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Methotrexate Analogs. 6. Replacement of Glutamic Acid by Various Amino Acid Esters and Amines

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A series of methotrexate (MTX) analogs was prepared in which the glutamic acid moiety is replaced by various amino acid esters and amines. The synthetic method consisted of the reaction of 4-amino-4-deoxy-N^{10}-methylpteroic acid with various reagents to form intermediate mixed anhydrides, which then reacted with amino acid esters or amines to give the MTX analogs. These compounds were tested for antibacterial activity against *Streptococcus faecium* and for antitumor activity against L1210 leukemia in mice. Several compounds showed significant antibacterial activity; the MTX homocysteinethiolactone and MTX aspartate analogs showed marginal in vivo antitumor activity.

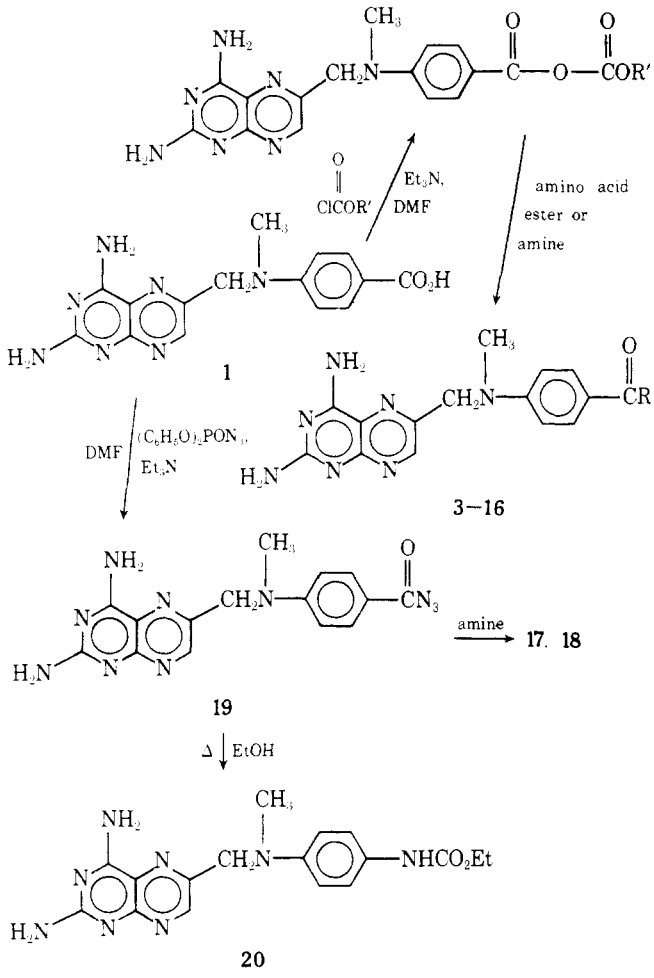
For the past several years, work has been conducted in this laboratory aimed at the structural modification of the antitumor agent methotrexate (4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid, amethopterin, MTX)[1] in order to prepare analogs with modified transport properties and improved biological activity. The initial work involved a total synthesis scheme whereby analogs were prepared in which the carboxyl groups of the glutamic acid moiety of MTX were replaced by less polar groups such as CH_2OH and CH_3 and analogs in which glutamic acid was completely replaced by adamantylamine.[2,3] Lipophilic alkyl esters of MTX and 3′,5′-dichloromethotrexate have also been prepared and investigated as "latent" forms of these drugs.[3,4] Other MTX analogs prepared in this laboratory include N^8-oxides,[3] 7-methyl derivatives,[5] and 7,8-dihydro-8-methylmethotrexate.[6]

During the course of this work, an efficient synthesis of 4-amino-4-deoxy-N^{10}-methylpteroic acid (1) was developed,[2,3] which allowed this compound to be prepared in large quantities for use as an intermediate for the preparation of other side-chain modified MTX analogs. We were interested in preparing additional MTX analogs for biological evaluation in which glutamic acid is replaced by various amino acid esters and amines. Many folate analogs and aminopterin analogs in which the glutamate moiety is replaced by other amino acids have been reported,[7,8] but relatively few MTX analogs of this type have been prepared and evaluated for biological activity. It has been shown that the MTX aspartate analog[9] and D-glutamate analog[10] exhibit antitumor activity against L1210 leukemia in mice.

Starting with the pteroic acid analog 1, the compounds listed in Table I were prepared and evaluated for biological activity. The synthetic method employed consisted of the reaction of 1 with various reagents to form intermediate mixed anhydrides,[11] which then reacted with amines or amino acid esters to give the products, in which the side-chain R was varied to include straight-chain, branched-chain, and cyclic structural features.

