phenone, 100-19-6; hydroxylamine amine hydrochloride, 5470-11-1; formaldehyde, 50-00-0; 4-formyl-*N*-(1-phenylethyl)benzamine, 108191-30-6; methylamine, 74-89-5; dimethylamine, 124-40-3; *N*-benzyl-α-methylbenzenemethanamine, 19302-20-6; *N*-(4-aminobenzyl)-α-ethylenzenemethanamine, 108191-31-7; *N*-(4-aminobenzyl)-α-propylbenzenemethanamine, 108191-32-8; *N*-(4-aminobenzyl)-α-butylbenzenemethanamine, 108191-33-9; acetophenone, 98-86-2; ethyl phenyl ketone, 93-55-0; propyl phenyl ketone, 495-40-9; butyl phenyl ketone, 1009-14-9; benzylamine, 100-46-9; *p*-aminobenzenemethanamine, 4403-71-8.

Quantitative Structure–Activity Relationship of Triazine–Antifolate Inhibition of *Leishmania* Dihydrofolate Reductase and Cell Growth

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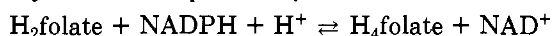
Quantitative structure–activity relationships have been formulated for the inhibition of *Leishmania major* dihydrofolate reductase (DHFR) and for inhibition of promastigote cell growth by a series of 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(3-substituted-phenyl)-*s*-triazines. The inhibition of DHFR is best correlated by a modified variable for hydrophobicity of the 3-X substituent (π'_3), an alkoxy group indicator variable (I_{OR}), a disposable parameter (β) obtained by iteration, and a variable that parameterizes steric effects (MR) in the equation, $\log 1/K_i = 0.65\pi'_3 - 1.22 \log(\beta \cdot 10^{\pi'_3} + 1) - 1.12 I_{OR} + 0.58\mathrm{MR_Y} + 5.05$ ($r = 0.965$). The EC_{50} values for triazine inhibition of *L. major* cell growth in culture are correlated by the equation $\log 1/EC_{50} = 0.21\pi_3 + 0.44 \log 1/K_i + 0.53$ ($r = 0.960$). When compared to DHFR from human, other vertebrates, and *E. coli*, *L. major* DHFR differs in that it optimally binds triazine congeners that are much more hydrophobic. Furthermore, in contrast to other DHFR's studied, triazine binding to *L. major* DHFR does not seem to be influenced by the electronic characteristics of the 3-X substituent of the parent triazine molecule. However, *L. major* DHFR is more sensitive to the steric effects and polarizability of the 3-X substituent. Our results indicate that triazines inhibit *L. major* promastigote growth via direct inhibition of DHFR as is shown by the good correlation between $\log 1/K_i$ values for inhibition of the purified enzyme and $\log 1/EC_{50}$ values for inhibition of cell culture growth. Two lipophilic, sterically large analogues of this triazine series showed selectivity for *L. major* DHFR over human DHFR. Further optimization of the MR and I_{OR} terms in the above QSAR equations may provide even more selective inhibitors.

Dihydrofolate reductase (DHFR, EC 1.5.1.3) catalyzes the reduction of 7,8-dihydrofolate (H_2folate) to 5,6,7,8-tetrahydrofolate (H_4folate) by NADPH as follows:

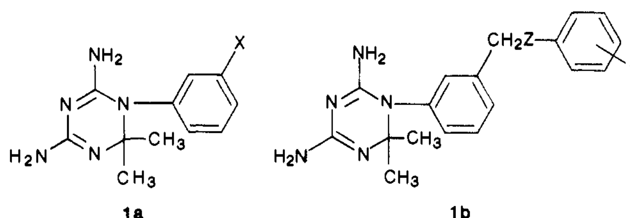


$$H_2\text{folate} + \text{NADPH} + H^+ \rightleftarrows H_4\text{folate} + \text{NAD}^+$$

Structural studies show that there are large differences in the primary sequences of DHFR from protozoan,[1a,b] bacteriophage,[2] bacterial,[3] and mammalian[3] sources, and indeed, the enzyme from different sources shows wide variation in its sensitivity to inhibitors. These differences, coupled with its biochemical importance in folate metabolism, make DHFR an attractive target for design of selective inhibitors in pathogens with respect to their hosts.[4] The clinical effectiveness of the antibacterial drug trimethoprim and the antimalarial drug pyrimethamine attest to the utility of selective DHFR inhibitors.

