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Methotrexate Analogues. 8. Synthesis and Biological Evaluation of Bisamide Derivatives as Potential Prodrugs

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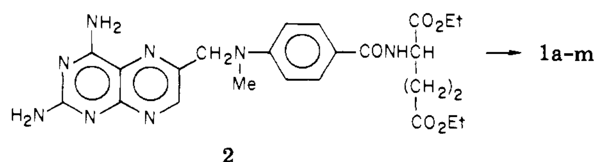
A series of heretofore unknown lipophilic bisamide derivatives (**1a–m**) of the antitumor agent methotrexate (MTX) was synthesized from MTX diethyl ester (**2**) by reaction with various amines. The amines were used in large excess, generally without solvent, at temperatures ranging from 50 to 100 °C and for periods of 24–72 h. Yields were in excess of 60% in most instances, and the products were stable and easily purified. The MTX bisamides proved significantly less active than MTX or MTX esters against human lymphoblastic leukemia (CCRF-CEM) cells in vitro (ID_{50} > 1.0 μg/mL vs. <0.05 μg/mL). However, some enhancement of activity was observed in two instances against rat basophilic leukemia (RBL) cells, which are myeloid rather than lymphoid in character. The bis(*n*-propylamide) **1b** was inactive in vivo against L1210 mouse leukemia even at doses of 525 mg/kg (q3d 1, 4, 7), but the bis(benzylamide) **1h** gave a +77% increase in median survival at 100 mg/kg (q3d 1, 4, 7). Neither compound was cleaved even after 24 h of incubation in whole rat serum at 37 °C, but the bis(benzylamide) **1h** was cleaved substantially in vivo, as evidenced by the detection of free MTX in the liver and plasma of mice 6 and 24 h after treatment with a single 100 mg/kg dose of "prodrug". These results suggest that MTX bis(benzylamide) (**1h**) may owe its in vivo activity against L1210 leukemia to the release of free MTX at sites other than the serum.

The pharmacologic and experimental therapeutic properties of esters of methotrexate (4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamate, MTX) have been studied in several laboratories.[1-10] While the mode of action of MTX esters is not yet established with certainty, they are believed to act as latent forms of MTX, i.e., "prodrugs", from which the parent acid is released on hydrolysis by extracellular and/or intracellular nonspecific esterases, whose levels are known in general to be species-variable.[11-13] Notwithstanding the fact that serum esterase levels in man are considerably lower than in rodents,[11-13] experimental trials with MTX esters have been confined thus far to mice[1,2,6,9] and, in a more limited way, to the dog.[2] Recent work in this laboratory[7] has shown that MTX esters cause marked inhibition of [^{3}H]-TdR incorporation into the DNA of mouse and human leukemic cells in serum-free short-term culture and that this effect is only partially prevented by leucovorin. Since MTX itself inhibits [^{3}H]-UdR but not [^{3}H]-TdR incorporation under these conditions, it was suggested that the esters may have a somewhat different mode of action than the parent acid at the biochemical level. As a logical extension of our overall program on lipophilic MTX derivatives, we became interested in MTX bisamides, a class of compounds about which very little could be found in the published literature.[14] We reported our first example of an MTX bisamide in 1975, in the form of the bis(*n*-propyl) derivative **1b**.[15] In this paper we wish to describe in detail the synthesis of this and 12 other MTX bisamides (**1a–m**) and to present data from some preliminary biological investigations. Structures of these heretofore unknown MTX bisamides are shown in Table I, along with other physical constants.

The ready availability of MTX diesters by direct HCl-catalyzed esterification[6] offered an attractive route to the desired bisamides, since amide groups could be introduced by nucleophilic displacement. The diethyl ester **2** was employed in most instances, although satisfactory results could also be achieved with the dimethyl analogue.



2

Ester **2** was heated with a variety of amines, generally for periods of 24–72 h, at temperatures ranging from 50 to 100 °C (Table I). Whenever the amine was used in

