Cyclopentylmethyl phenyl *N*-methyl-*N*-(2-chloroethyl)-phosphoramidate (3c) was prepared from **2c** (2.0 g, 7.4 mmol) as described above. The residue was purified by flash chromatography and was isolated as an oil (2.21 g, 85%): ^{1}H NMR ($CDCl_3$) 7.27 (m 5 H, phenyl), 3.98 (dd, 2 H, $-CH_2O-$), 3.54 (dt, 2 H, CH_2Cl), 3.40 (dt, 2 H, $-CH_2N-$), 2.81 (d, 3 H, CH_3) ppm; ^{31}P NMR ($CDCl_3$) -19.53 ppm.

Cyclopentylmethyl *N*-methyl-*N*-(2-chloroethyl)-phosphoramidate cyclohexylamine salt (4c) was prepared from **3c** (0.5 g, 1.5 mmol) as described above. The product was isolated as a white solid (0.4 g, 75%): mp 149-152 °C; ^{1}H NMR (DMSO-d_6) 3.74 (dd, 2 H, CH_2O), 3.55 (dt, 2 H, CH_2Cl), 3.27 (dt, 2 H, $-CH_2N-$), 2.55 (d, 3 H, CH_3) ppm; ^{31}P NMR (D_2O) -14.86 ppm; IR (KBr) 3200-3700, 2940, 2860, 2650, 2560, 2210, 1190, 1040 cm^{-1}. Anal. ($C_{15}H_{32}N_2O_3PCl\cdot{}^1/_2H_2O$) C, H, N.

Phenyl *N*-methyl-*N*-(2-bromoethyl)phosphoramidate chloride (2d) was prepared as described above from *N*-methyl-2-bromoethylamine hydrobromide (3.0 g, 14 mmol). The residue was purified by flash chromatography and the product was isolated as an oil (3.21 g, 75%): ^{1}H NMR ($CDCl_3$) 7.25 (m, 5 H, phenyl), 3.52 (dt, 2 H, CH_2Br), 3.35 (dt, 2 H, $-CH_2N-$), 2.81 ppm (d, 3 H, CH_3). ^{31}P NMR ($CDCl_3$) -12.67 ppm.

Cyclopentylmethyl phenyl *N*-methyl-*N*-(2-bromoethyl)-phosphoramidate (3d) was prepared from **2d** (3.0 g, 9.6 mmol) as described above. The residue was purified by flash chromatography and was isolated as an oil (3.25 g, 87%): ^{1}H NMR ($CDCl_3$) 7.25 (m, 5 H, phenyl), 3.97 (dd, 2 H, CH_2O), 3.52 (dt, 2 H, CH_2Br), 3.38 (dt, 2 H, $-CH_2N-$), 2.80 (d, 3 H, CH_3) ppm; ^{31}P NMR ($CDCl_3$) -19.57 ppm.

Cyclopentylmethyl *N*-methyl-*N*-(2-bromoethyl)-phosphoramidate cyclohexylamine salt (4d) was prepared from **3d** (0.5 g, 1.3 mmol) as described above. The product was isolated as a white solid (0.37 g, 72%): mp 113-116 °C; ^{1}H NMR (DMSO-d_6) 3.74 (dd, 2 H, $-CH_2O-$), 3.59 (dt, 2 H, CH_2Br), 3.26 (dt, 2 H, $-CH_2N-$), 2.56 (d, 3 H, CH_3) ppm; ^{31}P NMR (D_2O) -15.06 ppm; IR (KBr) 3250-3680, 2940, 2860, 2650, 2570, 2200, 1740, 1200, 1040 cm^{-1}. Anal. ($C_{15}H_{32}N_2O_3PBr\cdot{}^1/_2H_2O$) C, H, N.

Characterization of the Dimethyldithiocarbamate Trapped Product of 4c. When the reaction had gone to completion (^{31}P NMR), the solution was acidified and extracted with $CHCl_3$. The extracts were evaporated, and the crude product was characterized by NMR: ^{1}H NMR (DMSO-d_6) 3.70 (dd, 2 H, $-CH_2O-$), 3.55 (d, 6 H, $(CH_3)_2N$), 3.48 (dt, 2 H, $-CH_2S-$), 3.25 (dt, 2 H, $-CH_2N-$), 2.80 (d, 3 H, CH_3N) ppm; ^{31}P NMR (D_2O) -15.31 ppm.

In Vitro Cytotoxic Activity. Cultured mouse L1210 murine leukemia and B16 melanoma cells were obtained from the National Cancer Institute, Bethesda, MD. A soft agar clonogenic assay was used with L1210 cells as described previously.[16] Clonogenic survival of B16 cells was determined according to the method of Mirabelli et al.[17] L1210 and B16 cells, in exponential growth, were suspended in the appropriate unsupplemented medium (10 mL) at a final density of 2-3 × 10^5 cells/mL. Drug stock solutions (4 mM) were prepared with medium without supplement and sterilized by passage through a 0.22-μm filter. Appropriate volumes (0.125-1 mL) of drug stock solution were added to the cell suspension to give a final drug concentration of 5-400 μM. The drug-cell suspensions were then incubated for 2 h at 37 °C. The cells were washed (3×) with supplemented medium (3 mL) and then resuspended in medium (5 mL). Cells were counted (Coulter counter), and the number of cells required for plating was determined with 2 or 3 different dilutions per drug concentration.

L1210 cells were plated in soft agar (0.2%) at densities between 25 000 and 100 000 cells/mL, and colonies were counted 10 days later. B16 cells were plated by using the monolayer clonogenic assay at densities between 5000 and 20 000 cells/mL (5 mL of medium per 60-mm culture dish). Colonies were stained with crystal violet and counted 7 days later.