The DHFR's in *Leishmania major* and other parasitic protozoan are unique from mammalian and other sources in that the protozoan DHFR's exist coupled to thymidylate synthetase (TS, EC 2.1.1.45) as a bifunctional protein.[5a–c] This makes the enzyme a promising target for design of selective inhibitors. The parasite causes cutaneous leishmaniases, a disfiguring disease endemic to Latin and South American, Mediterranean, and Middle Eastern countries. Currently, treatment of the infection employs the use of antimonial compounds that have demonstrated cardiovascular and other toxicity as well as emerging resistance.[6]

Correlation analysis[7] has been used to develop quantitative structure–activity relationships (QSAR) for 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(3-substituted-phenyl)-*s*-triazine (triazine, **1a** and **1b**) inhibition of DHFR from human and other species.[8,9] A set of congeners is



used to probe the enzyme to obtain information about the active site in terms of its hydrophobic, steric, and electronic requirements for ligand interaction. Using the QSAR approach, one can quantify differences in the free energy of binding of less than 0.5 kcal/mol. Furthermore, the method allows a comparison of the enzyme–ligand effect

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determined from data obtained with the isolated enzyme and data obtained from cell cultures with the enzyme in its natural environment.[9,19]

In this paper, we discuss the QSAR equations obtained from a series of 3-substituted triazine compounds (**1a**, **1b**) that competitively inhibit isolated *L. major* DHFR and equations derived from the incubation of some of the same compounds to cell cultures of *L. major*.

Results

QSAR for the Inhibition of Isolated *L. major* DHFR by Triazines 1a and 1b. From the data in Table I, the following QSAR equations have been derived from the inhibition of *L. major* DHFR by triazines **1a** and **1b**:

$$\log 1/K_i = 0.31\ (\pm 0.16)\pi'_3 + 5.54\ (\pm 0.39) \quad (1)$$

$$n = 41 \quad r = 0.538 \quad s = 0.904 \quad F_{1,39} = 15.9$$

$$\log 1/K_i = 1.04\ (\pm 0.24)\pi'_3 - 1.20\ (\pm 0.35) \log (\beta \cdot 10^{\pi'_3} + 1) + 5.18\ (\pm 0.28) \quad (2)$$

$$n = 41 \quad r = 0.834 \quad s = 0.606 \quad \pi_0 = 2.51\ (\pm 1.38) \quad F_{1,37} = 24.8 \quad \log \beta = -1.680$$

$$\log 1/K_i = 0.99\ (\pm 0.17)\pi'_3 - 0.99\ (\pm 0.26) \log (\beta \cdot 10^{\pi'_3} + 1) - 1.19\ (\pm 0.42)I_{OR} + 5.27\ (\pm 0.21) \quad (3)$$

$$n = 41 \quad r = 0.917 \quad s = 0.445 \quad F_{1,37} = 32.7 \quad \pi_0 \simeq 5.0 \quad \log \beta = -1.790$$

$$\log 1/K_i = 0.65\ (\pm 0.08)\pi'_3 - 1.22\ (\pm 0.29) \log (\beta \cdot 10^{\pi'_3} + 1) - 1.12\ (\pm 0.29)I_{OR} + 0.58\ (\pm 0.16)MR_Y + 5.05\ (\pm 0.16) \quad (4)$$

$$n = 41 \quad r = 0.965 \quad s = 0.298 \quad \pi_0 = 4.54 \quad F_{1,35} = 45.2 \quad \log \beta = -4.491$$

