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Methotrexate Analogs. 3. Synthesis and Biological Properties of Some Side-Chain Altered Analogs†,1,2

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A versatile method for the unequivocal synthesis of 6-substituted pteridines was used for the preparation of several methotrexate analogs in which the glutamate moiety was modified in order to enhance lipophilic character. Two 8-oxido derivatives were also prepared. These compounds were tested for *in vitro* inhibitory activity against *Streptococcus faecium* and as enzyme inhibitors against dihydrofolate reductase derived from *Lactobacillus casei*, chicken liver, and L1210-FR8 cells. Several compounds showed significant bacterial inhibition, but all were found to be less inhibitory than methotrexate against these enzymes. Only the diethyl ester of methotrexate showed significant *in vivo* biological activity against L1210 leukemic mice, comparable to that of methotrexate. The pteridine 8-oxide function was found to have a detrimental biological effect in the two compounds of this type tested in these assays. An efficient synthesis of 4-amino-4-deoxy-N^{10}-methylpteroic acid and its ethyl ester is also described. The ester was found to have prophylactic antimalarial activity in the sporozoite-induced *Plasmodium gallinaceum* chick assay.

For more than 20 years, methotrexate (4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid, amethopterin, MTX) has been widely used as one of the most effective clinical antitumor agents, especially in the treatment of acute childhood leukemia.[3] Its mode of action as a folic acid antagonist[4] is recognized to involve strong inhibition of dihydrofolate reductase and interference with the synthesis of metabolites that play a role in important biochemical one-carbon transfer reactions, including the biosynthesis of nucleotides. Despite some remarkable successes in induction of remission in acute leukemia and in certain solid tumors, the clinical usefulness of MTX is limited by the development of drug resistance, due in part to increased levels of enzymes in the folic acid cycle and restricted cellular uptake of the drug.[5,6] Except when given intrathecally,[7] MTX is ineffective against tumors of the central nervous system. It is also ineffective against malarial parasites, which lack an active transport mechanism for folates.[8]

MTX is transported across tumor cell membranes by an active carrier-mediated mechanism, possibly sharing the same mechanism used for natural folates.[9-11] Since this mechanism probably involves the carboxyl groups of the glutamate moiety,[8] any significant chemical modification at this site should change the overall transport properties of the molecule without greatly affecting the enzyme-inhibitory properties of the 2,4-diaminopyrimido system, common to many types of folate antagonists.[12] Replacement of the carboxyl groups in MTX by less polar groups such as CH_2OH or CH_3 might result in a sacrifice of the active transport mechanism. However, this might be offset by the likelihood that the less ionic nature of the reduced species would enhance the lipophilic nature of the molecule, as would other structural modifications such as the complete replacement of the glutamate moiety by large alkylamine residues. Such molecules could still enter a cell by passive diffusion, as is the case with other small molecule antifolates,[8] and might be expected to penetrate the blood-brain barrier[7,13] more effectively than MTX.

We have been engaged in a program[1,2] aimed at the structural modification of MTX in order to prepare more effective analogs with improved transport across biological membranes. In this paper we describe the synthesis and some biological properties of analogs in which the glutamic acid portion of the molecule has been modified in order to bring about an enhancement in lipophilic character. The synthesis of these analogs is shown in Scheme I. Compounds **6a-c** are methotrexate analogs containing modifications only of the carboxyl groups, while in **6d** the glutamate moiety has been replaced by 1-adamantylamine. Since the synthetic scheme lends itself to the preparation of pteridine 8-oxides, two analogs of this type, **7a** and **7d**, were also prepared in order to assess the biological effect of this kind of substitution. An efficient preparation of 4-amino-4-deoxy-N^{10}-methylpteroic acid is also described.