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Antimalarial Polyamine Analogues

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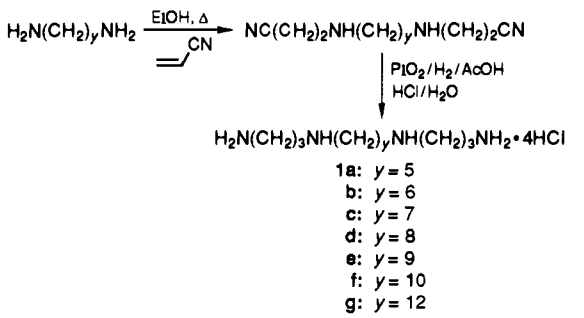
A series of novel tetraamines of the general formula $RNH(CH_2)_xNH(CH_2)_yNH(CH_2)_xNHR$ was synthesized and examined for activity against growth of *Plasmodium falciparum* in vitro. Within the series, dibenzyl analogues (R = benzyl) were found to be the most effective growth inhibitors, with IC_{50} values of about 10^{-6} M. Further modifications of the tetraamine provided the optimum chain length for antimalarial activity of $y = 7$, $x = 3$. Compound 8 (MDL 27,695) with the structure $y = 7$, $x = 3$, R = benzyl, in combination with the ornithine decarboxylase inhibitor α-(difluoromethyl)ornithine, resulted in radical cures when tested against experimental *Plasmodium berghei* infections in mice. The structure-activity relationships of the series are discussed.

Introduction

In spite of past progress in the prevention and treatment of malaria, half the world's population is still threatened by the disease. Its incidence is presently increasing due to the development of resistance of the malaria parasite to current drugs.[1,2] We have shown that parasitic protozoa are sensitive to polyamine biosynthesis inhibitors. Most notably, we found that α-(difluoromethyl)ornithine (DFMO, eflornithine),[3] an irreversible inhibitor of ornithine decarboxylase, is effective against African trypanosomiasis both in the laboratory[4] and in the clinic.[5]

Scheme I



Additionally, DFMO inhibits the growth of blood stages of the human parasite *Plasmodium falciparum* in vitro[6]

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Scheme II

$Br(CH_2)_yBr + H_2N(CH_2)_xNH_2$

↓ EtOH, Δ

$H_2N(CH_2)_xNH(CH_2)_yNH(CH_2)_xNH_2$

2

Table I. Activity of $RHN(CH_2)_3NH(CH_2)_xNH(CH_2)_3NHR_1$ against *P. falciparum* in Vitro

$RHN(CH_2)_3NH(CH_2)_yNH(CH_2)_3NHR_1 \cdot 4HCl$

no.[b]	y	R	R_1	mp, °C	IC_{50},[a] μM
1c	7	H	H	>310	4300
1d	8	H	H	312–313 dec	1800
5	4	Bn[c]	Bn	328–329 dec	14
6	5	Bn	Bn	329–330 dec	6.7
7	6	Bn	Bn	324–325 dec	5.3
8	7	Bn	Bn	319–320 dec	3.0
9	8	Bn	Bn	311–312 dec	0.8
10	9	Bn	Bn	319–19.5 dec	0.7
11	10	Bn	Bn	318–319 dec	0.5
12	12	Bn	Bn	318–319 dec	0.2
13	7	Bn	H	307–309 dec	330
14	8	Bn	H	304–305 dec	64

[a] *P. falciparum* (clone D6 of the Sierra Leone strain from Dr. Wilbur Milhous, Walter Reed Army Institute of Research) was maintained in continuous culture in human erythrocytes (O^+) by the method of Trager and Jensen.[20] The polyamine analogues were dissolved in culture medium and filter-sterilized prior to addition to cultures. The starting parasitemia was approximately 0.5% and in a 1% suspension of erythrocytes. After 24 h of growth in the presence or absence of the test compounds, [^{3}H]-hypoxanthine (1 μCi) was added and its incorporation was measured following a further 18–24 h incubation. Antimalarial activity (Table I) is expressed as the concentration of drug (μM) which inhibited [^{3}H]hypoxanthine incorporation by 50% (IC_{50}). [b] Compounds **1c,d**, were prepared by reduction of dinitrile as in Scheme I. Compound **5** was prepared by method B. Compounds **6–14** were prepared by method C. [c] Bn = Benzyl.

and of *Plasmodium berghei* in mice.[7]

Polyamine analogues, which may perturb polyamine biosynthesis and/or functions, have previously been shown to inhibit growth of tumor cells in vitro.[8-10] Our earlier success in treating parasitic diseases with inhibitors of polyamine biosynthesis led us to test polyamine analogues for activity against *P. falciparum* in vitro and on *P. berghei* in vivo. Recent findings have already shown that such analogues inhibit the growth of both organisms.[11]

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Table II. Activity of $BnNH(CH_2)_xNH(CH_2)_yNH(CH_2)_xNHBn$ against *P. falciparum* in Vitro

$BnNH(CH_2)_xNH(CH_2)_yNH(CH_2)_xNHBn \cdot 4HCl$

no.	x	y	method	mp, °C	IC_{50},[a] μM
15	2	6	A	332–334 dec	4.5
16	2	7	A	314–315 dec	1.4
17	2	8	A	306.5–308.5 dec	0.3
18	2	10	A	322–323 dec	0.3
19	4	7	A	327–328 dec	3.2
20	4	8	A	319–321 dec	5.3

[a] For test procedure see footnote *a*, Table I.

Table III. Activity of $RNH(CH_2)_3NH(CH_2)_yNH(CH_2)_3NHR$ against *P. falciparum* in Vitro

$RNH(CH_2)_3NH(CH_2)_yNH(CH_2)_3NHR \cdot 4HCl$

no.	y	R	method	mp, °C	IC_{50},[a] μM
21	8	4-CH_3OBn[d]	A	314–314.5 dec	1.3
22	8	4-HOBn	*b*	256–258	5.7
23	8	4-CH_3SBn	C	296–297 dec	0.6
24	8	4-CH_3SO_2Bn	*c*	328–329 dec	20
25	8	4-ClBn	A	315.5–317 dec	0.5
26	8	4-EtBn	A	322–324 dec	1.4
27	7	4-CH_3SBn	C	328–329 dec	<3
28	7	4-CH_3SO_2Bn	*c*	323–324 dec	86
29	7	4-ClBn	A	328–329 dec	1.4
30	7	4-CH_3OBn	A	322–323 dec	2.5
31	7	4-HOBn	*b*	259–262	27
32	7	4-EtBn	C	329–330 dec	1.8
33	7	cyclohexylmethyl	C	323–323 dec	1.8
34	7	2-thienylmethyl	C	311–313 dec	4.5
35	7	3-thienylmethyl	C	315–317 dec	3.9
36	8	*n*-Bu	A	320–322 dec	30
37	8	1-naphthylmethyl	A	262–264	0.34
38	7	Ph	D	264–267 dec	0.5
39	7	$Ph(CH_2)_2$	D	>300	1.9
40	7	$Ph(CH_2)_3$	D	>300	0.3

[a] For test procedure see footnote *a*, Table I. [b] Prepared from preceeding compound by treatment with aqueous HBr. [c] Prepared from preceeding compound by oxidation with *m*-chloroperbenzoic acid. [d] Tetrahydrobromide salt.

