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Methotrexate Analogues. 9. Synthesis and Biological Properties of Some 8-Alkyl-7,8-dihydro Analogues

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A series of 8-alkyl-7,8-dihydromethotrexate analogues was prepared by direct alkylation of 7,8-dihydromethotrexate, after pilot studies were performed with simpler pteridines. These compounds were tested for in vitro inhibitory activity against *Lactobacillus casei* and as enzyme inhibitors against dihydrofolate reductase and thymidylate synthetase derived from this organism. All of the analogues were less inhibitory toward dihydrofolate reductase than was methotrexate but were more inhibitory toward thymidylate synthetase. The analogues were also evaluated for in vitro inhibitory activity against the CCRF-CEM human lymphoblastic leukemia cells. In vivo against the L-1210 leukemia in mice, several of the analogues exhibited some antileukemic activity.

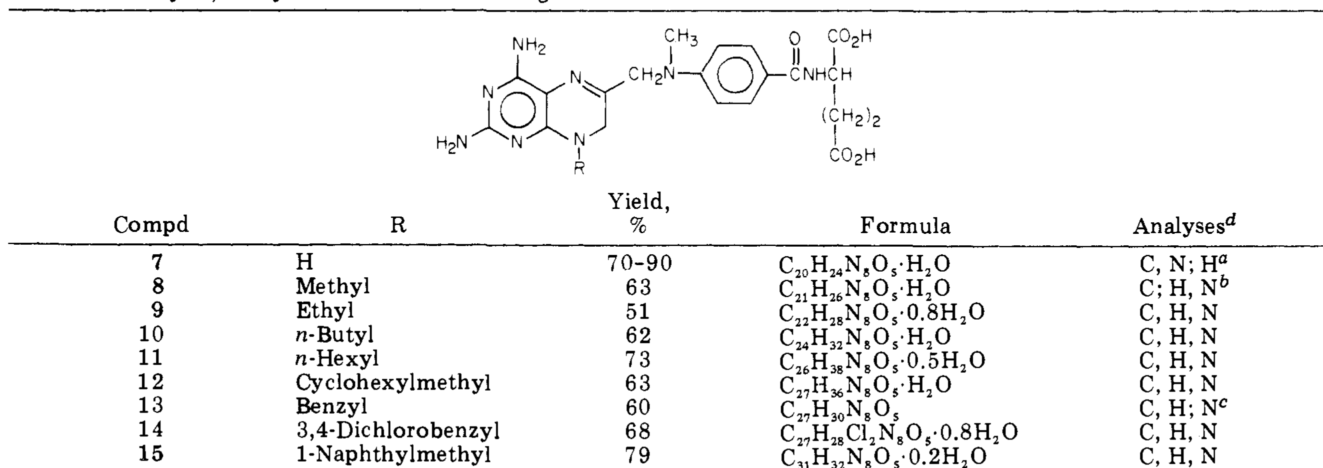
Dihydro- and tetrahydropteridines, in which the pyrazine ring is the reduced site, are intermediates in many biological reactions in diverse living organisms.[1] An important series of reactions is the biosynthetic reduction of folic acid and 7,8-dihydrofolic acid to tetrahydrofolic acid which is subsequently converted into metabolites that play a role in biochemical one-carbon transfer reactions, including the biosynthesis of nucleotides. Antimetabolites which inhibit these reactions are useful in the treatment of neoplastic diseases, a classical example of which is the antitumor agent methotrexate[2] (4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid; amethopterin; MTX), which binds very strongly to the enzyme dihydrofolate reductase and inhibits the formation of tetrahydrofolate. In doing so, MTX blocks the formation of most, if not all, of the coenzymes involved in folate metabolism, and this probably accounts for its high toxicity. An important goal in the search for new antifolates has been the design of agents which would be less inhibitory toward dihydrofolate reductase than is MTX, but which would inhibit other folate enzymes, such as thymidylate synthetase. This could lead to antineoplastic agents with more therapeutic specificity for tumor cells.[3]

