, glutamyl CH_2CH_2), 6.8 (s, 3 H, NCH_3), 6.7 (s, 2 H, CH_2NCH_3), 5.77 (m, 5 H, NHC*H* and $C_6H_5CH_2$), 3.5–1.4 (complex m, 20 H, 2- and 4-NH_2, CONH, aromatic protons, and C_7 pteridine proton).

When the above reaction was carried out without *N*-hydroxysuccinimide (i.e., via method A) the yield of bis(amide) was only 19%.

Preparation of MTX Bis(amides) via Mixed Anhydride Coupling (Method C). MTX Dianilide (8). A solution of DCC (7 g, 0.034 mol) in DMF (20 mL) was added to an ice-cold solution of *N*-carbobenzoxy-L-glutamic acid (4.5 g, 0.016 mol) and *N*-hydroxysuccinimide (3.8 g, 0.032 mol) in dry DMF (80 mL), the mixture was allowed to stand in the refrigerator for 22 h, and aniline (3 g, 0.032 mol) was added. After being stirred at room temperature for 48 h, the mixture was filtered to remove the *N,N*-dicyclohexylurea (6.9 g, 91% yield) and concentrated to dryness by rotary evaporation. The light brown semisolid was dissolved in EtOAc (300 mL), and the solution was washed consecutively with water (100 mL), 0.1 N $NaHCO_3$ (2 × 100 mL), 0.1 N HCl (2 × 100 mL), and water (100 mL). Drying and solvent evaporation left a solid, which was recrystallized from EtOAc in the form of fine white crystals (2.5 g): mp 225–229 °C; TLC (silica gel, 6:1 $CHCl_3$–MeOH) R_f 0.82; IR (KCl) ν 1700, 1665 cm^{-1}; NMR (Me_2SO-d_6) τ 8.0 (m, 4 H, glutamyl CH_2CH_2), 5.88 (m, 1 H, NHC*H*), 4.99 (s, 2 H, $C_6H_5CH_2$), 2.68 (m, 18 H, aromatic and CONH protons). A second crop of product (0.7 g) was also obtained, bringing the total yield to 3.1 g (45%). The *N*-carbobenzoxy-L-glutamic acid dianilide (2.4 g, 0.0056 mol) was suspended in absolute EtOH (200 mL) in a Parr hydrogenation bottle, a 1% solution of dry hydrogen chloride in absolute EtOH (20.3 mL, equivalent to 0.0056 mol of acid) and 10% Pd/C (0.24 g) were added, and the mixture was shaken under H_2 (ca. 3 atm) for 4 h. Filtration through Celite, rotary evaporation of the filtrate, and trituration of the foamy residue with ether gave L-glutamic acid dianilide hydrochloride as a white solid (1.9 g): TLC (silica gel, 3:1 $CHCl_3$–MeOH) R_f 0.67; positive ninhydrin test. This compound (1.8 g, 0.0053 mol) was suspended directly in DMF (15 mL) to which were then added consecutively *N*-methylmorpholine (0.54 g, 0.0053 mol) and DMF (20 mL) in which was dissolved the mixed anhydride (1.2 g, 0.0028 mol) from isobutyl chloroformate and 4-amino-4-deoxy-N^{10}-methylpteroic acid.[14] The reaction mixture was stirred at room temperature for 16 h and on a steam bath for 15 min, then cooled, and filtered. Rotary evaporation gave an amber-colored syrup, which solidified on trituration with pH 7.4 buffer (100 mL): IR (KCl) ν 1610 (amide C=O), 694 (monosubstituted phenyl) cm^{-1}; NMR (Me_2SO-d_6) τ 7.8 (br m, 4 H, glutamyl CH_2CH_2), 6.8 (s, 3 H, CH_2NCH_3), 6.58 (s, 2 H, CH_2NCH_3), 5.5 (m, 1 H, CHNH), 3.6–1.4 (complex m, 22 H, 2- and 4-NH_2, CONH, aromatic protons, and C_7 pteridine proton).

When compound 8 was prepared via the *N*-hydroxysuccinimide route (method B) the yield was 19%. IR and NMR spectra of the samples obtained by the two routes were indistinguishable.

Synthesis of MTX Dihydrazides from MTX Diethyl Ester (Method D). MTX Dihydrazide (9). A solution of MTX diethyl ester (0.1 g, 0.0002 mol) in a mixture of absolute EtOH (1 mL) and hydrazine (1 mL) was stirred at room temperature for 30 min. On addition of a mixture of absolute EtOH (15 mL) and ether (15 mL) a yellow solid was formed (0.092 g, 95% yield). The analytical sample was obtained by repeated crystallization from 1:1 MeOH–H_2O: IR (KCl) ν 1610 (hydrazide C=O) cm^{-1}.