Chemistry. Early methods for the synthesis of MTX and its analogs led to mixtures of compounds which were very difficult to purify.[14] Improved syntheses were later developed for folic acid,[15] folate analogs,[16-18] and MTX analogs[19,20] which involve multiple steps. In the work reported here, we chose to utilize a versatile synthetic method for the unequivocal synthesis of 6-substituted pteridines developed by Taylor and coworkers.[21,22] This method involves, in the present instance, the use of the key intermediate 2-amino-3-cyano-5-chloromethylpyrazine 1-oxide (**2**, Scheme I).[22]

The intermediate *p*-(methylamino)benzoyl derivatives **3a,c,d** used in this synthesis were prepared according to the method of Santi[23] by reaction of *N*-tosyl-*p*-(methylamino)benzoyl chloride with the appropriate amine to give **1a,c,d**, followed by detosylation with HBr in glacial AcOH in the presence of phenol. In order to prepare diol **3b**, attempts were made to selectively reduce the ester groups of **3a** with excess lithium borohydride[24] in refluxing THF. Solid salts separated during the reaction, and the reaction mixture upon work-up yielded viscous oils which were mixtures of diol **3b** and incompletely reduced products. However, when excess lithium aluminum hydride was used as the reducing agent, and was rapidly added in portions to a solution of **3a** in THF, complete reduction of

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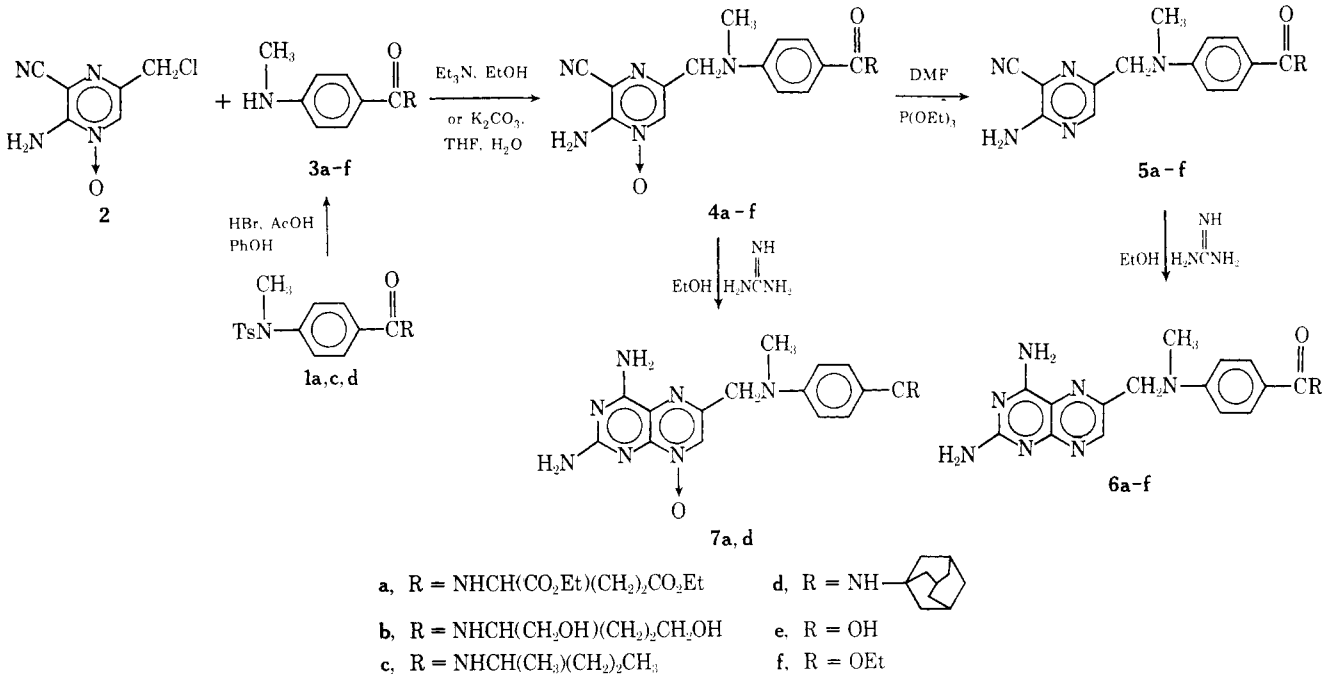
Scheme I



a, R = NHCH(CO$_2$Et)(CH$_2$)$_2$CO$_2$Et d, R = NH-adamantyl

b, R = NHCH(CH$_2$OH)(CH$_2$)$_2$CH$_2$OH e, R = OH

c, R = NHCH(CH$_3$)(CH$_2$)$_2$CH$_3$ f, R = OEt

the ester functions occurred, and work-up of the reaction mixture after 15 min gave crystalline diol **3b** in 80% yield. Separation of solid salts of the reduced product from solution as well as the low reactivity of the amide carbonyl (due to resonance with the *p*-methylamino moiety) combine to prevent the undesired reduction of the amide group.