Since all of the enzymes involved in folate metabolism utilize reduced folate coenzymes in their biosynthetic transformations, it became apparent that dihydro- and tetrahydrofolate analogues might inhibit these enzymes and might be useful as antineoplastic agents. The first work in this area began in the late 1950's and it continues to be an important area of research in cancer chemotherapy. Several studies[4–8] have shown that certain reduced folate analogues exhibit significant inhibition against thymidylate synthetase derived from *Escherichia coli*. For example, it was found[8] that 7,8-dihydromethotrexate was about 40 times more potent an inhibitor of this enzyme than was MTX and that it was only one-half as potent as MTX against dihydrofolate reductase (from L-1210 mouse leukemic cells).

It has long been recognized that the study of the chemical and biological properties of reduced pteridines and reduced folates is complicated by the susceptibility of these compounds to oxidative degradation, even upon standing in air.[1] However, their chemical stability can be greatly improved by substitution of alkyl groups at N^8 in 7,8-dihydropteridines[9] and at N^5 and/or N^8 in 5,6,7,8-tetrahydropteridines.[10] Direct chemical substitution at N^5 in tetrahydropteridines proceeds rather easily under mild conditions,[10–13] but N^8 has been found to be resistant to direct alkylation.[12] In a recent communication,[14] we described a method by which 2,4-diamino-7,8-dihydropteridines can be directly monoalkylated at N_8, without substitution either in the pyrimidine ring or on the 2- or 4-amino groups. The alkylated derivatives, unlike the parent compound, were stable under mild oxidative conditions.

With this chemical method in hand, we applied it to the preparation of several 8-alkyl-7,8-dihydromethotrexate analogues. Although these compounds are structurally more complex than the smaller pteridines, their preparation by direct alkylation of 7,8-dihydromethotrexate

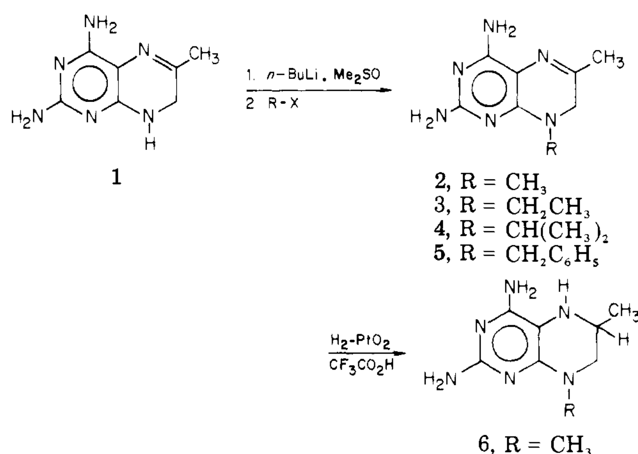
Table I. 8-Alkyl-7,8-dihydromethotrexate Analogues



Compd	R	Yield, %	Formula	Analyses[d]
7	H	70-90	$C_{20}H_{24}N_8O_5 \cdot H_2O$	C, N; H[a]
8	Methyl	63	$C_{21}H_{26}N_8O_5 \cdot H_2O$	C; H, N[b]
9	Ethyl	51	$C_{22}H_{28}N_8O_5 \cdot 0.8H_2O$	C, H, N
10	*n*-Butyl	62	$C_{24}H_{32}N_8O_5 \cdot H_2O$	C, H, N
11	*n*-Hexyl	73	$C_{26}H_{38}N_8O_5 \cdot 0.5H_2O$	C, H, N
12	Cyclohexylmethyl	63	$C_{27}H_{36}N_8O_5 \cdot H_2O$	C, H, N
13	Benzyl	60	$C_{27}H_{30}N_8O_5$	C, H; N[c]
14	3,4-Dichlorobenzyl	68	$C_{27}H_{28}Cl_2N_8O_5 \cdot 0.8H_2O$	C, H, N
15	1-Naphthylmethyl	79	$C_{31}H_{32}N_8O_5 \cdot 0.2H_2O$	C, H, N

[a] H: calcd, 5.52; found, 5.06. [b] H: calcd, 5.78; found, 5.35. N: calcd, 22.94; found, 22.25. [c] N: calcd, 20.50; found, 19.81. [d] All analyses were within ±0.4% of the theoretical values except where indicated otherwise.