Table I. Synthesis and Purification of Methotrexate Bisamides

Compd	R	Ester, g; amine, mL	Temp, °C; time, h	% yield[a]	Column chromatographic and TLC data[b]	Mp, °C[c]	Crystn solvent[d]	Analyses[e]
1a	NH_2	0.1; *f*	70-75; 75	41	15 g; 95:5 (11 × 20 mL), 9:1 (5 × 20 mL), 85:15 (5 × 20 mL), 4:1 (10 × 20 mL), 3:1 (10 × 20 mL) $CHCl_3$-MeOH; fractions 32-41; R_f 0.32 (1:1 $CHCl_3$-MeOH)	197-210 dec		C, H, N
1b	NH-*n*-C_3H_7	4.0; 200[g]	49; 24	78	150 g; 85:15 $CHCl_3$-MeOH; R_f 0.60 (9:1 $CHCl_3$-MeOH)	135-140	A	C, H, N[h]
1c	NH-*n*-C_4H_9	2.3; 40	78; 24	98	100 g; 95:5 (10 × 20 mL), 9:1 (9 × 120 mL) $CHCl_3$-MeOH; fractions 4-11; R_f 0.33 (3:1 $CHCl_3$-EtOH)	135-138		C, H, N
1d	NH-*s*-C_4H_9	0.1; *i*	95-100; 144	19	15 g; 49:1 $CHCl_3$-MeOH (133 × 15 mL); fractions 123-133; R_f 0.69 (3:1 $CHCl_3$-MeOH)	145-152		C, H, N[j]
1e	NH-*n*-C_6H_{13}	0.1; 5	85; 42	70	25 g; 95:5 (24 × 50 mL), 9:1 (14 × 50 mL) $CHCl_3$-MeOH; fractions 26-35; R_f 0.47 (4:1 CH_2Cl_2-EtOH)	145-150	B	C, H, N
1f	NH-c-C_6H_{11}	0.1; 5	95-100; 73	52	40 g; 100:0 (4 × 125 mL), 95:5 (18 × 110 mL) CH_2Cl_2-MeOH; fractions 10-20; R_f 0.42 (3:1 $CHCl_3$-EtOH)	163-166	B	C, H, N[k]
1g	c-NC_4H_8	0.1; 5	89; 45	76	12 g; 95:5 (8 × 45 mL), 9:1 (5 × 40 mL), 85:15 (5 × 40 mL) $CHCl_3$-EtOH; fractions 14-20; R_f 0.24 (3:1 $CHCl_3$-EtOH)	162-166	B	C, H, N
1h	$NHCH_2C_6H_5$	0.1; 8	90-95; 48	61	R_f 0.38 (3:1 $CHCl_3$-EtOH)	144-146	B	C, H, N
1i	$NHCH_2CH_2C_6H_5$	0.1; 8	83; 43	59	12 g; 95:5 (8 × 50 mL) $CHCl_3$-MeOH; fractions 4-8; R_f 0.40 (3:1 $CHCl_3$-MeOH)	124-126		C, H, N[l]
1j	$NHCH_2CH_2C_6H_3$-3,4-$(OMe)_2$	0.1; 5	90; 45	60	R_f 0.42 (3:1 $CHCl_3$-EtOH)	174-176	B, C	C, H, N
1k	$NHCH_2CH_2CH_2OH$	0.1; 5	87; 48	74	25 g; 20:1 (5 × 40 mL), 15:1 (8 × 40 mL), 10:1 (6 × 40 mL), 5:1 (6 × 40 mL) C_6H_6-MeOH; fractions 14-25; R_f 0.40 (3:1 $CHCl_3$-EtOH)	139-144	D	C, H, N[m]
1l	$NHCH_2CH_2NMe_2$	0.1; 5	84; 46	38	30 g; 9:1 (10 × 50 mL), 4:1 (10 × 50 mL), 7:3 (8 × 50 mL), 1:1 (20 × 45 mL) C_6H_6-MeOH; fractions 29-48; R_f 0.45 (neutral alumina, 1:1:1 $CHCl_3$-EtOH-$PhNH_2$)	133-135	D	C, H, N[n]
1m	$NHCH_2CH_2CH_2NMe_2$	0.1; 8	83; 46	66	0.43 (neutral alumina, 1:1:1 $CHCl_3$-EtOH-$PhNH_2$)	120-123	D	C, H, N[o]

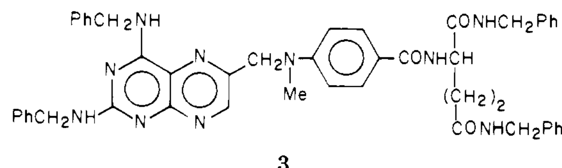
[a] Yields are for product which has been chromatographed and/or recrystallized as indicated. [b] Weight of silica gel (Baker 5-3405, 60-200 mesh), composition of the eluent, number and volume of individual fractions, fractions containing the product, R_f values, and TLC developing solvent are given. Unless otherwise specified, TLC data refer to silica gel sheets or plates. [c] Samples softened and became brown in color as the melting point was approached. [d] Solvent A, MeCN-MeOH (2:1); solvent B, absolute EtOH; solvent C, $CHCl_3$-EtOH mixtures; solvent D, EtOH-Et_2O mixtures. [e] C, H, and N analyses were all within 0.4% of calculated values. [f] The reaction was run in 100 mL of absolute EtOH which had been saturated with dry NH_3 gas. [g] Weight given is that of MTX disodium salt (cf. Experimental Section). [h] $C_{26}H_{36}N_{10}O_3 \cdot 0.75H_2O$. [i] The reaction was run in a mixture of *sec*-butylamine (25 mL) and absolute EtOH (5 mL) in a pressure-tight glass vessel. [j] $C_{28}H_{40}N_{10}O_3 \cdot 0.5CH_3OH$. [k] $C_{32}H_{44}N_{10}O_3 \cdot 1.1CH_3OH$. [l] $C_{36}H_{40}N_{10}O_3 \cdot 0.25CH_3OH$. [m] $C_{26}H_{36}N_{10}O_5 \cdot C_2H_5OH$. [n] $C_{28}H_{42}N_{12}O_3 \cdot 2H_2O$. [o] $C_{30}H_{46}N_{12}O_3 \cdot H_2O \cdot 0.4C_2H_5OH$.