Scheme III

method A

$H_2N(CH_2)_3NH(CH_2)_7NH(CH_2)_3NH_2 \cdot 4HCl$

1c

↓ THF, NaOH, H_2O, $O[CO_2C(CH_3)_3]_2$

$HN(CH_2)_3N(CH_2)_7N(CH_2)_3NH$
Boc Boc Boc Boc

3

↓ DMF, $KOC(CH_3)_3$, RX

$RN(CH_2)_3N(CH_2)_7N(CH_2)_3NR$
Boc Boc Boc Boc

4

↓ HCl/CH_3OH

$RNH(CH_2)_3NH(CH_2)_7NH(CH_2)_3NHR \cdot 4HCl$

8

Chemistry

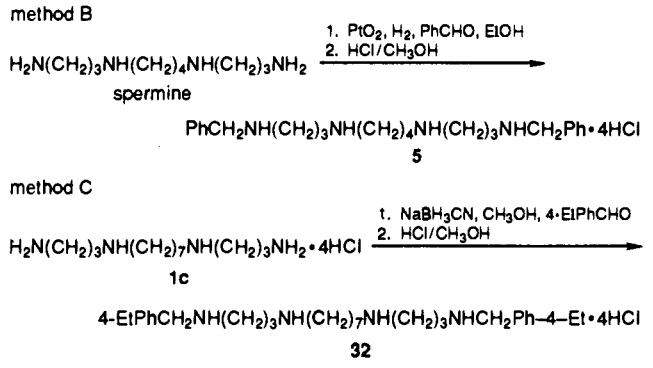
The bis(3-aminopropyl)alkyldiamines **1a–g** (Scheme I) required for the preparation of compounds **6–14**, **21–37**, and **41** were synthesized by a modification of the method of Israel et al.[12] Reaction of an α,ω-diaminoalkane with 2 equiv of acrylonitrile followed by reduction (PtO_2/H_2)

Table IV. Activity against *P. falciparum* in Vitro

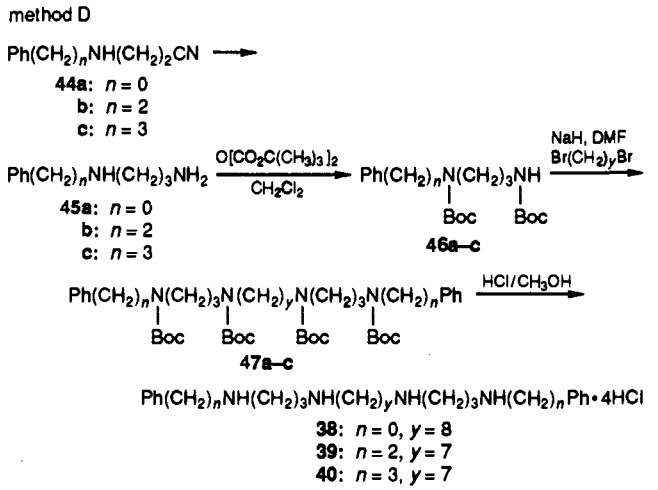
no.	structure	mp, °C	IC_{50},[a] μM
41	$PhCH(CH_3)NH(CH_2)_3NH(CH_2)_7NH(CH_2)_3NHCH(CH_3)Ph \cdot 4HCl$	244–246 dec	4.8
42	$BnNHCH(CH_3)(CH_2)_2NH(CH_2)_7NH(CH_2)_2CH(CH_3)NHBn \cdot 4HCl$	249–253 dec	2.6
43	$H_2NCH(CH_3)(CH_2)_2NH(CH_2)_7NH(CH_2)_2CHNH_2(CH_3) \cdot 4HCl$	209–211	>20

[a] For test procedure, see footnote *a*, Table I.

Scheme IV. Methods B and C



Scheme V. Method D



of the dinitrile product gave the desired tetraamines **1a–g**. Israel et al.[12] have described these compounds except **1c** (see the Experimental Section)[13] and **1d**.[19] The bis(2-aminoethyl)alkyldiamines and bis(4-aminobutyl)alkyldiamines (Scheme II) required for the preparation of compounds **15–20** were obtained by the method of van Alphen.[14]

Two general synthetic routes were used to convert the tetraamines to the α,ω-dibenzyl derivatives of Tables I–IV. The alkyl halide route (method A), an example of which is given in Scheme III, required conversion of the tetraamines into their tetra-*t*-Boc derivatives such as **3**. These tetra-*t*-Boc derivatives, treated with potassium *tert*-butoxide[15] and an alkyl halide in DMF, yield α,ω-disubstituted tetra-Boc-tetraamines **4**. Both the unsubstituted and substituted tetra-Boc-tetraamines are clear, viscous oils that resisted all attempts at crystallization and decomposed upon distillation. Removal of the Boc groups with alcoholic HCl gave the α,ω-disubstituted tetraamines (**6–21**, **23–30**, **36–40**) as hydrochloride salts. In the presence of the appropriate aldehyde, the tetraamines also could be reductively alkylated either by hydrogenation over Adam's catalyst[16] (method B) or by sodium cyanoborohydride[17] (method C). Examples of these reductive alkylations are given in Scheme IV. Compounds **38–40** of Table III were prepared (method D) from diamines **45a**[18]**–c**[19] via alkylation with sodium hydride/alkyl dihalide in DMF of the corresponding Boc derivatives **26a–c** (Scheme V).