The chloride **2** condensed readily with **3a–d** in the presence of base to yield substituted pyrazine 1-oxides **4a–d.** Use of K_2CO_3 as the base, in aqueous THF, gave cleaner products for **4a–c** than did triethylamine in ethanol, which served well for the less soluble adamantylamine analog **4d.** These compounds were difficult to crystallize but were isolated in a fairly pure state as glasses or semisolids and used directly in subsequent reactions. Deoxygenation of the *N*-oxides was carried out by heating for 45 min at 125–130° with excess triethyl phosphite (for **5c,** and **5d,** isolated as crystalline solids) or with triethyl phosphite in DMF as solvent (for **5a** and **5b,** isolated as glasses). The progress of the latter reactions could be followed by thin-layer chromatography on silica gel (5–20% EtOH–benzene). Reaction of the substituted pyrazines **5a–d** with excess guanidine in refluxing ethanol gave MTX analogs **6a–d.** Saponification of **6a** constitutes a new synthesis of MTX. Similarly, starting with the substituted pyrazine 1-oxides **4a** and **4d,** reaction with guanidine gave two analogs, **7a** and **7d,** which contain an 8-oxide function. Overall yields of the MTX analogs which contained ester groups, **6a** and **7a,** were much lower than those for the other analogs, as a result of side reactions of the ester groups with guanidine in the last step of the synthesis. All the analogs were isolated as solids and purified by recrystallization from various solvents.

Following the same reaction scheme, starting with **2** and the readily available *p*-(methylamino)benzoic acid **3e** or its ethyl ester **3b,** efficient syntheses of the pteroic acid analog **6e**[14,19] (overall yield 60%) and the ethyl ester **6b** (overall yield 38%) were developed. These products, and their intermediates, were all crystalline solids which were easily isolated and purified. The synthesis of **6e** by this method makes this compound readily available in large quantities for use as an intermediate for the synthesis of other methotrexate analogs. For instance, we have found that **6e** reacts rapidly with various alkyl chloroformates in dimethylformamide to give mixed anhydrides (some of which can be isolated as stable crystalline solids), which can then react with various amines or amino acid esters to give side-chain altered analogs. In these reactions the amino groups of **6e** need not be protected. This latter observation has been made independently by Nair and Baugh.[25] Further work in this area will be reported shortly from this laboratory.

Biological Results. All the MTX and pteroate analogs synthesized in this work were evaluated for growth-inhibitory activity against the folate-dependent organism *Streptococcus faecium* (ATCC No. 8043) by the method of Foley and coworkers,[26] and the results are reported in Table I. Compounds **6a–f** had significant activity against this organism. On the other hand, the 8-oxido analogs **7a** and **7d** showed no activity. Comparison of the ID_{50} values for the 8-oxides with the values for the corresponding nonoxygenated species indicates the large, unfavorable effect on inhibition produced by this type of structural change. This effect may possibly be attributed to the lowering of the basicity of the diaminopteridine ring system by the polar 8-oxide function, which may alter the enzyme-binding properties of the molecule. On the other hand, it may be related to the detrimental geometrical effect observed for substituents in the 7 position of pteridine antifolates.[12,27]

Enzyme inhibition studies were performed on the same compounds with purified dihydrofolate reductase from three different sources. The results are tabulated in Table II. All of the compounds were less inhibitory than MTX by about 1–4 orders of magnitude. It is noteworthy that glutamate replacement (*i.e.*, compounds **6b–f**) tends to decrease activity against *Lactobacillus casei* and chicken liver dihydrofolate reductases more than against the enzyme from L1210-FR8. Also, it can be seen that the 8-oxido analogs **7a** and **7d** are less inhibitory than their nonoxygenated counterparts **6a** and **6d** against all three enzymes.

The compounds listed in Table I were assayed *in vivo* against two transplantable murine leukemias in ascites form: L1210 leukemia in BDF/1 hybrid mice and P1534

Table I. Inhibition of *S. faecium* (ATCC No. 8043) by MTX and Pteroate Analogs

Compd	ID_{50}, μg/ml[a]	Compd	ID_{50}, μg/ml[a]
6a	0.017	6e	0.002
6b	0.37	6f	0.001
6c	0.002	7a	1.0+
6d	0.003	7d	1.0+

[a] Folate = 0.001 μg/ml; under these conditions MTX had ID_{50} = 0.002 μg/ml.