substantial excess no other solvent was necessary. If the boiling point of the amine was low enough, the mixture was simply refluxed. In other instances the reaction was conducted in a constant temperature oil bath. Sometimes, as in the reaction of ester **2** with ammonia, a glass pressure bottle had to be used. After evaporation of excess amine (and solvent where appropriate) under reduced pressure, the bisamides were isolated in pure form by column chromatography and/or recrystallization from a suitable solvent. For the most part the yields were 60–80%. The highest yield (98%) was obtained with *n*-butylamine and the lowest (19%) with *sec*-butylamine.

Secondary amines reacted less readily with **2** than primary amines. Thus, although pyrrolidine gave a 76% yield of **1g** (Table I), diethylamine (63-h reflux) and di-*n*-butylamine (42 h at 85–90 °C) both left the starting ester unchanged. In the latter reaction a small amount of DMF was also added in the hope of promoting displacement, but to no avail. Hindered primary amines were likewise less reactive than unhindered amines, as evidenced by the reaction of **2** with *sec*-butylamine which gave only a 19% yield of **1d** even after 6 days at 95–100 °C. The facile reaction of **2** with pyrrolidine is probably due to the five-membered cyclic structure of this particular amine, whose nitrogen is less hindered than in noncyclic secondary amines.

The reaction of **2** with piperidine (70 h at 90 °C) produced a complex mixture from which we were unable to isolate a pure bisamide. Further work revealed substantial amounts of a product whose solubility in dilute base and slow migration on silica gel indicated a free α- or γ-carboxyl on the glutamate moiety. The yield of this product (probably MTX γ-monoethyl ester) could be increased to about 60% by carrying out the piperidine reaction in 95% ethanol (45-h reflux). Though the lack of bisamide formation from piperidine was consistent with the low reactivity observed with diethylamine and di-*n*-butylamine, we had not anticipated to see such facile hydrolysis. Possible advantageous uses of this cleavage reaction in the synthesis of MTX monoesters will be reported in due course.[9]

It should be noted that attempts to improve yields or shorten the time of reaction by raising the temperature above 100 °C met with failure. We observed in at least one instance that the amino groups on the pteridine are displaced at elevated temperature. Thus, when **2** was allowed to react with benzylamine under reflux (185 °C) for 38 h a product was isolated (15% yield) which was fast moving on silica gel TLC, showed the expected number of aromatic protons in the NMR spectrum, and gave correct microanalytical values for structure **3**.



3

All the bisamides synthesized in this work were evaluated for growth-inhibitory activity against human lymphoblastic leukemia (CCRF-CEM) cells[16] in culture, but none showed activity at concentrations below 1.0 μg/mL (see Table II). This was in marked contrast to the esters of MTX, many of which have been found to have ID_{50} values of 0.1 μg/mL or less in this test system. For example, compound **1b** had an ID_{50} of 7.0 μg/mL whereas the ID_{50} value for MTX di-*n*-butyl ester was 0.032 μg/mL, approximately a 200-fold difference in activity. When assayed as inhibitors of dihydrofolate reductase from MTX-resistant *Lactobacillus casei* ATCC 7469, compound **1b** and MTX di-*n*-butyl ester gave ID_{50} values of 1.8×10^{-7} and 5×10^{-7} M, respectively, i.e., a less than threefold difference.

Table II. Growth Inhibitory Activity of Selected MTX Bisamides against Human and Rat Leukemic Cells in Culture

	ID_{50}, μg/mL	
Compd	Human lymphoblastic leukemia (CCRF-CEM)[a]	Rat basophilic leukemia (RBL)
MTX	0.003	0.003
1b	7.0	
1e	10.0	
1f	1.0	0.22
1h	7.6	2.5

[a] See ref 16 for details of the assay procedure; bisamides not listed in this table were all found to have ID_{50} values greater than 10.0 μg/mL against CCRF-CEM cells.