MTX Bis(*N*-methylhydrazide) (10). A solution of MTX diethyl ester (0.2 g, 0.0004 mol) in a mixture of absolute EtOH (4 mL) and *N*-methylhydrazine (4 mL) was stirred at room temperature for 48 h. Rotary evaporation yielded a yellow-orange glass which was taken up in a minimum volume of 3:1 $CHCl_3$–MeOH and separated into three fractions by preparative TLC (silica gel, 1000-μm thickness) using 1:2 $CHCl_2$–MeOH as the developing solvent. Extraction of the major band (R_f 0.26) with 3:1 $CHCl_3$–MeOH gave a yellow solid (0.056 g, 28% yield). Passage of this material through a cellulose column [10 g, stepwise elution with 100:0 (100 mL), 95:5 (100 mL), 90:10 (100 mL), and 80:20 (200 mL) $CHCl_3$–MeOH] and preparative TLC of the pooled 80:20 eluates gave the analytical sample as a bright yellow powder: IR (KCl) ν 1610 (hydrazide C=O) cm^{-1}; NMR (Me_2SO-d_6) τ 7.95 (m, 4 H, glutamyl CH_2CH_2), 7.02 (m, 1 H, NHN*H*CH_3), 6.80 (s, 3 H, NCH_3), 6.52 (br m, 5 H, NHNHCH_3 and CH_2NCH_3), 6.35–5.20 (m, 5 H, glutamyl NHC*H* and pteridine NH_2), 3.80–1.90 (complex m, 6 H, aromatic protons, CON*H*CH, and CON*H*NHCH_3), 1.42 (s, 1 H, pteridine C_7 proton).

MTX γ-Monohydroxamic Acid (12). A. Synthesis from MTX Diethyl Ester. To a solution of clean Na metal (0.46 g, 0.02 g-atom) in MeOH (15 mL, dried over Linde 4A molecular sieves) was added a solution of hydroxylamine hydrochloride (0.70 g, 0.01 mol) in the same dry solvent (10 mL). After 5 min the NaCl precipitate was allowed to settle and a portion of the supernatant (20 mL) was transferred to a flask containing solid MTX

diethyl ester (1 g, 0.00185 mol). After being stirred at room temperature for 44 h, the reaction mixture was diluted with ether, and the solid was filtered, washed with ether, and dried in vacuo over P_2O_5. The crude product (1.27 g) gave a single major TLC spot (R_f 0.43, cellulose, pH 7.4 phosphate buffer), and the infrared spectrum revealed the complete disappearance of ester absorption at 1740 cm^{-1}. The solid was taken up in a small volume of dilute base (pH 9.2 buffer), a small amount of insoluble material was filtered off, and the solution was applied to a DEAE-cellulose column (Whatman DE-52, 90 g) which was eluted with pH 7.4 phosphate buffer (120 mL/h). The pooled fast-moving eluates were lyophilized, the residue was taken up in a minimum of water, and 12 N HCl was added dropwise until, at pH ~6, a dense solid appeared. Filtration, washing with water, and drying in vacuo over P_2O_5 afforded the γ-monohydroxamic acid as a yellow-orange solid (0.74 g, 81% yield): mp 197–204 °C dec (foam ~225 °C); TLC (cellulose, pH 7.4 phosphate buffer) R_f 0.61; positive $FeCl_3$ test (reddish-violet color) for the hydroxamic acid function; IR (KCl) ν 1640, 1615 cm^{-1}.