Table II. Inhibition of Dihydrofolate Reductase by MTX and Pteroate Analogs[a]

	Concn (*M*) for 50% inhibition of dihydrofolate reductase from		
Compd	*L. casei*	Chicken liver	L1210-FR8
MTX	3×10^{-9}	3×10^{-8}	1.5×10^{-9}
6a	7×10^{-7}	5×10^{-6}	5×10^{-7}
6b	1×10^{-6}	2×10^{-5}	1×10^{-7}
6c	3×10^{-7}	1×10^{-5}	9×10^{-8}
6d	2×10^{-7}	2×10^{-7}	3×10^{-8}
6e	3×10^{-7}	3×10^{-6}	1×10^{-7}
6f	7×10^{-7}	6×10^{-6}	3×10^{-8}
7a	2×10^{-5}	8×10^{-5}	8×10^{-6}
7d	3×10^{-5}	$>4 \times 10^{-5}$	5×10^{-6}

[a] Assay conditions are listed in the Experimental Section.

leukemia in DBA/2 inbred mice. Each animal received daily intraperitoneal injections of the test compound, suspended in 10% Tween 80, for 4 days beginning on the first day after tumor implantation (qd 1-4). Dose levels were regularly spaced, usually ranging from 32 to 250 mg/kg per injection. In these experiments, significant antitumor activity was found only for the diethyl ester of methotrexate **6a**, which showed activity against L1210 leukemic mice (51% increase of survival; optimal dose 32 mg/kg, qd 1-4) comparable to that of MTX under the same conditions. The 8-oxido analog of diethyl MTX **7a** was only marginally active, showing a 22% extension of survival against the same tumor at an optimal dose of 64 mg/kg, qd 1-4. This result again reflects the detrimental biological effect of the 8-oxido substituent. Antitumor activity of MTX esters such as **6a** in transplantable mouse tumor systems is undoubtedly due, at least in part, to esterase hydrolysis to free MTX. Preliminary results indicate efficient hydrolysis of **6a** and other MTX esters by mouse serum and mouse ascites fluid as determined by both dihydrofolate reductase assay and solvent extraction after incubation with serum or ascites fluid.‡ These observations are in accord with preliminary data by Johns, *et al.*[28,29] The other compounds tested showed little or no activity against both tumors. Thus, although the *S. faecium* data were encouraging for several of these compounds, the preliminary observations indicate that drastic modification (aside from esterification) or deletion of the glutamate moiety in MTX does not improve *in vivo* antitumor activity against the two tumors tested. This may be due in part to impaired active transport properties of these molecules or to their poor solubility in aqueous media. Preliminary biological data on esters of MTX and dichloro-MTX have already been reported from this laboratory,[2] and more detailed studies are in progress to evaluate these compounds as latent or modified transport forms of these drugs.

Standard antimalarial evaluation of the compounds synthesized in this work was carried out against *Plasmodium berghei* in the mouse and *Plasmodium gallinaceum* in the chick as previously described.[30] None of the compounds proved to have activity in the mouse assays. However, the pteroate ester analog **6f** was found to have prophylactic activity in the sporozoite-induced *p. gallinaceum* assay when the compound was administered as a single subcutaneous dose, in oil, to leghorn chicks on the day of intravenous infection. Dose levels of 15 and 30 mg/kg resulted in cures (survival at 60 days postinfection; controls die at 6-11 days) in 4/5 chicks. Doses from 60 to 480 mg/kg resulted in cures in 5/5 chicks.

Experimental Section§

N-[[*p*-(*N'*-*p*-**Toluenesulfonyl)methylamino]benzoyl]-2-aminopentane (1c).** A mixture of *N*-tosyl-*p*-(methylamino)benzoyl chloride[23] (16.2 g, 0.05 mol), 2-aminopentane (4.35 g, 0.04 mol) (Columbia Organic Chemicals), and powdered $KHCO_3$ (5 g, 0.05 mol) in dry benzene (250 ml) was stirred under reflux for 2 hr, cooled, and filtered. The filtrate was added to 2% Na_2CO_3 (600 ml) and the two-phase mixture was stirred vigorously at room temperature for 1 hr. The organic layer was separated, washed with 2% HCl, rinsed with H_2O, dried (Na_2SO_4), and evaporated under reduced pressure. The pale yellow oily residue crystallized to a white solid (12.5 g, 67%) on trituration with *i*-Pr_2O. The analytical sample, mp 104-106°, was prepared by recrystallization from $CHCl_3$-*i*-Pr_2O: nmr ($CDCl_3$) δ 0.8-1.6 (m, 10 H, aliph CH_3 and CH_2), 2.40 (s, arom CH_3), 3.17 (s, NCH_3), 3.9-4.4 (m, NHC*H*-), 5.9-6.2 (m, NH), 7.0-7.9 (m, 8 H, arom protons). *Anal.* ($C_{20}H_{26}N_2O_3S$) C, H, N, S.