Chemistry. The pteroic acid analog 1 is not appreciably soluble in most organic solvents. However, as its partial hydrochloride hydrate ($C_{15}H_{15}N_7O_2 \cdot 0.5HCl \cdot 1.5H_2O$)[3] it is sufficiently soluble in DMF (1–2 g/100 ml) to permit this solvent to be used as a reaction medium. It was found that 1 reacted rapidly with an excess of isobutyl or isopropyl chloroformate, in DMF at room temperature and in the presence of Et_3N, to form mixed anhydrides (Scheme I).

Scheme I



These reactions were complete within 5–10 min and could be followed on TLC (silica gel, 10% MeOH–$CHCl_3$). The mixed anhydrides were usually not isolated but could be made to react with the amino nitrogen of an amino acid ester or amine at room temperature, or at elevated temperatures, forming a peptide linkage to yield the desired products. In these reactions, the amino groups of **1**, which are only weakly nucleophilic, need not be protected. This observation has been made independently by Nair and Baugh in their work on the synthesis of poly-γ-glutamyl derivatives of MTX.[12] In one instance, the mixed anhydride **2** was isolated and characterized and found to be a fairly stable yellow solid.

Reaction of a nucleophilic amine with the mixed anhydride can occur at either of the carbonyl groups. If attack occurs at the carbonyl group adjacent to the phenyl ring, the desired MTX analog is produced. On the other hand, if attack occurs at the other carbonyl group, a urethane and the pteroate analog **1** are the products. In the present cases, nucleophilic attack of the amino acid esters and amines occurred at both of the carbonyl groups in the mixed anhydrides. The MTX analogs were usually isolated by concentration of the reaction mixture under vacuum, followed by addition of dilute NH_4OH and extraction of the product with $CHCl_3$. Urethanes and other by-products were removed from the concentrated extracts by trituration with various solvents. In most cases, acidification of the basic aqueous phase led to recovery of fairly pure acid **1**. Although the yields of the MTX analogs were only low to moderate, they may be considered good if recovered **1** is taken into account.

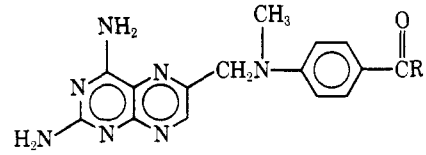
The alkyl chloroformate most frequently used for the preparation of the mixed carboxylic–carbonic anhydride was the isopropyl rather than the isobutyl derivative, since it was found that the former gave slightly higher yields of the MTX analog. This is illustrated in the preparation of compound **4** (Table I). In the preparation of the prolinate analog **12**, the mixed carboxylic–carbonic anhydrides gave very low yields of product. Higher yields of **12** were obtained when the carboxylic acid chlorides, 2-ethylbutyryl chloride or pivaloyl chloride, were used instead of the alkyl chloroformates to form intermediate carboxylic–carboxylic anhydrides. The bulky alkyl groups of these reagents probably diminish by-product formation by a combination of steric and electronic effects on the adjacent carbonyl group in the mixed anhydride. The use of these reagents did not lead to increased yields for several of the other MTX amino ester analogs.

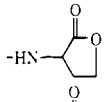
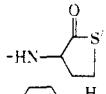
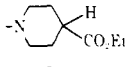
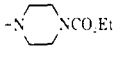
During the course of this investigation a communication appeared describing the use of a new reagent, diphenylphosphoryl azide, in peptide synthesis.[13] This method consists of the reaction of an acylamino acid or peptide and an amino acid or peptide ester hydrochloride, in DMF, in the presence of the phosphoryl azide and Et_3N to form a peptide linkage. Application of this method to the present work (Scheme I) did not lead to improvements in the yields of MTX analogs in which glutamate is replaced by a primary α-amino ester. However, it worked fairly well for the synthesis of MTX analogs **17** and **18** in which a peptide linkage is formed from acid **1** and a secondary cyclic amino function. When **1** was treated with diphenylphosphoryl azide and Et_3N alone in DMSO for 20 hr at room temperature, the carbonyl azide **19** precipitated from solution in good yield. This compound was fairly stable at room temperature although it was somewhat photosensitive. It could be stored at 0° in the dark for several months without appreciable decomposition. This compound is of great interest for potential use in photoaffinity labeling studies[14] on the enzymes involved in folate metabolism. Reaction of **19** in refluxing EtOH resulted in the formation of the urethane **20**.