B. Synthesis via Mixed Anhydride Coupling. To a stirred suspension of 4-amino-4-deoxy-N^{10}-methylpteroic acid (0.28 g, 0.00075 mol based on the empirical formula $C_{15}H_{15}N_7O_2 \cdot 0.5HCl \cdot 1.5H_2O$)[14] in dry DMF (25 mL) was added triethylamine (0.15 g, 0.00011 mol). After 30 min of stirring at room temperature the mixture was concentrated to dryness by rotary evaporation and the amber-colored residue was triturated with pH 7.4 phosphate buffer (6 mL) and rinsed with water. The base-insoluble portion was then dried in vacuo over P_2O_5 to obtain the mixed anhydride as a yellow-orange solid (0.31 g). This was redissolved in DMF (8 mL) and a solution of L-glutamic γ-monohydroxamic acid (0.19 g, 0.0012 mol, Sigma Chemical Co., St. Louis, MO) and 1,1,3,3-tetramethylguanidine (0.14 g, 0.0012 mol) in water (1 mL) was added. After 4 h at room temperature the DMF and water were removed by rotary evaporation, and the residue was taken up in pH 7.4 phosphate buffer. The solution was passed through Sephadex A-25 (10 g) and adjusted to pH 5 with 12 N HCl. The precipitate was collected by centrifugation and rechromatographed on DEAE-cellulose (Whatman DE-52, 80 g) using pH 7.4 phosphate buffer (100 × 20 mL). Fractions 11–27, which were TLC homogeneous, were pooled and lyophilized, and the residue was taken up in a minimum volume of water, which was then adjusted to pH 5 with 12 N HCl. Filtration of the precipitate, washing with water, and drying (P_2O_5) gave a yellow-orange solid (0.028 g, 8% yield) whose melting point, TLC mobility, and infrared spectral characteristics were the same as those of the product obtained via procedure A.

Reaction of MTX Dimethyl Ester with *N,N*-Dimethylhydrazine. A mixture of MTX dimethyl ester (0.1 g, 0.00018 mol) and *N,N*-dimethylhydrazine (10 mL) was stirred under reflux for 48 h. Rotary evaporation under reduced pressure (35–45 °C bath) yielded a yellow foam, which was taken up in a minimum volume of 9:1 $CHCl_3$–MeOH and applied to a column of silica gel (20 g). The column was eluted successively with 90:10 (20 × 10 mL), 80:20 (20 × 10 mL), 70:30 (20 × 10 mL), and 65:35 (80 × 10 mL) $CHCl_3$–MeOH. Evaporation of tubes 4–7 gave unchanged starting material (0.017 g, 17% recovery); evaporation of tubes 54–90 gave a bright yellow solid (0.055 g, 57% yield): mp 200–215 °C dec; TLC (cellulose, pH 7.4 phosphate buffer) R_f 0.71. The product was soluble in dilute $NaHCO_3$ and its IR spectrum was superimposable on that of an authentic specimen of MTX γ-monomethyl ester.[5a] Anal. ($C_{21}H_{24}N_8O_5 \cdot 3H_2O \cdot 0.5CH_3OH$) C, H, N.

Dihydrofolate Reductase Binding. The ability of compounds 1–9 and 12 to compete with [^{3}H]MTX for binding to dihydrofolate reductase was measured via a radioactive ligand-binding assay based on published procedures,[9,10] with some modifications as detailed below. [^{3}H]MTX (sp act. 19.2 mCi/mmol) was obtained from Amersham Corp., Arlington Heights, IL, and purified by column chromatography on DEAE-cellulose.[15] Partially purified dihydrofolate reductase from L1210 cells was prepared by the method of Arons and co-workers.[10] The enzyme extract was diluted in 0.1 M KH_2PO_4 buffer (pH 6.2) containing 0.1% bovine serum albumin (BSA) and 1 mM NADPH (Sigma Chemical Co., St. Louis, MO), and the solution was divided into 0.5-mL aliquots which were kept frozen until use. The adsorbant mixture employed to remove nonbound ligand prior to scintillation counting consisted of 5 g of activated carbon (Norite), 1.25 g of BSA, and 0.05 g of dextran (molecular weight 161 000) in 100 mL of deionized water. Assays were performed at 0 °C in rigid polystyrene microtiter plates (8 × 12 wells, Cooke Laboratory Products, Alexandria, VA). All reagents were made up in deionized water and kept ice-cold during use. Prior to each experiment, thawed enzyme was diluted 8-fold with buffer, fresh NADPH was added to a final concentration of 1 mM, and the mixture was kept on ice (preincubation step) for 5 min. In a typical experiment, a 100-μL aliquot of phosphate buffer–BSA solution containing approximately 1 × 10^4 counts/min of [^{3}H]-MTX was added to each of eight wells of the microtiter plate, 25 μL of a 6 μM solution of the test compound was added to the first well, and serial 5-fold dilutions were made through the next six wells with the aid of a 25-μL microdiluter. The eighth well (control solution) contained no test compound. A 50-μL aliquot of the preincubated enzyme solution was then added to each well, and 5 min later 50 μL of the Norite mixture was added. After centrifugation of the plate at 0 °C for 7 min at 640*g*, 100-μL aliquots of the supernatant were transferred into scintillation vials, 5.0 mL of Biofluor (New England Nuclear Corp., Boston, MA) was added, and enzyme-bound radioactivity was determined by scintillation counting. Results were plotted on a logit-log scale and found to be linear over a 4-log concentration range. ID_{50} values, i.e., the concentrations of unlabeled ligand required to decrease enzyme-bound radioactivity by 50%, were obtained from the logit-log plot.