Scheme I



proved to be almost as straightforward, resulting in derivatives which were stable to air oxidation. The novel features of these new MTX analogues are the "fixed" dihydro nature of the pyrazine ring and the lipophilic alkyl groups attached to that ring. We were interested in studying the growth-inhibitory and enzyme-inhibitory properties of these compounds and in their in vitro and in vivo antitumor evaluation.

Chemistry. 2,4-Diamino-7,8-dihydro-6-methylpteridine (1) (Scheme I) was prepared by the method of Taylor and co-workers.[15] The NMR spectrum of this compound in Me_2SO-d_6 (Me_4Si internal standard) showed that the N^8-H (δ 6.27) was deshielded with respect to the hydrogens on the two amino groups (δ 5.53 and 5.65). We anticipated that a powerful nucleophilic base might react with 1, abstracting a proton from the 8 position, to generate a resonance-stabilized anion. When a solution of 1 in dry Me_2SO was treated with *n*-butyllithium in hexane (which reacts with Me_2SO to generate lithium methylsulfinylcarbanion[16]), the initial almost colorless solution became orange-red. Addition of methyl iodide changed the color to pale yellow after a few minutes. Further processing led to the isolation of a single new compound (71% yield), which was identified as the N^8-alkylated product 2. This compound was stable in air and was unchanged upon treatment with hydrogen peroxide in DMF for 30 min, conditions which rapidly oxidized 1 to the fully aromatic pteridine. Catalytic hydrogenation of 2 gave the tetrahydropteridine derivative 6, isolated as the hydrochloride. In similar reactions compounds 3–5 were prepared from 1 by alkylation with ethyl bromide, isopropyl bromide, and benzyl chloride, respectively.

For the preparation of the 8-alkyl-7,8-dihydromethotrexate analogues (Table I), we required gram quantities of 7,8-dihydromethotrexate. Futterman[17] has reported the preparation of 7,8-dihydrofolic acid by reduction of a dilute solution of folic acid with excess sodium dithionite, at room temperature for 5 min, in the presence of potassium ascorbate. When this procedure was applied to the reduction of MTX, we obtained very low yields of 7,8-dihydromethotrexate, contaminated with unreacted MTX. Longer reaction times under these conditions did not improve the yields and, in fact, gave a product even more contaminated with impurities. We found that good yields of fairly pure 7,8-dihydromethotrexate, uncontaminated with MTX, could be prepared by dithionite reduction of MTX at 100 °C for 15 min. In this preparation (see Experimental Section), samples of sodium MTX were used as the starting material. Reduction was carried out in water, in the presence of 10 molar equiv of sodium hydroxide. This amount of sodium hydroxide was necessary to prevent the pH of the solution from falling below 7 during the reduction, since extensive reductive cleavage at the 9–10 bond occurs under acidic conditions.

The 8-alkyl-7,8-dihydromethotrexate analogues were prepared by direct alkylation of 7,8-dihydromethotrexate in Me_2SO, in a manner similar to that described above for the pteridines. In these cases, however, a large excess (4.5–5.0 molar equiv) of *n*-butyllithium had to be used because of the presence of the carboxyl groups and some water in the 7,8-dihydromethotrexate. The lithiated anionic intermediate precipitated from solution as a yellow solid, but it slowly dissolved after the addition of the alkyl halide. Initial experiments indicated that, in addition to alkylation at N^8, a substantial amount of esterification of the carboxyl groups had taken place. Therefore, in subsequent experiments, after the precipitate had dissolved, aqueous sodium hydroxide was added to the reaction mixture to hydrolyze the ester functions. The MTX analogues were isolated by precipitating them from the aqueous Me_2SO mixture with dilute HCl. All of the compounds were obtained as light tan solids in a good state of purity and were shown by TLC and NMR to be free of 7,8-dihydromethotrexate and MTX. The NMR spectrum of each compound indicated that alkylation had occurred at the 8 position. In order to further verify this, compounds 8 and 13 were subjected to reductive cleavage by sodium dithionite to give compounds 2 and 5, respectively.