Biological Results. All the compounds synthesized in this work were tested for antibacterial activity against the folate-dependent organism *Streptococcus faecium* (ATCC No. 8043) by the method of Foley and coworkers[15] and the results are reported in Table II. Several of these compounds showed significant activity toward this organism. These same compounds were assayed in vivo against L1210 murine leukemia (ascites form) in BDF/1 hybrid mice. Since esterase activity in mice is significantly higher than in larger animals,[16] it may be reasonable to assume that the MTX analogs which contain ester (or lactone) functional groups were at least partially hydrolyzed in vivo to the free acids. It has been shown that ordinary alkyl esters of MTX are rapidly hydrolyzed in vivo and in vitro by mouse serum and ascites fluid.[3,17] The test compounds, suspended in 10% Tween 80, were injected intraperitoneally into each animal beginning 24 hr after tumor implantation (ip 10^5 cells). Additional injections were given either daily until day 4 (qd 1–4) or spaced 3 days apart until day 7 (q3d 1, 4, 7). Nontoxic dose levels were regularly spaced, usually ranging from 10 to 80 mg/kg per injection. The thiolactone analog **16** showed marginal antitumor activity (20–30% increase in survival time) at dose levels of 80–160 mg/kg, and the aspartate analog **11** was less active (10–20% increase in survival time at 20–80 mg/kg). All of the other compounds were inactive against this tumor. The prolinate analog **12** was peculiar in that it was toxic at a dose level of as low as 30 mg/kg, causing paralysis to the hind legs of the animals.

These results reaffirm our earlier observations[3] that drastic changes in the glutamic acid portion of MTX do not

Table I. MTX Analogs



Compd	R	Mixed anhyd[a] forming reagent	Yield, %[b]	Mp, C°[c]	Recrystn solvent	Formula	Analyses[d]
2	$-OC(=O)OCH_2CH(CH_3)_2$	A	53	170–180	EtOH	$C_{20}H_{23}N_7O_4$	C, H, N
3	Ethyl 4-aminobutyrate	A	13	228–230	EtOH	$C_{21}H_{26}N_8O_3$	C, H, N
4	Methyl DL-2-amino-butyrate	A, B	30, 41	207–210	CH_3CN–Et_2O	$C_{20}H_{24}N_8O_3$	C, H, N
5	Ethyl L-leucinate	B	20	134–138	CH_3CN	$C_{23}H_{30}N_8O_3$	C, H, N
6	Methyl DL-norleucinate	B	40	194–196	CH_3CN	$C_{22}H_{28}N_8O_3$	C, H, N
7	Ethyl L-methioninate	B	33	142–144	CH_3CN	$C_{22}H_{28}N_8O_3S$	C, H, N, S
8	Ethyl L-tryptophanate	B	31	150–165	CH_3CN	$C_{28}H_{29}N_9O_3$	C, H, N
9	Methyl L-tyrosinate	B	12	160–170	EtOH	$C_{25}H_{26}N_8O_4$	C, H, N
10	Methyl ϵ-carbobenzyloxy-L-lysinate	B	37	110–114	CH_3CN	$C_{30}H_{35}N_9O_5$	C, H, N
11	Dimethyl DL-asparate	B	25	135–140,[e] 178–180	CH_3CN	$C_{21}H_{24}N_8O_5$	C, H, N
12	Methyl L-prolinate	A, C, D	10, 27, 40	210–212	CH_3CN	$C_{21}H_{24}N_8O_3$	C, H, N
13	Cyclohexanamine	A	45	305–308	DMF–H_2O	$C_{21}H_{26}N_8O$	C, H, N
14	Morpholine	A	30	259–261	DMF–H_2O	$C_{19}H_{22}N_8O_2$	C, H, N
15		B	36	220–230	DMF–H_2O	$C_{19}H_{20}N_8O_3$	C, H, N
16		B	12	230–240	CH_3CN	$C_{19}H_{20}N_8O_2S$	C, H, N, S[d]
17		E	51	175–179,[e] 210–215	CH_3CN	$C_{23}H_{28}N_8O_3$	C, H, N
18		E	50	220–222,[e] 230–233	CH_3CN	$C_{22}H_{27}N_9O_3$	C, H, N
19	$-N_3$ (azide)	E	86	280–290	*g*	$C_{15}H_{14}N_{10}O$	C, H, N

[a] A = isobutyl chloroformate, B = isopropyl chloroformate, C = 2-ethylbutyryl chloride, D = pivaloyl chloride, E = diphenylphosphoryl azide. [b] Yields are calculated on homogeneous products. [c] Melting point is on recrystallization product. [d] Analyses were within ±0.3% of the theoretical value except for compound 16; N: calcd, 25.86; found, 26.40. [e] Double melting point. [f] α-Amino-γ-butyrolactone hydrobromide and DL-homocysteinethiolactone hydrochloride were purchased from Aldrich Chemical Co. [g] See Experimental Section.