Inhibition of dihydrofolate reductase by compounds 1–9 and 12 was also evaluated by means of a kinetic spectrophotometric assay at 340 nm.[11] Partially purified dihydrofolate reductase from MTX-resistant *L. casei* was obtained from the New England Enzyme Center, Boston, MA. The enzyme (20.2 units) was diluted in 10.1 mL of 0.1 M KH_2PO_4 buffer (pH 6.2) containing 0.1% BSA and 1 mM NADPH and then divided into aliquots as described above for the L1210 enzyme. Fresh NADPH and dihydrofolate reductase solutions were prepared daily in deionized water and kept on ice during use. Prior to each assay, the stock solution of enzyme was diluted with fresh buffer (containing 0.1% BSA and 1 mM NADPH) and preincubated on ice for 5 min. The amount of diluent was adjusted so that a final volume of 0.05 mL added to the reaction mixture gave an uninhibited ΔA_{340} of approximately 0.8 optical density units/min at 37 °C. In a typical experiment, 0.45 mL of 0.2 mM NADPH, 0.45 mL of 0.2 mM dihydrofolate, and 0.05 mL of solution containing varying concentrations of inhibitor were added to a 1-mL cuvette, and the reaction was initiated by adding 0.05 mL of preincubated enzyme solution. Changes in absorbance were measured at 37 °C over a 5-min period. Appropriate corrections for nonspecific background absorbance were made, and the data were plotted on a logit-log scale and found to be linear over a 2-log range of inhibitor concentrations. ID_{50} values, i.e., the concentrations of inhibitor required to decrease the uninhibited rate by 50%, were determined from the logit-log plot.

Pharmacology of MTX γ-Monohydroxamic Acid (12) and MTX in Tumor-Bearing Mice. Drug Administration and Tissue Sampling. Male B6D2F1 mice weighing 20–25 g were injected subcutaneously with 1 × 10^6 P388 lymphocytic leukemia cells and 11 days later, when tumors were palpable, the animals were injected intraperitoneally with 6.25 mg/mL of compound 12 (or MTX) in 0.5 mL of a 1:1 mixture of phosphate-buffered saline (PBS, pH 7.0) and propylene glycol. Compound 12 formed a fine suspension in this vehicle, whereas MTX was completely soluble. Blood was collected at 0.5, 2, and 4 h by orbital puncture with heparinized 250-μL glass capillary tubes, and the animals were immediately sacrificed. The heparinized blood was centrifuged for 2 min at 8000*g* (0 °C), and the plasma was removed. Tumors and brains were weighed and homogenized in an equal volume of ice-cold PBS, the debris was removed by centrifugation (0 °C, 2 min, 8000*g*), and the supernatant was assayed immediately

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or stored frozen until further use.

Qualitative Analysis by Thin-Layer Chromatography. Plasma was processed for TLC by placing a 50-μL aliquot into a 175-μL plastic tube designed for use in an airflo-type ultracentrifuge (Beckman Airfuge) and carefully layering 50 μL of PBS on top of the plasma. Centrifugation for 2 h at 165000g, followed by removal of the top 50 μL from the tube by means of a micropipet, yielded a solution which was nearly protein free. The same procedure was followed in order to obtain deproteinized tumor extracts. TLC was performed on cellulose sheets (Eastman 13254) by spotting 20-μL aliquots, developing with 0.1 M potassium phosphate buffer (pH 7.2), and visualizing the spots in an ultraviolet viewing chamber at 254 nm. The R_f values for authentic reference samples of compound **12** and MTX in this solvent system were 0.60–0.65 and 0.80–0.85, respectively; deliberately prepared mixtures of the two compounds were readily separable. Only minute traces of MTX were detected in the sample of **12** used in this experiment. The 0.5-h plasma samples from mice treated with MTX or compound **12** contained enough drug to allow qualitative analysis by TLC, whereas the levels in tumor and brain extracts were too low for this method to be used. The only UV-absorbing spot in the 0.5-h plasma samples from MTX-treated mice according to TLC analysis was unchanged MTX. Similarly, there was no evidence of significant bioconversion to MTX in the 0.5-h plasma sample from the animal treated with compound **12**. Thus, it appears that drug levels measured by radioimmunoassay (see below), at least in plasma at 0.5 h, represent mainly compound **12**. However, because of the limited sensitivity of the TLC system at the very low concentrations involved in this experiment, it is possible that up to 10% hydrolysis to MTX could fail to be detected in dilute tissue samples.