N-[[*p*-(*N'*-*p*-**Toluenesulfonyl)methylamino]benzoyl]-1-aminoadamantane (1d).** To a rapidly stirred suspension of *N*-tosyl-*p*-(methylamino)benzoyl chloride[23] (69 g, 0.213 mol) and 1-aminoadamantane hydrochloride (40 g, 0.273 mol) (a gift from E. I. du Pont de Nemours Co., Inc., Wilmington, Del.) in CH_2Cl_2 (1 l.) was added a solution of $KHCO_3$ (100 g, 1 mol) in H_2O (700 ml). After vigorous overnight mechanical agitation, the layers were separated, and the organic layer was washed with 1 *N* HCl (1 l.), dried (Na_2SO_4), and evaporated under reduced pressure. Recrystallization of the residue from EtOH gave a colorless solid (89 g, 95%): mp 158-159°. *Anal.* ($C_{25}H_{30}N_2O_3S$) C, H, N, S.

***N*-(*p*-Methylaminobenzoyl)-2-aminopentane-1,5-diol (3b).** Lithium aluminum hydride (3.8 g, 0.1 mol) was added in portions over a period of 2 min to a stirred solution of **3a**[23] (13.4 g, 0.02 mol) in dry THF (300 ml). After stirring for 15 min the thick slurry was carefully treated with H_2O (3.8 ml), 15% aqueous NaOH (3.8 ml), and finally with H_2O (11.4 ml). The mixture was filtered, and the filter cake washed with THF. Evaporation of the filtrate and washings under vacuum gave a pale yellow oil which crystallized when scratched. Trituration with 1:1 EtOAc-hexane (50 ml) and filtration gave a white solid (8.1 g, 80%): mp 132-136°. The analytical sample was obtained as colorless needles (from CH_3CH): mp 144-145°; nmr (CD_3CO_2D) δ 1.66 (m, $-CH_2CH_2-$), 2.83 (s, CH_3N), 3.72 (m, 4 H, $-CH_2O$), 4.20 (m, CH), 7.22 (m, C_6H_4). *Anal.* ($C_{13}H_{20}N_2O_3$) C, H, N.

***N*-(*p*-Methylaminobenzoyl)-2-aminopentane (3c).** A solution of **1c** (17.7 g, 0.047 mol) in 88.4 ml of 30-32% HBr in AcOH (Fisher Chemical Co.) was treated with phenol (8.8 g, 0.094 mol) and stirred at room temperature for 5 hr. Et_2O (850 ml) was added and stirring continued for another 20 min. The mixture

‡A. Rosowsky, M. H. N. Tattersall, and E. J. Modest, unpublished results.

§Ir spectra were taken with a Perkin-Elmer Model 137B double-beam recording spectrophotometer. Quantitative uv spectra were measured on Cary Model 11 and 15 spectrophotometers. Nmr spectra were determined on a Varian A-60 instrument with Me_4Si as internal standard. Melting points were measured in Pyrex capillary tubes in a Mel-Temp apparatus (Laboratory Devices, Inc., Cambridge, Mass.) and are uncorrected. Microanalyses were performed by Galbraith Laboratories, Knoxville, Tenn. Where analyses are indicated only by a symbol of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical value.

was filtered, the solid was dissolved in a minimal volume of H_2O, a small amount of insoluble gum was filtered off, and the aqueous solution was basified with 1 *N* $KHCO_3$ until a dense white precipitate appeared. The product was collected, washed with H_2O, and dried *in vacuo*: yield 8.2 g (79%). For microanalysis, a sample was recrystallized from benzene–hexane: mp 90–92°; uv λ max (EtOH) 290 nm (ε 19,500); nmr ($CDCl_3$) δ 0.8–2.7 (m, 10 H, aliph CH_3 and CH_2), 2.90 (s, NCH_3), 3.9–4.4 (m, NHC*H*–), 5.6–6.1 (m, NH), 6.63 (d, 2 H, arom), 7.66 (d, 2 H, arom). *Anal.* ($C_{13}H_{20}N_2O$) C, H, N.