In these equations, n represents the number of data points used to derive the equation, r is the correlation coefficient, s is the standard deviation from the regression, F is the F statistic for significance of each additional variable, and the values in parentheses are for construction of the 95% confidence intervals; K_i is the Michaelis inhibition constant. The disposable parameter β is obtained by an iterative procedure for the bilinear structure–activity model.[10]

The squared correlation matrix ($\mathbf{r}^2$) for the variables of eq 1–4 is

	π'_3	I_{OR}	MR_Y
π'_3	1	0.15	0.00
I_{OR}		1	0.11
MR_Y			1

The eigen values for the parameter matrix and the fraction of variance (in parentheses) accounted for by each are 1.53 (51%), 0.96 (32%), and 0.51 (17%). Equation 2 in π'_3 alone accounts for 70% of the variance, I_{OR} accounts for an additional 14%, and MR_Y accounts for 9%. Unexplained is 7%.

The hydrophobicity variable π, superscribed with a prime (π'), denotes that in **1b**, for substituents of type $CH_2ZC_6H_4$-Y, where Z = O, NH, Se, or S, the π value for Y is set equal to zero (i.e., $\pi_{CH_2ZC_6H_4\text{-}Y} = \pi_{CH_2ZC_6H_5}$). This same parameterization is also applied to groups of the type $ZCH_2C_6H_4$-Y, where Z = O, S. This technique for QSAR is used for triazine inhibition of DHFR from a variety of sources[8] when $\log 1/K_i$ does not correlate with the hydrophobicity of the Y group in **1b**. For one congener acting on chicken DHFR, it has been directly established via X-ray crystallography[11] that the Y group of **1b** does not contact the enzyme and thus no parameterization of any kind was required for Y. However, with *Leishmania* DHFR, Y appears to have both steric and polar effects with the enzyme since it is found that a term in molar refractivity[12] (MR_Y) considerably improves the correlation (compare eq 3 to eq 4). If π is used in place of MR_Y in eq 4, a much poorer correlation results: $r = 0.923$. This value of the correlation coefficient is not much improved over that of r for eq 3.

The indicator variable[8] (I_{OR}) takes the value of 1 for all substituents where X = $O(CH_2)_nCH_3$. The negative coefficient for I_{OR} indicates that, other factors being equal, alkoxy groups are about 10-fold less inhibitory than are other substituents.

The optimal lipophilicity (π_0) cannot be firmly established for eq 3 because the right-hand side of the bilinear relationship is not significantly lower than the left (it has been rounded to 0.99).

The following analogues were tested but not included in the derivation of eq 1–4: **42–45** (Table I). The K_i for analogue **42** (**1a**, X = 3-$C(CH_3)_3$) is 3.6 standard deviations outside the correlation. This is in contrast to QSAR equations calculated from triazine inhibition of DHFR from many species where the bulky $C(CH_3)_3$ group is only 1 standard deviation less than expected.[8] This evidence suggests steric interference of *L. major* DHFR with bulky substituents attached directly to the 3-position of the phenyl ring of **1a**. The data for analogues **43** and **45** (**1a**, X = 3-$CH(OH)C_6H_5$ and 3-$COOCH_2CH_3$, respectively) were also not included in the derivation of eq 4 because these analogues invariably show a poor correlation in the QSAR equations derived from inhibition of DHFR from every source tested.[8] We believe this is due to untoward steric effects that arise from branching at the α-carbon on the substituent attached to the phenyl group of the parent triazine structure. It is not apparent why the 3-$CH_2OC_6H_4$-4'-$(CH_2)_4CH_3$ group (**44**, Table I) is not well correlated by the above equations. When analogues **42–45** (Table I) are included in the correlation analysis, eq 5 is obtained:

$$\log 1/K_i = 0.64\ (\pm 0.12)\pi'_3 - 1.26\ (\pm 0.46) \log (\beta \cdot 10^{\pi'_3} + 1) + 1.01\ (\pm 0.43)I_{OR} + 0.60\ (\pm 0.21)MR_Y + 4.92\ (\pm 0.22) \quad (5)$$

$$n = 45 \quad r = 0.922 \quad s = 0.451 \quad \pi_0 = 4.64 \quad \log \beta = -4.635$$

Although the experimental data does not correlate as well with eq 5 ($r = 0.922$) as with eq 4 ($r = 0.965$), the parameters in the two equations do not differ significantly. Other parameters that were examined but failed to yield significant correlations include the hydrophobic (π_3 of the whole substituent), electronic (σ, F, R) and steric (E_s, ν) constants.