The sulfone derivatives **24** and **28** were obtained by *m*-chloroperbenzoic acid oxidation of the tetra-Boc derivatives of **23** and **27** followed by deprotection in the usual manner. Phenol derivatives **22** and **31** were prepared by cleavage of the corresponding methyl ethers **21** and **30** with 48% HBr at reflux and were isolated as the tetrahydrobromide salts. Compound **43** was synthesized as described in the previous paper in this series.[13] Compound **41** and **42** were prepared by methods B and C, respectively, from the free base of compound **43**.

Biological Results and Structure–Activity Relationships

The polyamine analogues were tested for antimalarial activity in vitro by following the incorporation of [^{3}H]-hypoxanthine into *P. falciparum* growing in asynchronous cultures in human erythrocytes as described.[20,21] From the results summarized in Tables I–IV, the in vitro structure–activity relationships of these polyamine analogues may be analyzed in terms of variations of the length of the central chain (*y*) (Table I), the outer chain (*x*) (Table II), and the substituent on the terminal nitrogen atoms (R) (Tables III and IV).

Compounds without *N*-benzyl groups (**1c,d**) are much less active than the α,ω-dibenzyltetraamine derivatives (**8**, **9**) (see Table I), whereas, compounds with only one *N*-benzyl group are of intermediate potency. The central alkyl chain of the α,δ-dibenzyltetraamines could be varied from eight to 12 carbon atoms without a significant change in growth inhibitory activity against *P. falciparum* in vitro. Similarly, the terminal aminopropyl chain of these structures can be substituted by aminoethyl and aminobutyl without any notable modification of the inhibitory activity (see Table II). Again, a wide variety of substituents could be added on the α,ω-dibenzyl groups without loss of antimalarial activity in vitro. Two exceptions were hydroxyl and methyl sulfone (**22**, **24**, **28**, **31**) substitutions, which caused a decrease in potency. Replacement of the benzyl

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Table V. In Vivo Activity of Selected Compounds against *P. berghei* in Mice

no.	% max increase[a] in survival	dose,[b] mg/kg	% cured[c]
5	30	30[2]	
6	30	30[2]	
7		30[2]	
8		15[3]	100 (14/14)
9		10[3]	80 (4/5)
10		10[3]	60 (3/5)
11		3[1]	40 (2/5)
12		10[1]	
17	45	30[2]	
25		3[1]	60 (3/5)
32		30[2]	
42		100[2]	

[a] The standard 4-day suppression test of Peters et al.[22] was used. Mice (groups of five) were infected intravenously with *P. berghei* on a Monday morning and treatment with polyamine analogues and DFMO was begun that afternoon (4:30 pm), approximately 4–6 hr after infection. DFMO was given as a 2% solution in drinking water for four nights (Monday–Thursday) along with the administration of polyamine analogues intraperitoneally at 8:30 a.m., 12:30 p.m., and 4:30 p.m. on three consecutive days (Tuesday–Thursday). When the compound was found to be curative, the results are given as percent cured. [b] (1) Highest dose tolerated, (2) highest dose tested, or (3) dose that gave maximum response. [c] Cured animals lived longer than 4 months and were parasite-free upon periodic examination of Giemsa-stained blood smears.

groups with thienylmethyl (**34**, **35**), cyclohexylmethyl (**33**), or *n*-butyl (**36**) gave less active analogues. Modification of the terminal *N*-benzyl groups to give *N*-phenyl (**38**), phenethyl (**39**), and phenylpropyl (**40**) analogues gave little variance in IC_{50} values.

In an in vitro assay using polyamine oxidase (PAO) from rat liver, the dibenzylpolyamines (**8**, **9**) were debenzylated to give the desbenzyl compounds.[23] As these debenzylated analogues (**1c**,**d**) are inactive, derivatives (**41–43**) of **8** were prepared in an attempt to resist PAO metabolism. None of these analogues were found to be more potent against *P. falciparum* in vitro than compound **8**. The antimalarial activity of the dibenzyl compounds correlates with selective uptake by red blood cells. The intracellular concentration of drug can reach up to 20 times that of the medium.[24] This accumulation in red blood cells may be related to the lipophilicity of the compounds, and may account for the fact that a large number of compounds in the series was essentially equipotent in the in vitro assay. The IC_{50} values obtained for the polyamine analogues against *P. falciparum* in vitro suggest that the compounds are sufficiently potent to be considered for further development. Although not as potent as chloroquine (IC_{50} = 0.014 μM for *P. falciparum* clone D_6), the polyamine analogues are significantly more effective than tetracycline (IC_{50} = 62 μM), an antibiotic which is used successfully to treat malarial infections, especially infections of multiply resistant *P. falciparum*.[25]

On the basis of the activity in the in vitro assay, a group of analogues was evaluated in vivo using a model *P. berghei* infection in mice.[11] Compound **8** was found to be the most effective of the analogues evaluated in vivo. This is in contrast with the in vitro test results and may be explained by the different strain of *Plasmodium* used for the in vitro test. In addition, factors such as metabolism, distribution, and absorption may account for the differences in the results in vivo and in vitro. At the optimum dose of 15 mg/kg (ip), when given in combination with α-DFMO, compound **8** was found to be curative in the in vivo test.

The activity of these compounds against murine malaria did show a relationship with structure (Table V). Curative activity was essentially associated with dibenzyltetraamines (**8–11**). These tetraamines differed only in the length of the central carbon chain. Compounds in which this chain was greater than seven carbons demonstrated greater toxicity in the mouse. With the exception of **25**, which cured 60% of the animals at a dose of 3 mg/kg, substituted benzyl analogues were less active and not well tolerated in vivo. Doses of **25** higher than 3 mg/kg proved to be toxic. In summary, the α,ω-dibenzylpolyamine **8** was found to be curative for *P. berghei* infections in vivo, when coadministered with α-DFMO. Further evaluation of this compound is under way.