***N*-(*p*-Methylaminobenzoyl)-1-aminoadamantane (3d).** Starting with **1d** and following the procedure as outlined above for **3c**, **3d** was obtained in 93% yield. The analytical sample was recrystallized from EtOH (charcoal) in the form of colorless needles: mp 175–177°. *Anal.* ($C_{18}H_{24}N_2O$) C, H, N.

Diethyl *N*-[*p*-[[(2-Amino-3-cyano-5-pyrazinyl)methyl]methylamino]benzoyl]glutamate (5a). To a stirred solution of **2**[22] (9.2 g, 0.05 mol) and **3a**[23] (16.8 g, 0.05 mol) in THF (150 ml) was added a solution of K_2CO_3 (10.35 g, 0.075 mol) in H_2O (150 ml) with slight external cooling. After stirring at room temperature for 1 hr, H_2O (400 ml) was added and the mixture was extracted with CH_2Cl_2. The extracts were washed with H_2O, dried (Na_2SO_4), and evaporated to leave crude **4a** as an orange glass, which was dissolved in a mixture of DMF (50 ml) and triethyl phosphite (75 ml) and heated at 125° for 45 min. Evaporation under reduced pressure left a red glass, which was chromatographed on silica gel (300 g) with 3:7 EtOAc–benzene as the eluent. The main fraction yielded **5a** (tlc on silica gel, R_f ~0.3) as a yellow oil (10.5 g, 45%): ir ($CHCl_3$) λ 2.90 (NH_2), 4.50 (CN), 5.80 μ (CO_2Et).

***N*-[*p*-[[(2-Amino-3-cyano-5-pyrazinyl)methyl]methylamino]benzoyl]-2-aminopentane (5c).** Following the procedure outlined above, condensation of **2** and **3c** yielded **4c** as a yellow glass in 63% yield after chromatography on silica gel (5% EtOH–benzene): ir ($CHCl_3$) λ 2.80, 2.93, 3.23, 4.48, 6.10, 6.22, 6.50, 6.70 μ. Treatment with excess triethyl phosphite at 125° for 45 min and evaporation under vacuum gave a solid which was dissolved in hot EtOH, treated with charcoal, and evaporated. Recrystallization of the residue from EtOAc–hexane gave **5c** as a yellow solid (72%). The analytical sample had mp 184–185°: uv λ max (EtOH) 247.5 nm (ε 20,110), 295.5 (25,600), 355 (7410). *Anal.* ($C_{19}H_{24}N_6O$) C, H, N.

***N*-[*p*-[[(2-Amino-3-cyano-5-pyrazinyl)methyl]methylamino]benzoyl]-1-aminoadamantane (5d).** Triethylamine (10 ml) was added dropwise to a stirred suspension of **2** (6.8 g, 0.053 mol) and **3d** (10 g, 0.035 mol) in EtOH (100 ml). The mixture was refluxed for 1 hr and cooled, and the solid (**4d**) was filtered, washed with aqueous EtOH, and dried (14.2 g, 94%). The crude product (10 g, 0.024 mol) was heated with triethyl phosphite (160 ml) at 130° for 45 min. Upon cooling and filtration, a cream-colored solid was obtained (8.5 g, 88%). Recrystallization from EtOH afforded analytically pure off-white needles: mp 211–213° dec: uv λ max (EtOH) 249 nm (ε 15,900), 296 (23,500), 356 (6750). *Anal.* ($C_{24}H_{28}N_6O$) C, H, N.