QSAR for Growth Inhibition of *L. major* Cell Cultures by Triazines 1a and 1b. Table II shows the $\log 1/EC_{50}$ values for growth inhibition of *L. major* promastigotes in cell culture by triazines **1a** and **1b**. EC_{50} refers to that concentration of drug resulting in a growth rate equal to 50% of the rate in a drug free medium. We

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have derived eq 6, which correlates these EC_{50} values with the single parameter π_3. Since these compounds were not

$$\log 1/EC_{50} = 0.29\ (\pm 0.09)\pi_3 + 3.33\ (\pm 0.31) \quad (6)$$

$$n = 12 \quad r = 0.917 \quad s = 0.251 \quad F_{1,10} = 53$$

sufficiently potent as inhibitors of cell growth to warrant extensive investigation, we did not examine all of the analogues listed in Table I. When $\log 1/K_i$ for the inhibitors is added as a variable to eq 6 to account for binding to DHFR, a much improved correlation is obtained in the resultant eq 7; it is noted that K_i contains all of the parameters in eq 4, established for inhibition of purified DHFR.

$$\log 1/EC_{50} = 0.21\ (\pm 0.09)\pi_3 + 0.44\ (\pm 0.32) \log 1/K_i + 0.53\ (\pm 2.1) \quad (7)$$

$$n = 12 \quad r = 0.960 \quad s = 0.186 \quad F_{1,9} = 9.29$$

When the values from analogues 1a ($X = 3\text{-}(CH_2)_{11}CH_3$) and **1b** ($X = CH_2NHC_6H_3\text{-}3',5'\text{-}(CONH_2)_2$ (**12** and **23**, Table II) are omitted from the data set, eq 8 is obtained; note the excellent correlation ($r = 0.989$). We excluded

$$\log 1/EC_{50} = 0.52\ (\pm 0.06)\pi_3 + 2.64\ (\pm 0.19) \quad (8)$$

$$n = 10 \quad r = 0.989 \quad s = 0.079$$

the data of the $3\text{-}(CH_2)_{11}CH_3$ substituent (**12**, Table II) in the derivation of eq 8 because its π value does not correlate with its inhibitory potency. Very likely its π value is at π_0, but since not enough such compounds were tested, π_0 cannot be defined. When we compare the $\log 1/EC_{50}$ values for the $3\text{-}(CH_2)_{11}CH_3$ and $3\text{-}(CH_2)_8CH_3$ (**11**, Table II) substituents, we see that they are nearly the same despite the much larger π value of the former analogue. This result conflicts with eq 6, which suggests that, the greater the π value of the triazine 3-X substituent, the more potent will be the inhibition of DHFR, giving a larger $\log 1/EC_{50}$. It may be that the alkyl side chain of analogue **12** folded back on itself in solution. The data from the $3\text{-}CH_2NHC_6H_3\text{-}3',5'\text{-}(CONH_2)_2$ substituent (**23**, Table II) was excluded because this compound contains the CH_2NH bridge found in folic acid, and the QSAR of DHFRs from other sources[13] have shown that, in general, compounds of this type are more inhibitory to cell culture growth than is predicted by their QSAR equation. Note that in eq 6–8 π is needed to parameterize hydrophobicity rather than π'. This indicates that the hydrophobic interaction of the entire substituent in **1a** and **1b** must be considered in the QSAR for binding to intracellular *L. major* DHFR. However, we are unable to distinguish between hydrophobicity requirements for DHFR binding and for cell membrane penetration.

Discussion

It is interesting to compare eq 4 with other QSARs correlating the action of similarily substituted sets of triazine 1 on DHFR from other sources.