Table II. Inhibition of *L. casei* Growth and Dihydrofolate Reductase and Thymidylate Synthetase Derived from *L. casei* by MTX Analogues[a]

	Molar concn for 50% inhibn		
Compd	*L. casei* growth ($\times 10^{-11}$)	Dihydrofolate reductase ($\times 10^{-8}$)	Thymidylate synthetase ($\times 10^{-6}$)
7	2	2	1
8	10	14	4
9	35	90	20
10	120	38	19
11	27	180	43
12	350	87	17
13	35	74	8
14	1940	360	13
15	47	450	16
MTX	4	0.3	>100
Tetrahydro-methotrexate	14	20	5
8-Methyltetrahydro-methotrexate	140	250	39

[a] Microbiological assays[8] and enzyme assays[18] were carried out as described previously. See Experimental Section for substrate concentration in the enzyme assays.

Tetrahydromethotrexate and 8-methyltetrahydromethotrexate were prepared by catalytic reduction of MTX and compound 8.

Biological Results. The compounds listed in Table II were evaluated for growth-inhibitory activity against the folate-dependent organism *Lactobacillus casei* (ATCC 7469) and also for inhibition against the enzymes dihydrofolate reductase and thymidylate synthetase, derived from this organism. Of the dihydro analogues, only 7,8-dihydromethotrexate (7) and 8-methyl-7,8-dihydromethotrexate (8) were comparable to MTX in their inhibition of *L. casei* growth. All of the dihydro analogues were less inhibitory toward dihydrofolate reductase than was MTX, whereas with thymidylate synthetase the converse is true. However, none of the 8-alkyl analogues were better inhibitors of thymidylate synthetase than was 7,8-dihydromethotrexate. Conversion of 8-methyldihydromethotrexate to the tetrahydro derivative resulted in a lowering of the inhibition potency in all three assay systems.

As we pointed out above, thin-layer chromatography and NMR spectroscopy were used to determine the purity of all the compounds studied. Although these analytical methods indicated that the desired compounds were uncontaminated with the starting materials, MTX and 7,8-dihydromethotrexate, we would not have been able to detect these impurities in concentrations of less than 1%. Therefore, although we are confident of our data, we cannot exclude the possibility that some of the biological results obtained for the poorer enzyme inhibitors may be attributable to very small amounts of these impurities. For example, it is conceivable that the inhibition results for compounds 14 and 15 against dihydrofolate reductase could have been due to the presence of as little as 0.1% of MTX in these samples. On the other hand, it would have taken a much larger contamination (5–10%) of these compounds with 7,8-dihydromethotrexate (7) for this potent inhibitor to have been responsible for the inhibition against thymidylate synthetase, and we would have detected such an amount.

The compounds listed in Table III were evaluated for inhibitory activity, in vitro against the CCRF-CEM human lymphoblastic leukemic cell line[19] and in vivo against L-1210 leukemia in mice. 7,8-Dihydromethotrexate (7) was about as active as MTX in the in vitro assay. However,

Table III. In Vitro and in Vivo Antitumor Evaluation of MTX Analogues

		In vivo (L-1210)[b]	
Compd	In vitro (CCRF-CEM),[a] ID_{50}, μg/mL	Optimal nontoxic dose, mg/kg × 3	% ILS[c]
7	0.021	30	9
8	0.23	300	27
9	0.27	400[d]	18
10	0.23	100	25
11	0.58	400[d]	0
12	0.14	300	27
13	0.78	400[d]	25
14	>1.0	400[d]	36
15	>1.0	400[d]	0
8-Methyltetrahydro-methotrexate	0.14	200	9
MTX	0.018	15	70