Several of the bisamides were also tested against rat basophilic leukemia (RBL) cells[17] in culture. These cells were selected on the basis that, since they are of myeloid rather than lymphoid origin, they might contain higher levels of amidases and other catabolic enzymes.[18] In at least two instances (Table II), a three- to fivefold enhancement of activity was observed relative to CCRF-CEM cells. One obviously has to be cautious in interpreting these preliminary results because (a) CCRF-CEM and RBL cells may contain different amounts of dihydrofolate reductase, (b) RBL dihydrofolate reductase may be more sensitive to inhibitors than CCRF-CEM dihydrofolate reductase, and (c) RBL cells may take up the amides more readily than CCRF-CEM cells by virtue of differences in cell membrane permeability. On the basis of these initial results it is hoped that further testing of MTX bisamides against normal and myeloid human cells in culture will allow us to uncover new MTX analogues with improved therapeutic characteristics or a qualitatively altered spectrum of activity.

Five of the bisamides synthesized in this work (**1b,f,g,h,m**) were tested in vivo against L1210 leukemia in mice. Ip injections were given in 10% Tween 80 on days 1, 4, and 7 following tumor implantation (10^5 cells ip). Doses ranged from 15 to 525 mg/kg. MTX was used as a positive control and was administered as the disodium salt in aqueous solution. Only one compound (**1h**) showed significant and reproducible activity (+77% ILS at 100 mg/kg), another (**1b**) had borderline activity (+27% ILS at 225 mg/kg), and the rest were inactive or toxic. The data for bisamides **1b** and **1h** are given in Table III.

In order to determine whether the divergent activities of **1b** and **1h** might be due to differences in serum stability, we incubated these compounds in whole rat serum at 37 °C for up to 24 h and looked for the appearance of metabolites by TLC (see Experimetal Section). MTX di-*n*-butyl ester[6,7] was used as a positive control. To our surprise, whereas the diester was barely detectable by TLC after just 1 h, both amides remained unchanged even after 24 h. In another experiment a mouse was injected ip with 100 mg/kg of compound **1h** and sacrificed 24 h later. Under these conditions, TLC analysis of the plasma clearly revealed the presence of MTX. Because we suspected that hepatic cleavage might be responsible for the appearance of free MTX in vivo, we removed the liver of the mouse after sacrifice and again found a substantial content of free MTX, along with a second product which may be a monoamide. Thus, bisamide **1h** probably does not owe its

Table III. Effect of MTX Bis(*n*-propylamide) (**1b**) and MTX Bis(benzylamide) (**1h**) against L1210 Lymphatic Leukemia in Mice

Expt	Compd	Dose, mg/kg (q3d 1, 4, 7)	Survival^a Range, days	T/C	% ILS
1	None		10-12 (11)		
	MTX	15	10-27 (21)	21/11	+90
	1b	150	10-12 (11)	11/11	0
		225	11-14 (14)	14/11	+27
		350	12-14 (13)	13/11	+18
		525	13-17 (13)	13/11	+18
2	None		8-9 (9)		
	MTX	15	14-18 (15)	15/9	+66
		25	13-21 (18)	18/9	+100
	1h	50	11-14 (13)	13/9	+44
		100	12-18 (13)	13/9	+44
		200	7-13 (9)	9/9	0
		400	7-10 (8)	8/9	-11
3	None		8-10 (9)		
	MTX	15	14-16 (15)	15/9	+66
	1h	25	11-14 (12)	12/9	+33
		50	13-15 (14)	14/9	+55
		100	15-19 (16)	16/9	+77
		150	12-19 (15)	15/9	+66

^{a} T/C = treated/control; figures in parentheses are median values; % ILS = 100 × (T − C)/C; mice were inoculated ip with 10^5 cells on day 0 and compounds were injected ip in 10% Tween 80 on days 1, 4, and 7; test and control groups contained 5 and 10-15 animals, respectively.

in vivo activity against L1210 leukemia to serum hydrolysis but rather to the action of amide bond-cleaving enzymes in the liver and/or perhaps other tissues. To our knowledge this is the first reported instance of a rodent-active "prodrug" derivative of MTX in which "activation" takes place at a site other than the serum.

It is interesting to note that the closely related folate antagonist aminopterin and its bis(benzylamide) have been reported to be approximately equipotent (30–40% ILS) against late L1210 mouse leukemia at doses of 0.18 and 7.0 mg/kg, respectively (subcutaneous injection, daily until death, starting on day 5 after tumor implantation).[14] This dose ratio of 40:1 may be compared with a ratio of about 8:1 in the case of bisamide **1h** and MTX. Whether the difference in ratios is related to the fact that the two treatment schedules are not the same is uncertain. It seems noteworthy, however, that *N*-benzyl substitution is the only kind, thus far, to give retention of antitumor activity in the amide series. The concept of "amide latentiation" has been applied previously with some success in the design of latent nitrogen mustard alkylating agents,[19,20] but progress in this area has been hampered by the paucity of systematic data on amidase levels in various tumors and, just as importantly, in normal tissues.[21] Additional work is in progress in our laboratory to help clarify the role of this particular type of N-substitution in the "amide latentiation" of MTX.