Experimental Section

Melting points were determined in a Thomas-Hoover melting point apparatus and are uncorrected. Elemental analyses were performed by the Merrell Dow Research Institute Analytical Department and, unless otherwise indicated, agree with theoretical values within ±0.4%. NMR spectra were obtained on a Varian VXR-300 or a Varian EM-360L spectrometer. Chemical shifts are reported downfield from TMS in spectra obtained in $CDCl_3$ and from DSS in spectra obtained in D_2O. IR spectra were obtained on a Perkin-Elmer 1800 FT IR spectrometer. MS were obtained on a Finnigan MAT 4600 spectrometer. All spectra were consistent with structure. Thin-layer chromatography (TLC) were developed on Merck silica gel 60 F254 analytical plates, visualized with I_2 and/or UV light.

Modified Reduction of Bis[(cyanoethyl)amino]alkanes. 1,5,13,17-Tetraazaheptadecane Tetrahydrochloride (1c). α,ω-Bis[(cyanoethyl)amino]heptane (35.8 g, 0.092 mol) was combined with concentrated hydrochloric acid (132 mL), AcOH (132 mL), and PtO_2 (0.5 g) and treated with hydrogen at 45 lb $in.^{-2}$ until hydrogen uptake ceased. The catalyst was removed by filtration and the solvent removed in vacuo to yield a white solid. Crystallization from CH_3OH/H_2O yielded 22.0 g (61%) of **1c**: mp >310 °C; ^1H NMR (D_2O) δ 3.2–3.0 (m, 12 H), 2.1 (m, 4 H), 1.7 (m, 4 H), 1.4 (m, 6 H); IR (KBr) 2950, 1610, 1460 cm^{-1}; MS (CI, CH_4) *m/z* 245 (M + H); TLC (50% NH_4OH/CH_3OH) R_f = 0.33. Anal. ($C_{13}H_{32}N_4 \cdot 4HCl$) C, H, N; Cl: calcd, 36.34; found, 34.91.

1,5,13,17-Tetrakis(*tert*-butoxycarbonyl)-1,5,13,17-tetraazaheptadecane (3). Compound **1c** (22.0 g, 0.056 mol) and NaOH (9.48 g, 0.237 mol) were combined in water (100 mL) and were allowed to stir until homogeneous. THF (400 mL) was added followed by di-*tert*-butyl dicarbonate (51.66 g, 0.237 mol). The mixture was allowed to stir overnight and diluted with EtOAc (1000 mL) and water (500 mL). The organic layer was separated, dried ($MgSO_4$), and evaporated in vacuo. Flash chromatography on silica gel eluted with 25% EtOAc/hexane yielded 14.1 g (40%) of **3** as an analytically pure clear viscous oil: ^1H NMR ($CDCl_3$) δ 5.3 (2 H, m), 3.25 (4 H, m), 3.1 (8 H, m), 1.65 (4 H, t), 1.5 (4 H, m), 1.45 (36 H, s), and 1.25 (6 H, m); IR (thin film) 3460, 2980, 2940, 1700, 1175, and 735 cm^{-1}; MS (CI, CH_4) *m/z* 645.6 (M + H); TLC 25% EtOAc/hexane, R_f = 0.22. Anal. ($C_{33}H_{64}N_4O_8$) C, H, N.

1,17-Bis(phenylmethyl)-1,5,13,17-tetrakis(*tert*-butoxycarbonyl)-1,5,13,17-tetraazaheptadecane (4). Method A. To a solution of **3** (14.0 g, 0.0217 mol) in DMF (42 mL) was added freshly sublimed potassium *tert*-butoxide (5.35 g, 0.0478 mol). The mixture was allowed to stir until homogeneous and cooled in an ice bath before benzyl bromide (5.7 mL, 0.0478 mol) was added. The mixture was allowed to warm to room temperature and stirred for 24 h. The DMF was removed in vacuo and the viscous gum thus obtained was dissolved in EtOAc (700 mL). The solution was washed 2× with water (100 mL), dried ($MgSO_4$), and evaporated in vacuo. Flash chromatography on silica gel (20% EtOAc/hexane) yielded 5.8 g (33%) of **4** as an analytically pure clear viscous oil: ^1H NMR ($CDCl_3$) δ 7.25–7.05 (10 H, m), 4.2 (4

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H, s), 3.1–2.98 (12 H, m), 1.6 (4 H, m), 1.35 (4 H, m), 1.32 (36 H, s), and 1.15 (6 H, m); IR (thin film) 2980, 2940, 1700, and 1425 cm^{-16}, MS (CI, CH_4) m/z 825 (M + H); TLC 20% EtOAc/hexane R_f = 0.39. Anal. ($C_{47}H_{76}N_4O_8$) C, H, N.

1,14-Bis(phenylmethyl)-1,5,10,14-tetraazatetradecane Tetrahydrochloride (5). Method B. Spermine (Sigma, 2.02 g, 0.01 mol), benzaldehyde (2.13 mL, 0.021 mol), and PtO_2 (0.1 g) were combined in ethanol (40 mL) and treated with hydrogen in a Parr apparatus until the uptake of hydrogen ceased. The catalyst was removed by filtration and the filtrate was treated with 1 N HCl in ethanol (50 mL). Water was added until the solution cleared, the solution was heated to boiling, and 2-propanol was added until the solution became turbid (total volume of 150 mL). Cooling and filtration yielded 2.0 g (38%) of **5** as a white solid: mp 328–329 °C dec; ^1H NMR ($CDCl_3$/TFA, 1:1) δ 7.95 (8 H, br m), 7.35 (10 H, m), 4.2 (4 H, s), 3.3–3.05 (12 H, m), 2.47 (4 H, m), and 1.95 (4 H, m); IR (KBr) 3420, 2950, 2760, and 1450 cm^{-1}; MS (CI, CH_4) m/z 383 (M + H). TLC 20% NH_4OH/CH_3OH R_f = 0.5. Anal. ($C_{24}H_{38}N_4$·4HCl) C, H, N, Cl.