[a] The assay method has been previously described.[19] [b] Male BDF/1 hybrid mice were inoculated ip with 10^5 L-1210 leukemic cells. Test compounds in 0.1 M phosphate buffer (pH 8) were injected ip 24 h after tumor implantation (day 1) and on days 4 and 7 (q3d 1, 4, 7). [c] % ILS = 100 × (T/C − 1) where T and C are median survival times of treated and control animals. [d] Highest nontoxic dose tested.

it was inactive against the L-1210 mouse leukemia and had toxicity comparable to MTX. All of the 8-alkyl analogues were much less toxic than 7,8-dihydromethotrexate. Several of these compounds showed some antitumor activity (ILS 25–36%) in this system at relatively high dose levels.

Experimental Section

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.

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

General Method for the N^8-Alkylation of 2,4-Diamino-7,8-dihydro-6-methylpteridine (1). To a stirred solution of 1 (1.78 g, 10 mmol) in dry Me_2SO (50 mL), at room temperature under nitrogen, was added a solution of *n*-BuLi in hexane (11 mmol; Alfa, Beverly, Mass.). After 1 min, the neat alkyl halide (12 mmol of methyl iodide, ethyl bromide, isopropyl bromide, or benzyl chloride) was added to the red solution, and the reaction mixture was then stirred until red color changed to pale greenish yellow and then for an additional 15 min (15–30 min total). The reaction mixture was then diluted with water (150 mL) and the precipitated solid was filtered and washed with water, aqueous ethanol, and finally with ether. Compound 4 did not precipitate when water was added, but it was extracted from the solution with $CHCl_3$. The yield was low in this case due to extensive dehydrohalogenation of the hindered isopropyl bromide, regenerating 1, most of which remained in the Me_2SO–water mixture.

The products were purified by digestion with hot ethanol, then cooled and filtered, and were obtained as almost colorless needles. Pertinent physical data for these compounds are listed below.

2: 71% yield; mp 260–280 °C dec; UV (0.1 N HCl) 236 nm (ϵ 26 700), 263 (8130), 292 (13 600); NMR (CF_3CO_2H) δ 2.61 (s, C^6-CH_3), 3.24 (s, N^8-CH_3), 4.86 (s, 7-CH_2). Anal. ($C_8H_{12}N_6$) C, H, N.

3: 60% yield; mp 255–260 °C dec; UV (0.1 N HCl) 237 nm (ε 25200), 263 (9000), 293 (13000). Anal. ($C_9H_{14}N_6$) C, H, N.

4: 24% yield; mp 220–230 °C dec; UV (0.1 N HCl) 237 nm (ε 25400), 263 (11900), 293 (13100). Anal. ($C_{10}H_{16}N_6$) C, H, N.

5: 80% yield; mp 215–220 °C dec; UV (0.1 N HCl) 238 nm (ε 29200), 265 (8500), 293 (13800). Anal. ($C_{14}H_{16}N_6$) C, H, N.

2,4-Diamino-6,8-dimethyl-5,6,7,8-tetrahydropteridine (6). A mixture of platinum oxide (100 mg) and trifluoroacetic acid (40 mL) was shaken under hydrogen at 25 psi for 30 min in a Parr apparatus. Compound 1 (384 mg, 2.0 mmol) was then added and the mixture was reduced with hydrogen at the same pressure for 45 min. The mixture was filtered to remove the catalyst and the filtrate was evaporated to dryness under vacuum. HCl (10 mL of 1 N) was added and the solution was then lyophilized to leave 470 mg (82.5%) of the hydrochloride as a white solid: mp ~230 °C dec; UV (0.1 N HCl) 277 nm (ε 16300). Anal. ($C_8H_{14}N_6 \cdot 2HCl \cdot H_2O$) C, H, N.