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 T-60A instrument, with tetramethylsilane as the reference. TLC was performed on 250-μ Analtech silica gel GF plates, Eastman 13181 silica gel sheets, or Eastman 13523 neutral alumina sheets. Spots were visualized under ordinary fluorescent light or 254-nm ultraviolet light or by iodine staining. Column chromatography was carried out on Baker 5-3405 silica gel (60–200 mesh). Melting points were measured in Pyrex capillary tubes in a Mel-Temp apparatus (Laboratory Devices, Inc., Cambridge, Mass.) and are not corrected. Microanalyses were performed by Galbraith Laboratories, Knoxville, Tenn., and were within ±0.4% of the calculated C, H, and N values.

Bisamide Formation from MTX Diesters. General Procedure. The ester was stirred in excess amine, generally 50 mL of amine per gram of ester. When ammonia was used, the reaction was conducted in ethanol solution. Aminations were carried out in a round-bottom or pear-shaped flask heated by means of an electric mantle or, preferably, a thermostatically regulated silicone oil bath. With the exception of the reaction with ammonia, where a pressure-tight glass vessel was used, the heating flask was fitted with a reflux condenser and $CaCl_2$ drying tube, and the contents was stirred magnetically at 50–100 °C (internal temperature) for periods of 24 h to several days. In most instances heating at 80–90 °C for 48 h sufficed to consume the ester according to TLC analysis. Excess amine was removed by evaporation under reduced pressure (water aspirator or vacuum pump with dry ice–acetone in the trap) at a bath temperature not exceeding 45 °C, and the remaining gummy solid was purified (Table I) by recrystallization from appropriate solvents, by chromatography on silica gel columns, or by a combination thereof. With 2-phenylethylamine and homoveratrylamine, removal of excess amine was achieved by simply pouring the reaction mixture into a large volume of Et_2O and filtering off the crude product. In most preparations the bisamide was accompanied by small amounts of one or two other yellow products which were easily seen on visual examination of analytical TLC plates. Other spots were sometimes present which were not yellow but absorbed iodine and were therefore assumed not to be pteridines. These byproducts were removed readily from the bisamides by chromatography or recrystallization. Detailed aspects of the preparation and purification of all the bisamides described in this paper are given in Table I. The following specific examples are illustrative.

Methotrexate Bisamide (1a). Procedure 1. Absolute EtOH (100 mL) was saturated with dry NH_3 gas at 0 °C and the solution was transferred to a pressure-tight glass bottle (250-mL capacity), to which was then added MTX diethyl ester (**2**, 0.1 g, 0.0002 mol). The bottle was stoppered securely and kept at 70–75 °C (bath temperature) for 75 h. After being cooled to room temperature, the ethanolic ammonia was evaporated under reduced pressure. TLC of the yellow residue (silica gel, 1:1 $CHCl_3$–MeOH) revealed two major spots (R_f 0.85 and 0.32) and two very faint spots (R_f 0.70 and 0.62). The crude product was dissolved in a minimum volume of 3:1 $CHCl_3$–MeOH, and the solution was applied to a column of silica gel (15 g, 300 × 19 cm) which was eluted successively with the following $CHCl_3$–MeOH combinations: 95:5 (11 × 20 mL), 9:1 (5 × 20 mL), 85:15 (5 × 20 mL), 4:1 (10 × 20 mL), and 3:1 (10 × 20 mL). Evaporation of the pooled 3:1 $CHCl_3$–MeOH eluates gave a bright yellow solid (0.036 g, 41%): R_f 0.32 (silica gel, 1:1 $CHCl_3$–MeOH); mp 197–210 °C dec.

Methotrexate Bis(*n*-hexylamide) (1e). Procedure 2. A mixture of MTX diethyl ester (**2**, 0.1 g, 0.0002 mol) and *n*-hexylamine (5 mL) was stirred at 85 °C (internal temperature) for 42 h. Evaporation of the solution at 35–40 °C (0.25 mm) left a yellow-orange solid whose TLC (silica gel, 4:1 CH_2Cl_2–EtOH) showed two major yellow spots (R_f 0.82 and 0.47) and two minor iodine-absorbing spots (R_f 0.91 and 0.04). The crude solid was applied to a silica gel column (25 g, 35 × 2.2 cm) which was eluted successively with 95:5 $CHCl_3$–MeOH (24 × 50 mL) and 9:1 $CHCl_3$–MeOH (14 × 50 mL). Individual fractions were examined by TLC and fractions 26–35, which contained only one spot (R_f 0.47), were pooled and evaporated to a bright yellow solid (0.085 g, 70%): mp 145–150 °C (EtOH).