Diethyl *N*-[*p*-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]glutamate (6a). Guanidine hydrochloride (1.91 g, 0.02 mol) was added to a stirred solution of sodium ethoxide prepared from sodium metal (460 mg, 0.02 g-atom) and EtOH (200 ml). After being stirred for 5 min the mixture was filtered to remove NaCl and the filtrate was refluxed with **5a** (8.2 g, 0.0175 mol) for 1.5 hr. The hot solution was treated with charcoal and filtered, and the filtrate was evaporated under vacuum to leave a gummy solid. The solid was dissolved in a warm mixture of 1:4 EtOH–$CHCl_3$ (100 ml) and filtered through a column containing silica gel (100 g), the same solvent mixture being used as eluent. Evaporation of the combined eluents gave a yellow solid which was triturated with cold CH_3CN and filtered (3.0 g, 33.6%): mp 153–158°. Recrystallization from CH_3CN gave yellow prisms: double mp 159–161 and 226–230°. The ir spectrum was identical with that of the compound prepared by direct esterification of commercial methotrexate.[2] *Anal.* ($C_{24}H_{30}N_8O_5$) C, H, N.

***N*-[*p*-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-2-aminopentane-1,5-diol (6b).** Compounds **2** and **3b** were condensed in aqueous THF as outlined above. The crude diol **4b** was isolated as an orange glass by addition of excess saturated aqueous NaCl solution to the reaction mixture and separation of the THF layer, which was then evaporated under vacuum. After treatment of this product with DMF–triethyl phosphite at 125° for 45 min and evaporation under vacuum, the residue was refluxed with excess EtOH for 1 hr to cleave any diol–phosphite ester which may have formed by transesterification. Condensation of this product with guanidine as outlined above gave crude **6b** which was purified by chromatography on silica gel with 1:4 MeOH–$CHCl_3$ as the eluent (tlc on silica gel, R_f ~0.5). A yellow solid was obtained (35% overall yield): mp 223–226° dec (H_2O). *Anal.* ($C_{20}H_{26}N_8O_3 \cdot 0.5H_2O$) C, H, N.

***N*-[*p*-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-2-aminopentane (6c).** Condensation of **5c** with excess guanidine in refluxing EtOH for 1 hr, cooling, and filtration of the precipitated yellow solid gave **6c** (80%): mp 264–266° dec; uv λ max (EtOH) 261 nm (ε 25,000), 291 (23,800), 375 (7830). *Anal.* ($C_{20}H_{26}N_8O$) C, H, N.

***N*-[*p*-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-1-aminoadamantane (6d).** Condensation of **5d** with excess guanidine in refluxing EtOH for 1 hr, cooling, and filtration gave **6d** (94%): mp 265–268° dec. Recrystallization from EtOH gave the analytical sample as a bright yellow solid: mp 273–275° dec; uv λ max (EtOH) 262 nm (ε 29,500), 286 (28,200), 374 (10,200). *Anal.* ($C_{25}H_{30}N_8O \cdot 0.3H_2O$) C, H, N.

Diethyl *N*-[*p*-[[(2,4-Diamino-8-oxido-6-pteridinyl)methyl]methylamino]benzoyl]glutamate (7a). Following the same procedure as outlined above for **5a**, but with omission of the triethyl phosphite deoxygenation step, crude **4a** was isolated and purified by chromatography on silica gel with 7:3 EtOAc–benzene as the eluent. The product was obtained as a yellow glass (46% yield; tlc on silica gel, R_f ~0.45). Condensation with guanidine, as outlined above for **6a**, gave **7a** as a yellow solid (25%): mp 158–164° (EtOH–H_2O). *Anal.* ($C_{24}H_{30}N_8O_6 \cdot H_2O$) C, H, N.

***N*-[*p*-[[(2,4-Diamino-8-oxido-6-pteridinyl)methyl]methylamino]benzoyl-1-aminoadamantane (7d).** Crude **4d** (as prepared above under **5d**) was condensed with excess guanidine in refluxing EtOH for 1 hr. The crude product (92% yield, mp 225–230° dec) was recrystallized from EtOH (charcoal) to give orange-yellow microcrystals: mp 262–264° dec. *Anal.* ($C_{25}H_{30}N_8O_2 \cdot 0.55H_2O$) C, H, N.

2-Amino-3-cyano-5-[(*p*-carboxy-*N*-methylanilino)methyl]pyrazine 1-Oxide (4e) and the Ethyl Ester 4f. Triethylamine (10.1 g, 0.1 mol) was added to a stirred slurry of **2** (18.4 g, 0.1 mol) and **3e** (15.1 g, 0.1 mol) in EtOH (175 ml). After 1 hr the precipitated orange solid was filtered, washed with 1:1 EtOH–H_2O, and dried (26.5 g, 88.5%): mp 235–242° dec. Recrystallization from DMF–H_2O gave orange needles: mp 246–250° dec. *Anal.* ($C_{14}H_{13}N_5O_3$) C, H, N.