***N*-[*p*-[[(2,4-Diamino-7,8-dihydro-6-pteridinyl)methyl]-methylamino]benzoyl]-L-glutamic Acid (7,8-Dihydro-MTX, 7).** Sodium methotrexate (equivalent to 2.0 g, 4.4 mmol of MTX; Ben Venue Laboratories, Bedford Ohio) and sodium dithionite (7.65 g, 44 mmol) were dissolved in a solution of 1 N sodium hydroxide (22 mL) and water (50 mL). The stirred solution was immediately immersed in an oil bath previously heated to 100 °C. After exactly 15 min, the pale yellow solution was briefly cooled in an ice bath and then filtered with suction through a bed of Celite filter acid. The pH of the filtrate was adjusted with dilute HCl from approximately 7 to 3.5 (pH meter). The mixture was cooled in ice and the solid was then filtered and washed with water. The wet solid was redissolved in 0.1 N sodium hydroxide (70 mL) and the solution was again adjusted to pH 3.5. The cooled mixture was filtered and the almost white solid was washed with water, ethanol, and finally with ether: mp 180–185 °C dec; UV (0.1 N HCl, 1% 2-mercaptoethanol) 292 nm (ε 23400); NMR (CF_3CO_2H) δ 2.3–2.9 (m, side-chain CH_2CH_2), 3.66 (s, N^{10}-CH_3), 4.57 (s, 9-CH_2), 4.76 (s, 7-CH_2), 5.10 (m, side-chain CH), 8.15 (m, C_6H_4).

The yield of product in this reaction varied between 70 and 90%. Lower yields were obtained if the pH of the solution fell to 6.3–6.5 during the reduction, and the product was contaminated with fluorescent impurities (cleavage products). This occurred during several runs in which different batches of sodium dithionite and sodium methotrexate were used. This situation could be rectified by adding a larger amount (24–26 mL) of 1 N sodium hydroxide to the initial reaction mixture.

***N*-[*p*-[[(2,4-Diamino-7,8-dihydro-8-methyl-6-pteridinyl)methyl]methylamino]benzoyl]-L-glutamic Acid (8).** To a stirred solution of 7 (1.42 g, 3.0 mmol), in dry Me_2SO (40 mL) under nitrogen, was added a solution of *n*-BuLi in hexane (13.5 mmol). A yellow solid gradually precipitated from the solution. After stirring for 5 min, methyl iodide (1.28 g, 9.0 mmol) was rapidly added, which resulted in complete solution of the solid after about 4 min. Stirring was continued for exactly 1 min longer and then aqueous NaOH (30 mL of 1 N) was added with slight external cooling in a cold water bath. The mixture was stirred for 15 min and poured into water (120 mL), and the stirred solution was adjusted to pH 4.5 with dilute HCl to precipitate the product. The mixture was cooled in ice for 20 min and filtered, and the solid was washed with water and then redissolved in 0.1 N NaOH (75 mL). The product was precipitated again by adjusting the solution to pH 4.5, cooled, filtered, and then washed with water, 2-propanol, and ether. Drying gave 920 mg (63%) of a light tan solid: mp 185–195 °C dec; UV (0.1 N HCl) 295 nm (ε 23700); NMR (CF_3CO_2H) δ 2.3–2.9 (m, side-chain CH_2CH_2), 3.24 (s, N^8-CH_3), 3.64 (s, N^{10}-CH_3), 4.46 (s, 9-CH_2), 4.72 (s, 7-CH_2), 5.10 (m, side-chain CH), 8.0 (m, 4 H, phenyl CH's); TLC (5% $NaHCO_3$; Eastman cellulose) showed the product as a single spot, R_f 0.68, free of any detectable amount of 7, which had R_f 0.55.

Reductive Cleavage of 8. A mixture of 8 (415 mg, 0.85 mmol), sodium dithionite (1.74 g, 10 mmol), 1 N NaOH (2 mL), and water (15 mL) was heated at reflux for 45 min. The cooled solution (pH 6.0) was adjusted to pH 10 with concentrated NH_4OH and extracted with $CHCl_3$ (5 × 50 mL). The extracts were evaporated to give 75 mg (46%) of a single compound as a light tan solid, which was identified as 2 by TLC (8% MeOH–$CHCl_3$; silica gel), IR, and NMR. The presence of any 1 in this solid, or in the aqueous phase, was not detected.