Methotrexate Bis(phenethylamide) (1i). Procedure 3. A mixture of MTX diethyl ester (**2**, 0.1 g, 0.0002 mol) and 2-phenylethylamine (8 mL) was heated at 83 °C (internal temperature) for 43 h, cooled, and poured into Et_2O (70 mL). Filtration gave a yellow solid (0.15 g) whose TLC (silica gel, 3:1 $CHCl_3$–EtOH) showed two major yellow spots (R_f 0.40 and 0.01) and three minor iodine-absorbing spots (R_f 0.72, 0.59, and 0.37). Recrystallization of this material from absolute EtOH produced a solid (0.076 g, 59%) whose TLC contained only one spot (R_f 0.40). The analytical sample was obtained by applying this solid to a silica gel column (12 g, 25 × 1.5 cm) and eluting with 95:5 $CHCl_3$–MeOH (8 × 50 mL). Fractions 4–8 were pooled and

evaporated to obtain 0.026 g (59%) of bright yellow solid: mp 124–126 °C.

Methotrexate Bis[2-(*N*,*N*-dimethylaminoethyl)amide] (1l). Procedure 4. A mixture of MTX diethyl ester (2, 0.1 g, 0.0002 mol) and 2-(*N*,*N*-dimethylaminoethyl)amine (5 mL) was stirred at 84 °C (internal temperature) for 46 h. Excess amine was removed under reduced pressure to obtain an amber-colored syrup whose TLC (silica gel, 4:1 CH_2Cl_2–EtOH) showed two major yellow spots (R_f 0.36 and 0.06). This material was applied to a silica gel column (30 g, 35 × 2.2 cm) which was eluted successively with 9:1 C_6H_6–MeOH (10 × 50 mL), 4:1 C_6H_6–MeOH (10 × 50 mL), 7:3 C_6H_6–MeOH (8 × 50 mL), and 1:1 C_6H_6–MeOH (20 × 45 mL). Evaporation of the pooled 1:1 C_6H_6–MeOH eluates gave an orange-yellow semisolid (0.16 g) which was purified further by recrystallization from mixtures of EtOH and Et_2O. The final product (0.045 g, 38%) was a yellow solid: mp 133–135 °C; R_f 0.06 (silica gel, 4:1 CH_2Cl_2–EtOH), R_f 0.45 (neutral alumina, 1:1:1 $CHCl_3$–EtOH–$PhNH_2$); NMR ($CDCl_3$) τ 8.79 [m, -NHCH(CONHR)CH_2-], 7.78 (s, NCH_3), 7.60 [m, -NHCH(CONHR)CH_2CH_2- and -CH_2NMe_2], 6.97 (m, -$CH_2CH_2NMe_2$), 6.57 (br, NH_2), 5.88 [m, -NHC*H*(CONHR)CH_2-], 5.46 (br, CH_2NMe), 3.34 (m, CONH), 2.28 (m, phenyl protons), 1.68 (br s, C_7 pteridine proton).

Methotrexate Bis[3-(*N*,*N*-dimethylaminopropyl)amide] (1m). Procedure 5. A mixture of MTX diethyl ester (2, 0.1 g, 0.0002 mol) and 3-(*N*,*N*-dimethylaminopropyl)amine (8 mL) was stirred at 83 °C (internal temperature) for 40 h. Excess amine was removed under vacuum, the residue was extracted with H_2O (2 × 10 mL), and the combined H_2O layers were washed with Et_2O (3 × 15 mL). The aqueous phase was treated with decolorizing carbon and filtered (Celite), and the filtrate was evaporated [40–50 °C (0.25 mm)] to a yellow-orange semisolid, from which the last traces of water were removed by azeotropic distillation with C_6H_6 and MeOH. Recrystallization of the dried residue from mixtures of EtOH and Et_2O gave a bright yellow solid (0.081 g, 66%): mp 120–123 °C; R_f 0.05 (silica gel, 3:1 $CHCl_3$–EtOH), R_f 0.43 (neutral alumina, 1:1:1 $CHCl_3$–EtOH–$PhNH_2$). The recrystallized material, in this instance, consisted of microcrystals which were collected most conveniently by centrifugation and decantation of the solvent.

Direct Synthesis of Methotrexate Bis(*n*-propylamide) (1b) from Methotrexate Disodium Salt. Procedure 6. The finely powdered disodium salt (4.0 g, 0.0088 mol) was suspended in absolute EtOH (300 mL), in which had been dissolved previously 3.0 g of dry HCl gas. After 72 h of stirring at room temperature, the solvent and HCl were removed on a rotary evaporator connected to a water aspirator, the residue was suspended directly in *n*-propylamine (200 mL), and the mixture was stirred under reflux for 24 h and left at room temperature for another 18 h. Ether (400 mL) was added and the solid was filtered, washed with Et_2O, and chromatographed on a column of silica gel (150 g). Elution with 85:15 $CHCl_3$–MeOH followed by recrystallization of the chromatographed product from 2:1 MeCN–MeOH yielded a bright yellow solid (4.3 g, 78%): mp 135–140 °C; R_f 0.60 (silica gel, 9:1 $CHCl_3$–MeOH).