1,17-Bis(phenylmethyl)-1,5,13,17-tetraazaheptadecane Tetrahydrochloride (8). A solution of 0.5 N methanolic HCl (85 mL) was added to 4 (5.8 g, 0.0073 mol) and the mixture was stirred for 18 h. The white precipitate was collected by filtration and recrystallized from CH_3OH/H_2O/2-PrOH twice before the sample was collected and dried over P_2O_5 at 82 °C (0.05 mm) to yield 0.95 g (23%) of **8** as a white solid: mp 319–320 °C dec; ^1H NMR (D_2O) δ 7.45 (10 H, s), 4.27 (4 H, s), 3.18 (8 H, m), 3.05 (4 H, t), 2.2–2.05 (4 H, m), 1.75–1.62 (4 H, m), and 1.38 (6 H, m); IR (KBr) 3440, 2950, 2780, an 1450 cm^{-1}; MS (CI, CH_4) m/z 425 (M + H). TLC 20% NH_4OH/CH_3OH. R_f = 0.71. Anal. ($C_{27}H_{44}N_4$·4HCl) C, H, N, Cl.

1,17-Bis[(4-ethylphenyl)methyl]-1,5,13,17-tetraazaheptadecane Tetrahydrochloride (32). Method C. To a solution of **1c** (1.95 g, 0.005 mol) in methanol (distilled from Mg, 50 mL) was added 4-ethylbenzaldehyde (1.37 mL, 0.01 mol), sodium cyanoborohydride (0.62 g, 0.010 mol), and 1 drop of 1% bromocresol green in ethanol. The pH of the reaction was maintained with 1 N HCl in CH_3OH until the indicator no longer changed. The mixture was then evaporated in vacuo and the residue was partitioned between 1 N NaOH (50 mL) and EtOAc (100 mL). The organic layer was separated, dried ($MgSO_4$), and evaporated in vacuo. Treatment with HCl/CH_3OH followed by evaporation in vacuo gave a solid that was crystallized from water to yield 0.71 g (22%) of **32** as a white solid: mp 329–330 °C dec; ^1H NMR ($CDCl_3$/TFA, 8:1) δ 7.72 (4 H, br m), 7.47 (4 H, br m), 7.26 (10 H, s), 4.20 (4 H, m), 3.25 (8 H, m), 3.08 (4 H, m), 2.66 (4 H, m), 2.45 (4 H, m), 1.70 (4 H, m), 1.34 (6 H, m), and 1.22 (6 H, m); IR (KBr) 3420, 2940, 2790, and 1450 cm^{-1}; MS (CI, CH_4) m/z 473 (M + H). TLC 15% NH_4OH/CH_3OH. R_f = 0.47. Anal. ($C_{31}H_{52}N_4$·4HCl) C, H, N, Cl.

4,8,16,20-Tetraaza-1,23-diphenyltricosane Tetrahydrochloride (40). The tetra-Boc derivative **47c** (1.2 g, 1.5 mmol) was added to a chilled solution of HCl (15 mmol) in methanol (120 mL). The mixture was stirred 18 h at ambient temperature and filtered, and the precipitate was vacuum dried at 50 °C to give the product (**40c**; 0.6 g, 67%) as a white solid: mp >300 °C; NMR (D_2O) δ 7.50–7.30 (10 H, m), 3.35 (4 H, t), 3.13 (8 H, m), 3.04 (8 H, m), 2.08 (4 H, m), 1.65 (4 H, m), and 1.45 (6 H, m); IR (KBr) 2950, 2780, and 1460 cm^{-1}; MS (CI, CH_4) m/z 453 (M + H). Anal. ($C_{29}H_{48}N_4$·4HCl) C, H, N, Cl.

The following were similarly prepared.

38: mp 264–267 °C dec; NMR ($CDCl_3$/TFA) δ 7.95 (br m, 4 H), 7.50 (s, 10 H), 3.60 (m, 4 H), 3.20 (m, 8 H), 2.70 (m, 4 H), 1.80 (m, 4 H), and 1.40 (m, 8 H); IR (KBr) 3440, 2940, 2780, 2750, 2460, 1600, and 1475 cm^{-1}; MS (CI, CH_4) m/z 410 (M + H).

39: mp >300 °C; NMR (D_2O) δ 1.48 (s, 6 H), 1.6–1.7 (m, 4 H), 2.0–2.1 (m, 4 H), 3.0–3.17 (m, 16 H), 3.34 (t, J = 1 Hz, 4 H), and 7.3–7.48 (m, 10 H); IR (KBr) 2940, 2780, 1510, and 690 cm^{-1}; MS (CI) m/z 452 (M + H). Anal. ($C_{29}H_{48}N_4$·4HCl) C, H, N, Cl.

***N*-(2-Cyanoethyl)-3-phenyl-1-propylamine (44c). Method D.** A solution of 3-phenyl-1-propylamine (25 g, 0.185 mol) and acrylonitrile (11.8 g, 0.22 mol) in ethanol (700 mL) was heated at reflux for 18 h. The solution was evaporated and the residue was distilled to give 16.1 g (46%) of a clear liquid: $bp_{0.2}$ 121–124 °C; H^1 NMR ($CDCl_3$) δ 7.30 (2 H, m), 7.18 (3 H, m), 2.92 (2 H, t), 2.65 (4 H, m), 2.48 (2 H, t), 1.80 (2 H, m), and 1.12 (1 H, br m); IR (film) 2940, 2250, 1610, and 1130 cm^{-1}; MS (CI, CH_4) m/z 189. Anal. ($C_{12}H_{16}N_2$) C, H, N.

44b was similarly prepared: $bp_{0.3}$ 121 °C; ^1H NMR (DMSO-d_6) δ 1.85 (br s, 1 H), 2.5–2.6 (t, J = 1 Hz, 2 H), 2.65–2.8 (m, 6 H), and 7.1 (m, 5 H); IR (film) 3025, 2925, 2830, 2240, 1495, 1455, 1130, 750, and 700 cm^{-1}; MS (CI) m/z 174. Anal. ($C_{11}H_{14}N_2$) C, H, N.

44a was purchased from Aldrich.