***N*-[*p*-[[(2,4-Diamino-8-cyclohexylmethyl-7,8-dihydro-6-pteridinyl)methyl]methylamino]benzoyl]-L-glutamic Acid (12).** To a stirred solution of 7 (950 mg, 2.0 mmol), in dry Me_2SO (50 mL) under nitrogen, was added a solution of *n*-BuLi in hexane (10 mmol). After stirring for 5 min, cyclohexylmethyl bromide (2.48 g, 14 mmol) was added and the mixture was stirred at room temperature for 24 h (complete solution). Aqueous NaOH (30 mL of 1 N) was added with slight external cooling; the mixture was stirred for 1 h and then poured into water (200 mL). The cloudy solution was adjusted to pH 4.5 with dilute HCl and then cooled in ice, and the precipitated solid was filtered, washed with water, and then redissolved in a mixture of 0.05 N NaOH (200 mL) and saturated salt solution (50 mL). This solution was extracted with ether (2 × 150 mL) to remove the cloudiness and then readjusted to pH 4.5. The mixture was cooled in ice for 20 min and filtered, and the solid was washed with water, 2-propanol, and ether. There was obtained 720 mg (63%) of a light tan solid: mp 190–210 °C dec; TLC (5% $NaHCO_3$; cellulose) showed a single spot with R_f 0.62.

***N*-[*p*-[[(2,4-Diamino-8-benzyl-7,8-dihydro-6-pteridinyl)-methyl]methylamino]benzoyl]-L-glutamic Acid (13).** To a stirred solution of 7 (474 mg, 1.0 mmol), in dry Me_2SO (25 mL) under nitrogen, was added a solution of *n*-BuLi in hexane (5.0 mmol). After 5 min, benzyl chloride (632 mg, 5.0 mmol) was added and the mixture was stirred for 30 min (complete solution). Aqueous NaOH (10 mL of 1 N) was added with slight cooling, and after 20 min the mixture was poured into water (100 mL) and adjusted to pH 4.5 with dilute HCl and then cooled in ice. The precipitate was filtered and then redissolved in a mixture of 0.05 N NaOH (100 mL) and saturated aqueous salt solution (50 mL), and the solution was extracted with ether (100 mL). The aqueous solution was again adjusted to pH 4.5 and cooled in ice, and the solid was filtered and washed with water, acetone, and ether. There was obtained 327 mg of light tan solid: mp 175–195 °C dec; TLC (5% $NaHCO_3$; cellulose) showed a single spot at R_f 0.63; NMR (CF_3CO_2H) δ 2.35–3.0 (m, side-chain CH_2CH_2), 3.60 (s, N^{10}-CH_3), 4.23 (s, N^8-CH_2), 4.58 (s, 9-CH_2), 4.78 (s, 7-CH_2), 5.05 (m, side-chain CH), 7.36 (m, C_6H_5), 8.0 (m, C_6H_4).

Under similar conditions as described above for compound 8, compound 13 was cleaved with sodium dithionite to give 5 in 40% yield.

Compounds 9–11, 14, and 15. Under reaction conditions similar to those described above, these compounds were prepared by the alkylation of 7 with ethyl bromide (reaction time 1.5 h), *n*-butyl bromide (2 h), *n*-hexyl bromide (1 h), 3,4-dichlorobenzyl chloride (3 min), and 1-naphthylmethyl chloride (5 min), respectively. Each compound was found, by TLC and NMR, to be free of any compound 7 and MTX.

Tetrahydro-MTX and 8-Methyltetrahydro-MTX. These compounds were prepared by catalytic hydrogenation of MTX or compound 7 (~500 mg in each case) in glacial acetic acid (35 mL) with PtO_2 (100 mg) in a Parr apparatus at 30 psi for 3 h. The catalyst was removed by filtration and the solution was evaporated to dryness under vacuum. Addition of warm methanol (20 mL) caused the glassy residue to solidify. Ether (20 mL) was added to the mixture, which was then placed under nitrogen and cooled in a refrigerator. The white solid was filtered, washed with ether, and dried under vacuum (~80% yield). TLC (5% aqueous, $NaHCO_3$ containing 1% 2-mercaptoethanol; cellulose) showed each product as a major spot (R_f ~ 0.65–0.70) and only traces of fluorescent impurities (R_f ~ 0.25–0.35).