3-X-triazine Inhibition of Chicken Liver DHFR[11]

$$\log 1/K_i = 1.01\pi'_3 - 1.16 \log (\beta \cdot 10^{\pi'_3} + 1) + 0.86\sigma + 6.33 \quad (9)$$

$$n = 59 \quad r = 0.906 \quad s = 0.267 \quad \pi_0 = 1.89$$

3-X-triazine Inhibition of Human DHFR[15]

$$\log 1/K_i = 1.07\pi'_3 - 1.10 \log (\beta \cdot 10^{\pi'_3} + 1) + 0.50I + 0.82\sigma + 6.07 \quad (10)$$

$$n = 60 \quad r = 0.890 \quad s = 0.308 \quad \pi_0 = 1.84$$

3-X-triazine Inhibition of Bovine DHFR[16]

$$\log 1/K_i = 1.10\pi'_3 - 1.23 \log (\beta \cdot 10^{\pi'_3} + 1) + 0.61\sigma + 7.08 \quad (11)$$

$$n = 38 \quad r = 0.914 \quad s = 0.277 \quad \pi_0 = 1.72$$

3-X-triazine Inhibition of Rat Liver DHFR[17]

$$\log 1/K_i = 1.12\pi_3 - 1.34 \log (\beta \cdot 10^{\pi_3} + 1) + 6.80 \quad (12)$$

$$n = 18 \quad r = 0.977 \quad s = 0.171 \quad \pi_0 = 1.68$$

3-Triazine Inhibition of L5178Y Leukemia DHFR[18]

$$\log 1/K_i = 1.19\pi'_3 - 1.38 \log (\beta \cdot 10^{\pi'_3} + 1) + 0.50I + 0.90\sigma + 6.20 \quad (13)$$

$$n = 38 \quad r = 0.935 \quad s = 0.289 \quad \pi_0 = 1.56$$

3-X-triazine Inhibition of L1210 Leukemia DHFR[19]

$$\log 1/K_i = 0.98\pi'_3 - 1.14 \log (\beta \cdot 10^{\pi'_3} + 1) + 0.79\sigma + 6.12 \quad (14)$$

$$n = 58 \quad r = 0.900 \quad s = 0.264 \quad \pi_0 = 1.76$$

3-X-triazine Inhibition of *Lactobacillus casei* DHFR[9]

$$\log 1/K_i = 0.53\pi'_3 - \log (\beta \cdot 10^{\pi'_3} + 1) + 1.49I + 0.70\sigma + 2.93 \quad (15)$$

$$n = 44 \quad r = 0.953 \quad s = 0.319 \quad \pi_0 = 4.31$$

3-X-triazine Inhibition of *E. coli* DHFR[14]

$$\log 1/K_i = 1.16\pi'_3 - 1.10 \log (\beta \cdot 10^{\pi'_3} + 1) + 1.36\sigma + 0.41I + 5.08 \quad (16)$$

$$n = 31 \quad r = 0.930 \quad s = 0.280$$

The most important parameter in these QSAR equations is π'_3, a measure of the effect of hydrophobicity on inhibitor potency. The π'_3 coefficient for *L. major* DHFR (0.65) is similar to that of *L. casei* DHFR (0.53) but differs greatly from the *E. coli* (1.16) and vertebrate reductases, which form a relatively consistent group ranging from 1.01 to 1.19 with a mean of 1.08. The optimum lipophilicity (π_0) for the vertebrate enzymes range from 1.56 to 1.89 with a mean of 1.74; the π_0 for *L. major* (4.64) greatly differs from that of vertebrate DHFRs and resembles the *L. casei* value (4.31). It may be that highly lipophilic compounds are required for optimal binding to *L. major* and *L. casei* enzymes because, compared to other DHFR's, these reductases contain a more nonpolar environment at their active sites.