Similarly, **2** and **3f**[31] afforded the ethyl ester **4f** in 50% yield: yellow prisms; mp 164–165° (EtOAc–hexane); uv λ max (EtOH) 252 nm (ε 25,790), 303 (30,400), 380 (7700). *Anal.* ($C_{16}H_{17}N_5O_3$) C, H, N.

2-Amino-3-cyno-5-[*p*-carboxy-*N*-methylanilino)methyl]pyrazine (5e) and the Ethyl Ester 5f. A solution of **4e** (28.1 g, 0.094 mol), freshly distilled triethyl phosphite (100 ml), and DMF (250 ml) was heated at 125° for 1 hr. The reaction was monitored by tlc on silica gel with 20% MeOH–$CHCl_3$ as the developing solvent. Evaporation under vacuum left a yellow solid, which was triturated with 1:1 EtOH–Et_2O (100 ml) and filtered (22 g, 82.6%). Recrystallization from DMF–H_2O gave yellow prisms: mp 248–252° dec. *Anal.* ($C_{14}H_{13}N_5O_2$) C, H, N.

The ethyl ester **4f** was deoxygenated by heating in excess triethyl phosphite without DMF. Evaporation under vacuum, trituration with *i*-Pr_2O, and filtration gave **5f** in 88% yield. Recrystallization from EtOAc–hexane gave yellow prisms: mp 166–168° dec (softening at 158°); uv λ max (EtOH) 249 nm (ε 14,600), 306 (28,280), 355 (7160). *Anal.* ($C_{16}H_{17}N_5O_2$) C, H, N.

***p*-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoic Acid (6e)[14,19] and the Ethyl Ester 6f.** Guanidine hydrochloride (6.0 g, 0.063 mol) was added to a solution of sodium methoxide prepared from sodium metal (2.6 g, 0.113 g-atom) and MeOH (250 ml). Acid **5e** (14.15 g, 0.050 mol) was added and the mixture was refluxed for 2.5 hr, concentrated under vacuum to a volume of 100 ml, and then poured into H_2O (1 l.). The mixture was heated on a steam bath, treated with charcoal, and filtered with suction through a Büchner funnel containing a matting of infusorial earth, and the filter cake was washed with H_2O. The clear orange filtrate was acidified to pH 3 with dilute HCl, and the precipitated solid was filtered and washed with H_2O, EtOH, and finally ether: yield 15 g (81.3%); mp 246–250° dec; nmr (DMSO-d_6) δ 3.25 (s, NCH_3), 4.84 (s, CH_2N), 7.33 (m, C_6H_4), 8.66 (s, ring CH). *Anal.* ($C_{15}H_{15}N_7O_2 \cdot 0.5HCl \cdot 1.5H_2O$) C, H, N, Cl. Recrystallization from DMF gave a yellow powder: mp >300° dec. *Anal.* ($C_{15}H_{15}N_7O_2 \cdot 0.5H_2O$) C, H, N.

The ethyl ester **6f** was prepared by treatment of **5f** with excess

guanidine in refluxing ethanol for 3 hr. The product precipitated from solution as a tan powder: mp 293–296° dec; uv λ max (EtOH) 261 nm (ε 22,600), 305 (28,300), 377 (7630). *Anal.* ($C_{17}H_{19}N_7O_2$) C, H, N.

Dihydrofolate (DHF) Reductase Assays. In all cases an amount of enzyme was added which yielded a ΔOD_{340} of 0.02/min in the absence of inhibitor.

1. *L. casei:* dihydrofolate reductase derived from an MTX-resistant strain, DHF 50 μ*M*, NADPH 80 μ*M*, Tris HCl 0.05 *M*, 2-mercaptoethanol 0.01 *M*, EDTA 0.001 *M*, protein 0.0012 mg, pH 7.4, 30°. Reaction initiated with enzyme.

2. Chicken liver: conditions as above except protein 0.014 mg.

3. L1210-FR8: DHF 30 μ*M*, NADPH 60 μ*M*, Tris HCl 0.1 *M*, KCl 0.15 *M*, 2-mercaptoethanol 0.1 *M*, pH 7.5, 37°. Enzyme preincubated 2 min with reaction mixture plus inhibitor minus DHF. Reaction initiated with DHF.

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