Quantitative Radioimmunoassay Measurements. Levels of compound **12** or MTX were determined by means of an assay based on the competitive binding of [^{3}H]MTX to an anti-MTX antibody; the assay was adapted with some changes from a published procedure.[13] Goat anti-MTX antibody was obtained from Cappel Laboratories, Cochranville, PA, and was diluted 4000-fold prior to use. The ID_{50} for competitive binding of [^{3}H]MTX to the antibody was 7.6 ± 0.91 nM for MTX and 10.0 ± 0.1 nM for compound **12**, i.e., the antibody was almost completely cross-reactive toward these drugs. In a typical experiment, 100 μL of 0.1 M potassium phosphate buffer (pH 6.2) containing 0.1% BSA and approximately 1×10^4 counts/min of [^{3}H]MTX was added to each of eight wells of a microtiter plate. To each well was then added 25 μL of a 6 μM solution of compound **12** or MTX (or of an appropriately diluted unknown sample of plasma or tissue extract), and serial 5-fold dilutions were made as described above for the enzyme-binding assay. The eighth well (control) contained no competitive ligand. Buffer-diluted antibody (50 μL) was added to each well, and after 1 h at 37 °C the plate was cooled in ice for 5 min and 50 μL of Norite mixture was added to remove nonbound ligand. The charcoal was sedimented by centrifugation (7 min, 640g, 0 °C), and 100-μL aliquots of supernatant were removed and counted in 5.0 mL of Biofluor. Results were recorded on a logit-log scale, and the dilution of compound **12** or MTX causing a 50% decrease in binding was determined. Appropriate corrections were made for dilutions during workup of tissue samples in order to arrive at the concentration of drug in the original sample. The dose–response curve was linear over a 4-log range of concentration. Three mice were used for each time point in the MTX experiment, in order to compare interanimal differences with the experimental error in replicate measurements on a single sample. Interanimal variation was found to be equal to, or at most 3-fold greater than, this error.

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Analogues of the Cytostatic Cyclic Tetrapeptide Chlamydocin. Synthesis of N^{β}-(*N*-Maleoylglycyl) and N^{β}-(*tert*-Butyloxycarbonyl) Derivatives of *cyclo*(Gly-L-Phe-D-Pro-L-Dap)[1]

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The synthesis of analogues of the cytostatic cyclic tetrapeptide chlamydocin is described. *cyclo*(Gly-L-Phe-D-Pro-N^{β}-Boc-L-Dap) (**4**) was prepared from N^{β}-(*tert*-butyloxycarbonyl)-L-diaminopropionic acid methyl ester (Dap) and Cbz-Gly-L-Phe-D-Pro using DCC/HOBt as the coupling reagent. The methyl ester was saponified to the acid, which was converted to the 2,4,5-trichlorophenyl (Tcp) ester by reaction with trichlorophenol and DCC. The *N*-(benzyloxycarbonyl) group was removed by hydrogenolysis and the amine active ester cyclized at 95 °C in pyridine. The Boc-protected cyclic tetrapeptide **4** was isolated in 14% yield. Cyclic tetrapeptide **4** was converted to *cyclo*-[Gly-L-Phe-D-Pro-N^{β}-(*N*-maleoylglycyl)-L-Dap] (**5**) to test for a possible sulfhydryl group at the chlamydocin receptor. Removal of the *tert*-butyloxycarbonyl group, followed by reaction with *N*-maleoylglycine and DCC/HOBt in methylene chloride, gave cyclic tetrapeptide **5** in 68% yield. The maleoyl cyclic tetrapeptide **5** did not inhibit [^{3}H]thymidine incorporation into calf thymus lymphocytes at concentrations 1000-fold higher than the IC_{50} for chlamydocin (6 nM).

Chlamydocin, *cyclo*[α-aminoisobutyryl-L-phenylalanyl-D-prolyl-L-2-amino-8-oxo-9,10-epoxydecanoyl] (**1**), was isolated and characterized by Closse and Huguenin[2] as a result of their efforts to identify a cytostatic agent detected

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