All of the above QSAR except those for rat and *L. major* contain a term in σ, a measure of electronic effects of triazine 1 interaction with DHFR. If one poorly fit data point (**1a**, X = 3-d, 1-CH(OH)C_6H_5) is dropped from the

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Table I. Parameters[a] Used To Derive Eq 1–5 for the Inhibition of DHFR from *L. tropica* by Triazines 1

no.	X	log $1/K_i$, M obsd	calcd[b]	$\Delta \log 1/K_i$	π'_3	I_{OR}	MR_Y
1	H	4.90 ± 0.06	5.05	0.15	0.00	0	0
2	3-SO_2NH_2	3.44 ± 0.08	3.87	0.43	−1.82	0	0
3	3-$CONH_2$	3.93 ± 0.06	4.08	0.15	−1.49	0	0
4	3-$COCH_3$	4.40 ± 0.02	4.69	0.29	−0.55	0	0
5	3-OH	4.27 ± 0.05	4.61	0.34	−0.67	0	0
6	3-CF_3	5.66 ± 0.04	5.62	0.04	0.88	0	0
7	3-Cl	5.92 ± 0.03	5.51	0.41	0.71	0	0
8	3-NO_2	5.00 ± 0.04	4.86	0.14	−0.28	0	0
9	3-CN	5.22 ± 0.02	4.68	0.54	−0.57	0	0
10	3-C_2H_5	5.71 ± 0.08	5.71	0.00	1.03	0	0
11	3-$(CH_2)_8CH_3$	7.68 ± 0.05	7.57	0.11	4.79	0	0
12	3-$(CH_2)_{11}CH_3$	7.14 ± 0.08	6.84	0.30	6.41	0	0
13	3-OCH_3	4.64 ± 0.05	3.91	0.73	−0.02	1	0
14	3-OC_2H_5	4.42 ± 0.12	4.17	0.25	0.38	1	0
15	3-$O(CH_2)_5CH_3$	5.40 ± 0.03	5.65	0.25	2.67	1	0
16	3-$O(CH_2)_8CH_3$	6.52 ± 0.01	6.45	0.07	4.29	1	0
17	3-$O(CH_2)_{10}CH_3$	6.01 ± 0.04	6.26	0.25	5.37	1	0
18	3-$O(CH_2)_{13}CH_3$	5.23 ± 0.08	5.39	0.16	6.99	1	0
19	3-$O(CH_2)_2OC_6H_4$-3′-CF_3	6.60 ± 0.06	6.42	0.18	1.68	0	0.50
20	3-$O(CH_2)_4OC_6H_4$-3′-CF_3	7.12 ± 0.02	7.08	0.04	2.71	0	0.50
21	3-$OCH_2C_6H_5$	5.72 ± 0.03	6.18	0.46	1.66	0	0.10
22	3-OCH_2-1-adamantyl	5.51 ± 0.04	5.90	0.39	3.07	1	0
23	3-$CH_2NHC_6H_3$-3′,5′-$(CONH_2)_2$	6.29 ± 0.03	6.83	0.54	1.00	0	1.96
24	3-$CH_2NHC_6H_4$-4′-SO_2NH_2	6.12 ± 0.04	6.41	0.29	1.00	0	1.23
25	3-$CH_2OC_6H_4$-3′-Cl	6.65 ± 0.14	6.47	0.18	1.66	0	0.69
26	3-$CH_2OC_6H_4$-3′-CN	6.75 ± 0.07	6.49	0.26	1.66	0	0.63
27	3-$CH_2OC_6H_4$-3′-OCH_3	6.82 ± 0.04	6.58	0.24	1.66	0	0.79
28	3-$CH_2OC_6H_4$-3′-CH_2OH	6.19 ± 0.02	6.54	0.35	1.66	0	0.72
29	3-$CH_2OC_6H_4$-3′-CH_3	6.37 ± 0.04	6.45	0.08	1.66	0	0.57
30	3-$CH_2OC_6H_4$-3′-C_2H_5	6.90 ± 0.05	6.72	0.18	1.66	0	1.03
31	3-$CH_2OC_6H_4$-3′-$CH(CH_3)_2$	6.76 ± 0.08	6.99	0.23	1.66	0	1.50
32	3-$CH_2OC_6H_4$-3′-C_6H_5	7.77 ± 0.10	7.60	0.17	1.66	0	2.54
33	3-$CH_2OC_6H_4$-3′-$NHCOCH_3$	7.12 ± 0.05	6.99	0.13	1.66	0	1.49
34	3-$CH_2OC_6H_4$-3′-$NHCONH_2$	7.04 ± 0.09	6.92	0.12	1.66	0	1.37
35	3-CH_2O-1-naphthyl	7.40 ± 0.08	7.14	0.26	1.66	0	1.75
36	3-$CH_2SC_6H_5$	6.39 ± 0.09	6.59	0.20	2.30	0	0.10
37	3-$CH_2SeC_6H_5$	6.56 ± 0.02	6.64	0.08	2.37	0	0.10
38	3-S-$CH_2C_6H_5$	6.65 ± 0.05	6.59	0.06	2.30	0	0.10
39	3-$SCH_2C_6H_4$-4′-Cl	6.96 ± 0.02	6.88	0.08	2.30	0	0.60
40	3-$CH_2OC_6H_2$-2′,4′,5′-Cl_3	7.16 ± 0.04	7.17	0.01	1.66	0	1.80
41	3-$CH_2NHC_6H_4$-4′-Cl	6.18 ± 0.04	6.04	0.14	1.00	0	0.60
42	3-$C(CH_3)_3$[b]	5.24 ± 0.02	6.33	1.09	1.98	0	0
43	3-d,1-$CH(OH)C_6H_5$[b]	4.37 ± 0.03	5.40	1.03	0.54	0	0
44	3-$CH_2OC_6H_4$-4′-$(CH_2)_4CH_3$[b]	6.47 ± 0.03	7.53	1.06	1.66	0	2.42
45	3-$COOCH_2CH_3$[b]	3.95 ± 0.03	5.38	1.43	0.51	0	0
46	methotrexate[c,d]	9.89 ± 0.03					