Table II. Inhibition of *S. faecium* (ATCC No. 8043) by MTX Analogs

Compd	ID_{50}, μg/ml[a]	Compd	ID_{50}, μg/ml[a]
2	0.0001	12	0.030
3	0.003	13	0.002
4	0.020	14	0.018
5	0.003	15	0.350
6	0.010	16	0.010
7	0.001	17	0.005
8	0.006	18	0.010
9	0.010	19	0.005
10	0.035	20	0.005
11	0.005		

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

improve antitumor activity against the L1210 tumor. This could be due to poor penetration of these analogs into cells and/or to weak inhibition of enzymes involved in folate metabolism.

Experimental Section

Ir spectra were taken with a Perkin-Elmer Model 137B double-beam spectrophotometer. 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.

Detailed experimental procedures are given only for selected compounds, which will serve to illustrate the general synthetic methods employed.

4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl Isobutyl Carbonate (2). The pteroic acid analog 1 (1.11 g, 3.0 mmol; $C_{15}H_{15}N_7O_2 \cdot 0.5HCl \cdot 1.5H_2O$) was dissolved in hot DMF (100 ml) and the solution cooled to room temperature. With stirring, Et_3N (604 mg, 6.0 mmol) was added, followed immediately by isobutyl chloroformate (615 mg, 4.5 mmol). After stirring for 10 min, TLC (silica gel, 10% MeOH–$CHCl_3$) showed that the formation of the mixed anhydride was complete by the absence of any material at the origin and a single spot for the mixed anhydride at R_f 0.72. The DMF was removed under vacuum at 60° and the residue was treated with 0.3 *N* NH_4OH (100 ml) and extracted with $CHCl_3$ (2 × 100 ml). The extracts were washed with H_2O, dried (Na_2SO_4), and evaporated to leave a residue which was triturated with a mixture of CH_3CN (10 ml) and Et_2O (20 ml). Filtration gave 672 mg (53%) of yellow solid: mp 170–180° (foaming); ir (KCl) λ 5.5, 5.80, 6.10, 6.20, 6.40, 6.55, 6.90, 7.25, 7.35, 8.20, 8.60 μ.

Methyl *N*-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methyla-

mino]benzoyl]-DL-2-aminobutyrate (4). To a stirred solution of 1 (1.11 g, 3.0 mmol) in DMF (100 ml) at room temperature was added Et_3N (605 mg, 6.0 mmol) followed immediately by isopropyl chloroformate (550 mg, 4.5 mmol). After 10 min a solution of methyl DL-2-aminobutyrate hydrochloride (920 mg, 6.0 mmol) and Et_3N (605 mg, 6.0 mmol) in DMF (10 ml) was added and the solution heated at 80° for 20 min. The solution was then concentrated under vacuum to a volume of 5 ml, 0.3 *N* NH_4OH (100 ml) was added, and the mixture was extracted with $CHCl_3$ (2 × 100 ml). The extracts were washed with H_2O, dried (Na_2SO_4), and evaporated to leave a paste which was triturated with a mixture of CH_3CN (5 ml) and Et_2O (20 ml) and then filtered. There was obtained 520 mg (41%) of yellow solid, mp 207–210° dec. The aqueous portion was acidified with HCl to pH 3 and filtered to yield 400 mg (36%) of **1**, mp 250–253° dec.

Methyl *N*-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-L-tyrosinate (9). To a stirred solution of 1 (2.22 g, 6.0 mmol) in DMF (200 ml) at room temperature was added Et_3N (1.21 g, 12.0 mmol) followed immediately by isopropyl chloroformate (1.1 g, 9.0 mmol). After 10 min a solution of L-tyrosine methyl ester hydrochloride (2.78 g, 12.0 mmol) and Et_3N (1.21 g, 12.0 mmol) in DMF (20 ml) was added and the mixture stirred for 20 hr. The solution was then concentrated under vacuum to a volume of 10 ml and hot 0.5 *N* aqueous $NaHCO_3$ (200 ml) was added. After cooling to room temperature the aqueous portion was decanted and the gummy residue was heated for 15 min with a mixture of MeOH (50 ml) and 0.2 *N* aqueous $NaHCO_3$ (200 ml). After cooling, the aqueous portion was again decanted and the gummy residue was triturated with cold H_2O until it solidified. The brown solid was filtered, then dissolved in hot EtOH (40 ml), treated with charcoal, and filtered. Evaporation of the filtrate left a gum which was dissolved in a hot mixture of CH_3CN (50 ml) and MeOH (5 ml) and cooled overnight. The precipitated yellow solid was filtered to yield 360 mg (12%), mp 155–170° dec.