Reaction of Methotrexate Diethyl Ester (2) with Benzylamine at Elevated Temperature. Simultaneous Amidation and Replacement of the Pteridine Amino Groups. A mixture of MTX diethyl ester (2, 0.1 g, 0.0002 mol) and benzylamine (5 mL) was stirred under reflux (185 °C) for 38 h, cooled, and evaporated under reduced pressure [40–45 °C (0.25 mm)]. The TLC of the crude residue (silica gel, 5:1 $CHCl_3$–MeOH) showed a single yellow spot (R_f 0.74) and two minor iodine-absorbing spots (R_f 0.80 and 0.18). The product was applied to a column of silica gel (30 g) which was eluted with $CHCl_3$. Evaporation of the eluates and recrystallization of the residue from absolute EtOH yielded the 2,4-bis(benzylamino)pteridine 3 as a yellow solid (0.025 g, 15%). The analytical sample was prepared by further recrystallization from a mixture of MeOH and $CHCl_3$: mp 199–201 °C; NMR (Me_2SO-d_6) τ 7.81 [m, -NHCH(CONHR)CH_2CH_2CONHR], 6.80 (s, NCH_3), 6.63 (s, CH_2NMe), 5.6–5.9 [m, CONHCH_2Ph and -NHC*H*(CONHR)-CH_2-], 5.0–5.6 (m, NHCH_2Ph), 1.4–3.6 (complex m, aromatic protons, CONH protons, and C_7 pteridine proton). The observed ratio for the areas of the τ 7.81 and 1.4–3.6 signals was 6:1, in exact agreement with structure 3.

Incubation of MTX Bis(benzylamide) (1h) with Whole Rat Serum. A mixture of fresh rat serum (0.9 mL) and the bisamide (0.006 g in 0.1 mL of Me_2SO) was incubated at 37 °C with gentle shaking for 24 h, then cooled in ice, and diluted 1.5-fold with methanol in order to precipitate the serum proteins. After centrifugation (1600 rpm, 6 min), an aliquot was spotted on a cellulose TLC sheet (Eastman Chromagram 13254, with fluorescent indicator) which was developed with 0.5 M phosphate buffer (pH 7.4). A parallel incubation was performed with MTX di-*n*-butyl ester[6,7] in place of compound 1h. The supernatant derived from the bisamide contained only the starting compound and a barely perceptible trace of MTX, whereas the supernatant from the diester showed complete disappearance of the starting compound, substantial formation of MTX, and another product which we have shown to be MTX mono-*n*-butyl ester.[9]

Detection of MTX in the Liver and Plasma of Mice Treated with MTX Bis(benzylamide) (1h). Two normal mice each received a single ip injection of the bisamide (100 mg/kg, in 10% Tween 80). One animal was sacrificed after 6 h and the other after 24 h. Prior to sacrifice, approximately 1 mL of blood was withdrawn by ocular puncture with a heparinized syringe, the red cells were spun down (2000 rpm, 6 min), and the plasma (0.2 mL) was cooled in ice and diluted with methanol (0.4 mL) in order to precipitate the proteins. Centrifugation and TLC exactly as in the preceding experiment revealed substantial formation of MTX and disappearance of the starting material in both the 6- and 24-h specimens. Immediately after sacrifice, the livers were removed, washed gently with buffered saline in order to remove adhering peritoneal contents, and minced on a glass plate with a razor blade. The minced material was frozen and thawed repeatedly, triturated vigorously under MeOH (10 ml), left overnight in the refrigerator, and centrifuged (2000 rpm, 10 min). An aliquot of the supernatant was spotted on cellulose as in the previous experiment. There was no unchanged bisamide in either the 6- or 24-h specimen. A strong spot corresponding to MTX was evident, along with a second spot which may be a monoamide. Visual examination of the livers prior to mincing revealed yellow patches, indicating the presence of large amounts of drug in this organ.

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Synthesis of Cyclohexyl Carbocyclic Puromycin and Its Inhibition of Protein Synthesis