1,5-Diaza-1,5-bis(*tert*-butoxycarbonyl)-8-phenyloctane (46c). Method D. A solution of $AlCl_3$ (7.3 g, 0.054 mol) in ether (250 mL) was added slowly to a suspension of $LiAlH_4$ (2.1 g, 0.054 mol) in ether (250 mL). The mixture was stirred for 20 min and a solution of *N*-(2-cyanoethyl)-3-phenyl-1-propylamine (9.5 g, 0.054 mol) in ether (100 mL) was added. The reaction mixture was stirred at ambient temperature for 18 h. The reducing agent was decomposed by careful addition of water (20 mL) and 30% aqueous KOH (100 mL). The mixture was filtered and the filtrate was evaporated. The residue was redissolved in dichloromethane (400 mL), di-*tert*-butyl dicarbonate (22 g, 0.1 mol) was added, and the solution was stirred for 18 h at ambient temperature. The solution was evaporated and the residue was chromatographed (toluene/ethyl acetate 9/1) to give the product (**46c**; 8.7 g, 43%) as a thick gum: ^1H NMR ($CDCl_3$) δ 7.28 (2 H, m), 7.19 (3 H, m), 6.78 (1 H, br m), 3.11 (4 H, m), 2.89 (2 H, m), 2.52 (2 H, m), 1.76 (2 H, m), 1.57 (2 H, m), and 1.35 (18 H, s); IR (film) 2940, 1700, 1450, and 1210 cm^{-1}; MS (CI, CH_4) m/z 392. Anal. ($C_{22}H_{36}N_2O_4$) C, H, N.

46a: thick oil; ^1H NMR ($CDCl_3$) δ 7.35–7.00 (m, 5 H) 5.00 (br m, 1 H), 3.65 (t, 2 H, J_1 = 6.6 Hz), 3.15 (dt, 2 H, J_1 = 6.6 Hz, J_2 = 6.8 Hz), 1.65 (t, 2 H, J_r = 6.8 Hz), and 1.45 (s, 9 H).

46b: thick oil; ^1H NMR (DMSO-d_6) δ 1.35 (s, 18 H), 1.5–1.62 (m, 2 H), 2.7–2.8 (m, 2 H), 2.85–3.02 (m, 2 H), 3.05–3.15 (m, 2 H), 3.27–3.35 (m, 2 H), 6.7–6.8 (br s, 1 H), and 7.15–7.25 (m, 5 H); IR (film) 2990, 1700, 1515, 1505, 1480, 1460, 1420, 1370, 1280, 1255, and 1170 cm^{-1}; MS (CI) m/z 378. Anal. ($C_{21}H_{34}N_2O_3$) H, N; C: calcd, 66.64; found 67.15.

4,8,16,20-Tetraaza-4,8,16,20-tetrakis(*tert*-butoxycarbonyl)-1,23-diphenyltricosane (47c). Method D. A solution of compound **46c** (8.7 g, 23 mmol) in DMF (500 mL) was stirred at ambient temperature while sodium hydride (1 g, 23 mmol of 50% suspension in oil) was added in portions. The mixture was stirred an additional 2 h, then NaI (0.5 g) and 1,7-dibromoheptane (1.45 g, 6 mmol) were added. Stirring was continued another 70 h. Methanol (10 mL) was added to decompose any unreacted NaH and the solution was evaporated. The residue was redissolved in CH_2Cl_2 (600 mL) and the solution was washed with water. The organic layer was dried and evaporated and the residue was chromatographed (silica gel, toluene/ethyl acetate 4/1) to give the product (**47c**; 1.2 g, 23%) as a thick oil: ^1H NMR ($CDCl_3$) δ 7.30 (m, 4 H), 7.15 (m, 6 H), 3.15 (m, 16 H), 2.10 (t, 4 H), 1.85 (m, 4 H), 1.72 (m, 4 H), 1.45 (s, 36 H), and 1.32 (m, 6 H); IR (film) 2920, 1675, 1430, 1360, 1160, and 760 cm^{-1}; MS (CI, CH_4) m/z 880. Anal. ($C_{51}H_{84}N_4O_8$) C, H, N.

The following were similarly prepared.

47a: ^1H NMR ($CDCl_3$) δ 7.40–7.00 (m, 10 H) 3.75–3.50 (m, 8 H), 3.15 (m, 8 H), 1.95–1.65 (m, 4 H), 1.45 (s, 36 H), and 1.30 (m, 8 H).

47b: oil; ^1H NMR ($CDCl_3$) δ 1.2–1.3 (m, 6 H), 1.44 (s, 38 H), 1.65–1.8 (m, 6 H), 2.75–2.85 (br s, 4 H), 3.1–3.2 (br s, 12 H), 3.3–3.45 (m, 4 H), and 7.1–7.35 (m, 10 H); IR (film) 2950, 2910, 1690, 1470, 1460, 1410, 1360, 1240, and 1160 cm^{-1}; MS (CI, CH_4) m/z 853. Anal. ($C_{49}H_{80}N_4O_8$) C, H, N.

Registry No. **1c**, 131275-09-7; **1c** free base, 119341-65-0; **1d**, 82958-54-1; **1d** free base, 54443-83-3; **3**, 131275-10-0; **4**, 131275-11-1; **5**, 117654-79-2; **5** free base, 119493-76-4; **6**, 131275-12-2; **6** free base, 119493-77-5; **7**, 131275-13-3; **7** free base, 117654-72-5; **8**, 131275-14-4; **8** free base, 117654-71-4; **9**, 117654-74-7; **9** free base, 117654-73-6; **10**, 131275-15-5; **10** free base, 119493-78-6; **11**, 131275-16-6; **11** free base, 119508-64-4; **12**, 131275-17-7; **12** free base, 119508-65-5; **13**, 131275-18-8; **13** free base, 119341-66-1; **14**, 131275-19-9; **14** free base, 131275-20-2; **15**, 131275-21-3; **15** free base, 131275-23-5; **16**, 131275-23-5; **16** free base, 131275-24-6; **17**, 131275-25-7; **17** free base, 131275-26-8; **18**, 131275-27-9; **18** free base, 131275-28-0; **19**, 131296-21-4; **19** free base, 131275-29-1; **20**, 117654-77-0; **20** free base, 124827-68-5; **21**, 131275-30-4; **21** free base, 131275-31-5; **22**, 131275-32-6; **22** free base, 131275-33-7; **23**,