Methyl *N*-[4-[[2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-L-prolinate (12). The same procedure as described for **4** was used for the preparation of this compound in three separate experiments, with substitution of isobutyl chloroformate, 2-ethylbutyryl chloride, and pivaloyl chloride for the isopropyl chloroformate, and the use of L-proline methyl ester hydrochloride as the amino acid ester. The use of pivaloyl chloride to form the mixed anhydride led to the best yield (40%) of product.

***N*-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]cyclohexanamine (13).** To a stirred solution of 1 (1.11 g, 3.0 mmol) in DMF (120 ml) was added Et_3N (455 mg, 4.5 mmol) followed immediately by isobutyl chloroformate (615 mg, 4.5 mmol). After 10 min cyclohexanamine (1.19 g, 12.0 mmol) was added, the solution was stirred for 30 min, and the DMF was then removed under vacuum to leave a yellow solid. The solid was treated with 0.5 *N* NH_4OH (100 ml), heated on a steam bath for 10 min, and cooled, and the yellow solid was filtered and washed with EtOH. The yield was 550 mg (45%), mp 290–300° dec. The aqueous filtrate was acidified with HCl to pH 3 and the precipitated solid was filtered to give 500 mg (45%) of **1**, mp 251–253° dec.

Ethyl *N*-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]piperidine-4-carboxylate (17). A stirred solution of 1 (1.11 g, 3.0 mmol), diphenylphosphoryl azide (1.03 g, 3.75 mmol) (Willow Brook Labs, Inc., Waukesha, Wis.), Et_3N (1.01 g, 10 mmol), and ethyl isonipecotate (588 mg, 3.75 mmol) (Aldrich Chemical Co.) in DMF (75 ml) was heated at 50° for 30 min. The mixture was evaporated under vacuum to a volume of 5 ml, treated with 0.3 *N* NH_4OH (100 ml), and extracted with $CHCl_3$ (2 × 100 ml). The extracts were evaporated to leave a solid residue which was dissolved in a minimal volume of 10% EtOH–$CHCl_3$ and filtered through silica gel (30 g) using the same solvent mixture as eluent. Evaporation of the eluent left a yellow solid which was triturated with a mixture of CH_3CN (10 ml) and Et_2O (10 ml) and then filtered to give 714 mg (51%), mp 168–172°.

When compound **19** was used as the starting material and heated with ethyl isonipecotate, in the presence of Et_3N in DMF under the same conditions, the yield of **17** was about the same as above.

4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl Azide (19). A solution of 1 (7.38 g, 0.02 mol), diphenylphosphoryl azide (7.15 g, 0.026 mol), and Et_3N (3.64 g, 0.036 mol) in DMSO (120 ml) was stirred at room temperature for 20 hr, during which time a solid precipitated. The mixture was diluted with 0.3 *N* NH_4OH (200 ml) and filtered, and the solid was washed with H_2O, 50% EtOH, and finally with ether. After drying at 60° under vacuum for several hours there was obtained 6.0 g (86%) of dark yellow solid, mp 260–280° dec. For analysis a small sample was dissolved in hot 10% MeOH–$CHCl_3$ and filtered through silica gel using the same solvent mixture as eluent. Evaporation of the eluent gave a yellow solid: mp 280–290° dec; ir (KCl) λ 2.95, 4.63, 5.95, 6.10, 6.22, 6.38, 6.52, 6.88 μ.

Ethyl *N*-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]phenyl]carbamate (20). A mixture of **19** (1.4 g, 4.0 mmol) and EtOH (300 ml) was heated at reflux for 24 hr, then treated with charcoal while still hot, and filtered. The filtrate was evaporated to a volume of 75 ml and cooled for several hours, and the precipitated solid was filtered to yield 254 mg (17%) of orange solid: mp 270–290° dec (carbonizes); ir (KCl) λ 2.95, 3.10, 5.88, 5.95, 6.10, 6.27, 6.42, 6.54, 6.85 μ. Anal. ($C_{17}H_{20}N_8O_2$) C, H, N.

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