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In a continuation of our studies with puromycin analogues, four cyclohexyl carbocyclic puromycins, 6-dimethylamino-9-[(*R*)-[2(*R*)-hydroxy-3(*R*)-(*p*-methoxyphenyl-L-alanylamino)]cyclohexyl]purine (**1a**), 6-dimethylamino-9-[(*S*)-[2(*S*)-hydroxy-3(*S*)-(*p*-methoxyphenyl-L-alanylamino)]cyclohexyl]purine (**1b**), 6-dimethylamino-9-[(*R*)-[2-(*R*)-hydroxy-3(*S*)-(*p*-methoxyphenyl-L-alanylamino)]cyclohexyl]purine (**2a**), and 6-dimethylamino-9-[(*S*)-[2(*S*)-hydroxy-3(*R*)-(*p*-methoxyphenyl-L-alanylamino)]cyclohexyl]purine (**2b**), have been synthesized. *trans*-3-Amino-2-hydroxycyclohexanone ethylene ketal (**4**) was easily obtained by opening of epoxide **3** with liquid ammonia. Condensation of **4** with 5-amino-4,6-dichloropyrimidine and subsequent ring closure gave the 9-substituted 6-chloropurine **5** which was converted to the *O*-acetyloxime **8**. Reduction of **8** with diborane gave a mixture of *cis*- and *trans*-amino alcohols separated as their acetamides, **9** (7%) and **10** (33%), respectively. The amino alcohols were converted to **1a**,**b** and **2a**,**b** by a general method previously reported from our laboratory. In addition, the *trans*-cyclopentyl carbocyclic puromycins, 6-dimethylamino-9-[(*R*)-[2(*R*)-hydroxy-3(*S*)-(*p*-methoxyphenyl-L-alanylamino)]cyclopentyl]purine and 6-dimethylamino-9-[(*S*)-[2(*S*)-hydroxy-3(*R*)-(*p*-methoxyphenyl-L-alanylamino)]cyclopentyl]purine (**19a** and **19b**), were prepared from the previously described 2α-acetamido-5α-(6-dimethylamino-9-purinyl)cyclopentan-1β-ol. The puromycin analogues were evaluated as inhibitors of protein synthesis by their ability to inhibit the rate of poly(U,C)-directed L-[^{14}C]poly(phenylalanine) formation in an *Escherichia coli* cell-free ribosome system. The results of this study, in conjunction with our previous reports, suggest that maximum activity of puromycin analogues is obtained when the purine moiety, the amino acid, and the hydroxyl group are oriented about a five-membered ring. In addition, the amino acid and hydroxyl group must be in a cis orientation, and the absolute stereochemistry of the parent antibiotic must be conserved.

Previous reports relating to our studies on puromycin analogues have described the antimicrobial[1] and antitumor activity[2] of a carbocyclic puromycin analogue (**20a**) in which the furanosyl ring was replaced with a cyclopentyl moiety. In vitro testing demonstrated that **20a** inhibits the formation of poly(phenylalanine) in the *Escherichia coli* cell-free system[3] and that its mechanism of action involves the termination of protein synthesis by accepting the growing peptide chain from peptidyl-tRNA.[4] The inhibition is stereospecific with the diastereomer **20b** being only slightly active.

The relative ease of preparing the corresponding cyclohexyl series of intermediates has prompted the synthesis of cyclohexyl carbocyclic puromycins **1a**,**b** and **2a**,**b**. The synthesis followed a route similar to that previously described for the synthesis of the cyclopentyl analogues of puromycin[1] (Scheme I). The amino alcohol **4** was easily obtained by opening of the epoxide **3** with liquid ammonia. Condensation of **4** with 5-amino-4,6-dichloropyrimidine and subsequent ring closure with triethyl orthoformate gave the 9-substituted 6-chloropurine **5**, which was converted to the corresponding 6-dimethylaminopurine **6**. The ketal of **6** was hydrolyzed in the presence of hydroxylamine to give oxime **7**. The *O*-acetyloxime **8** was reduced with diborane to a mixture of amino alcohols and separated chromatographically as their acetamides, **9** (7%) and **10** (33%). The ratio of **9** to **10** is in contrast to that previously reported for the diborane reduction of the analogous cyclopentyl compound, where the *cis*-acetamido alcohol was the major product (45%) and the *trans*-acetamido alcohol was the minor product (4%).[1] It has been noted in the reduction of acetylated 2-oximino-α-D-hexopyranosides that there is an axially directed nucleophilic attack by the borane complex at C-2.[5] It is not surprising that the reduction of **8** gives the equatorial amine (*trans*-amino alcohol) as the major product. Possibly, in the case of the cyclopentyl analogue,[1] a borane complex at N-3 of the purine ring effects the reduction of the *O*-acetyloxime resulting in hydride attack selectively from the purine side of the ring. This situation would not be sterically feasible in the cyclohexyl analogue where the N-3 of the pseudoequatorial purine is quite far from C-3. It is also possible that hydride attack in the cyclopentyl case is directed primarily to the purine side of the ring by the 2-acetoxy group for steric reasons.

The structural assignments of **9** and **10** were confirmed by an alternate synthesis of **9**. Epoxide **11**[6] was opened with sodium azide and the azido alcohol **12** was obtained (87%). Catalytic reduction of **12** gave amine **13** (91%). The purine moiety was formed in the usual way by condensation of **13** with 5-amino-4,6-dichloropyrimidine, followed by ring closure with triethyl orthoformate to the 9-substituted 6-chloropurine. Aqueous dimethylamine was used to convert the chloropurine to the 6-dimethyl-