Enzyme Assays. In both 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 was derived from an MTX-resistant strain: DHF, 50 μM; NADPH, 80 μM; Tris HCl, 0.05 M; 2-mercaptoethanol, 0.01 M; EDTA, 0.001 M; pH 7.4; 30 °C.

(2) *L. casei* thymidylate synthetase was derived from an MTX-resistant strain: THF, 3×10^{-4} M; dUMP, 4×10^{-5} M; Tris HCl, 0.04 M; 2-mercaptoethanol, 0.11 M; EDTA, 0.001 M; CH_2O, 0.012 M; $MgCl_2$, 0.021 M; pH 7.4; 30 °C.

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Antifilarial Agents. 1,2-Cyclobutanediamines as Analogues of Diethylcarbamazine. Status of Structure–Activity Relationships among Diethylcarbamazine Analogues

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cis- and *trans*-1,2-cyclobutanediamines bearing appropriate *N*-methyl and *N*-acyl substituents were prepared as analogues of diethylcarbamazine (DEC). None displayed activity against *Litomosoides carinii* in the gerbil despite substantial structural and stereochemical similarities to the parent drug. The inactivity of these drugs is rationalized in terms of eclipsed pharmacophore configurations and the increased population of unfavorable rotational conformations made possible by the exocyclic position of both pharmacophores. To provide perspective for these conclusions, the literature on DEC analogues is briefly summarized and structure–activity data are discussed in terms of critical structural factors associated with microfilaricidal activity. Generalizations on structural principles governing activity are advanced which encompass test results for the large majority of DEC analogues.

In the preceding papers of this series[1,2] we presented several groups of active diethylcarbamazine (DEC, 1) analogues derived from 3-aminopyrrolidine and various bridged piperazines. These compounds were characterized by relatively rigid skeletons that held the pharmacophoric groups in spatial orientations that closely resembled those of DEC. Our rationalization for their biological activity was based on this similarity. We now report the synthesis of a group of *cis*- and *trans*-1,2-diaminocyclobutane derivatives in which the pharmacophores are again held by the ring system in orientations generally comparable to those of DEC but among which no antifilarial activity has been found. In this paper we attempt to rationalize these results in terms of conformational characteristics of the cyclobutanediamine derivatives. We also attempt to summarize the essential characteristics of active DEC analogues and to assess the validity of the resulting description by comparison with the accumulated results of structure–activity studies in this area.[1-20]

Results and Discussion

Table I presents the antifilarial assay results obtained from DEC and the substituted *cis*- and *trans*-1,2-diaminocyclobutanes (**7**, **10–17**). The procedure used to evaluate the compounds against *Litomosoides carinii* in the gerbil was described in our first publication.[1] None of the new compounds caused a reduction of microfilaremia or had an effect on the number of adult worms in this test system. The cyclobutane derivatives of this series are similar to previously described active DEC analogues in several significant respects. The spacing of the pharmacophores in the cis derivative **7** (2.7 Å between N atoms) is very similar to that of DEC (2.9 Å) according to Dreiding models. Although the spacing in the trans series is larger (3.5 Å) compounds with greater internitrogen separations but similar functional groups have provided reduced but readily demonstrable antifilarial activity according to prior work.[8] Both acyl groups used in this series have previously provided highly active derivatives,[1,7] and the lipid–water partition properties of the members of this series should not differ substantially from those of known active groups, according to estimates derived from partition additivity concepts.[21] A rigid orientation of the pharmacophores has proven favorable previously.[1-3,9] The lack of activity in the 1,2-diaminocyclobutane series must therefore be attributed to more subtle stereochemical differences.