117654-80-5; **23** free base, 117654-94-1; **24**, 131275-34-8; **24** free base, 131275-35-9; **25**, 131275-36-0; **25** free base, 131275-37-1; **26**, 131275-38-2; **26** free base, 131275-39-3; **27**, 131275-40-6; **27** free base, 131296-22-5; **28**, 131275-41-7; **28** free base, 131275-42-8; **29**, 131275-43-9; **29** free base, 131275-44-0; **30**, 131275-45-1; **30** free base, 131275-46-2; **31**, 131275-47-3; **31** free base, 131275-48-4; **32**, 131275-49-5; **32** free base, 131275-50-8; **33**, 131275-51-9; **33** free base, 131275-52-0; **34**, 131275-53-1; **34** free base, 131275-54-2; **35**, 131275-55-3; **35** free base, 131275-56-4; **36**, 117654-75-8; **36** free base, 125763-86-2; **37**, 117654-76-9; **37** free base, 131275-57-5; **38**, 131275-58-6; **38** free base, 131275-59-7; **39**, 131275-60-0; **39** free base, 131275-61-1; **40**, 131275-62-2; **40** free base, 131275-63-3; **41**, 131275-64-4; **41** free base, 131275-65-5; **42**, 131296-23-6; **42** free base, 131275-66-6; **43**, 131275-67-7; **43** free base, 131275-68-8; **44a**, 1075-76-9; **44b**, 1488-20-6; **44c**, 131275-69-9; **46a**, 117654-95-2; **46b**, 131275-70-2; **46c**, 131275-71-3; **47a**, 117654-96-3; **47b**, 131275-72-4; **47c**, 131275-73-5; α,ω-bis[(cyanoethyl)amino]heptane, 131275-74-6; benzyl bromide, 100-39-0; spermine, 71-44-3; benzaldehyde, 100-52-7; 4-ethylbenzaldehyde, 4748-78-1; 1,7-dibromoheptane, 4549-31-9.

Synthesis and Biological Activity of Methotrexate Analogues with Two Acid Groups and a Hydrophobic Aromatic Ring in the Side Chain[1]

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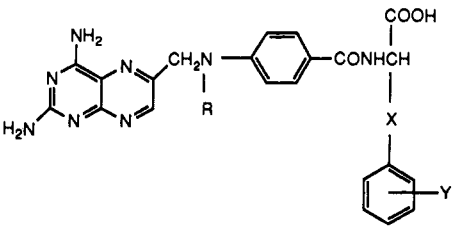
The heretofore unknown γ-(*m*-carboxyanilide) and γ-(*m*-boronoanilide) derivatives of methotrexate (MTX) and the γ-(*m*-carboxyanilide) derivatives of aminopterin (AMT) were prepared and tested as inhibitors of dihydrofolate reductase (DHFR) and as inhibitors of cell growth in culture with the aim of comparing their activity with that of N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine, a potent antifolate whose side chain likewise contains a hydrophobic aromatic ring with an acid group on the ring. All three anilides were potent DHFR inhibitors, with activity comparable to MTX and AMT. The γ-(*m*-boronoanilide) displayed growth inhibitory potency similar to that of the hemiphthaloylornithine analogue, with an IC_{50} of only 0.7 nM. This compound, which is the most potent of the γ-amides of MTX tested to date, is also the first reported example of an antifolate with a *$B(OH)_2$ group in the side chain* and is especially novel because of its potential to form a stable tetrahedral boronate complex by reaction with electron rich OH or NH_2 groups in the active site of DHFR or other folate enzymes. In antitumor assays against L1210 leukemia in mice, N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine gave a T/C of >263% at 20 mg/kg (qd×9) and 300% at 16 mg/kg (bid×10), whereas maximally tolerated doses of MTX of 8 mg/kg (qd×9) and 1 mg/kg (bid×10) gave T/C values of 213 and 188%, respectively. MTX γ-(*m*-boronoanilide) was also active, with a T/C of 175% at 32 mg/kg (qd×9), the highest dose tested.

This laboratory has been active for a number of years in the design and synthesis of analogues of the well-known anticancer drugs methotrexate (MTX) and aminopterin (AMT) for the purpose of systematically exploring the subtle relationships that exist between structure and biological activity in classical antifolates.[2] In two recent papers we reported the synthesis[3,4] and potent antifolate[3] activity of N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (**1**), an analogue of aminopterin (AMT) with three instead of two CH_2 groups in the side chain and the γ-COOH group replaced by a more liphophilic hemiphthaloyl group. Compound **1** had the unusual property of being not only more toxic than MTX to MTX sensitive cells but also more toxic to MTX resistant cells than MTX was to the parental cells from which the resistant sublines were derived.[3] With the aim of identifying the structural features that may contribute to this unusual level of activity, we wished to examine several possible factors: (a) whether the number of CH_2 groups in the side chain could be decreased from three to two, (b) whether the amide could be "reversed" from NHCO to CONH, (c) whether the COOH group on the phenyl ring could be moved from the ortho to the meta position, and (d) whether this group could be replaced by another acidic group. The present paper describes the synthesis of compounds **2**–**4** (Chart I) the first three analogues of **1** studied in this connection. Compounds **1**–**4** all inhibited DHFR from human leukemic cells with a potency similar to AMT and MTX and were also good inhibitors of cell growth in culture. Compound **4**, the first example of a folate analogue with a $B(OH)_2$ group in the side chain, was approximately ten times more potent than MTX as a cell

Chart I



	R	X	Y
1	H	$(CH_2)_3NHCO$	*o*-CO_2H
2	H	$(CH_2)_2CONH$	*m*-CO_2H
3	Me	$(CH_2)_2CONH$	*m*-CO_2H
4	Me	$(CH_2)_2CONH$	*m*-$B(OH)_2$

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(1) Paper 43 in this series; for previous paper, see: Rosowsky, A.; Forsch, R. A.; Moran, R. G.; Freisheim, J. H. *J. Med. Chem.*, in press.
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