[a] Substituent constants taken from ref 12. [b] Calculated by using eq 4. [c] These points omitted in the derivation of eq 1–4. [d] Reference 4.

equation for rat DHFR, the resultant equation, 12a, does contain a significant term in σ. The coefficients of σ then range from 0.46 to 0.90, with a mean of 0.73. Thus, the QSAR for *L. major* DHFR is unique in that it does not possess a σ term.

$$\log 1/K_i = 1.13\pi_3 - 1.30 \log (\beta \cdot 10^{\pi_3} + 1) + 0.46\sigma + 6.23 \quad (12a)$$

$$n = 17 \quad r = 0.987 \quad s = 0.126 \quad \pi_0 = 1.72$$

The QSAR for triazine inhibition of *L. major* DHFR is also unique among other DHFRs examined in that it contains a term in MR_Y. MR is primarily a measure of volume and secondarily a measure of the polarizability of the substituent.[12] The positive coefficient for the MR term in the *L. major* equation indicates that, the larger the Y of **1b** is, the more effective is the inhibitor. The QSAR for other DHFRs studied do not possess this parameter, suggesting that, as is the case for chicken DHFR,[11] bulky Y substituents do not make contact with these enzymes in solution, but do with *L. major* enzyme.

The indicator variable, I, which accounts for the better than predicted inhibitory potency of analogues containing the $CH_2ZC_6H_4$ or $ZCH_2C_6H_4$ moieties, does not seem to show a consistent pattern in the examples studied thus far. Likewise, alkoxy groups (parameterized by I_{OR}), behave erratically with DHFR from various sources.

In summary of the above QSAR equations, we find that *L. major* DHFR shares similar hydrophobic requirements with *L. casei* DHFR, but differs dramatically from other DHFRs that optimally bind triazine compounds that are much less hydrophobic. Furthermore, in contrast to other DHFRs, triazine binding to the *L. major* reductase does not seem to be influenced by the electronic characteristics of the 3-X substituent as evidenced by the lack of a σ term in eq 4. On the other hand, the *L. major* DHFR is more sensitive to the steric effects and polarizability of the 3-X substituent when compared to the other DHFR's studied. The QSAR for the *L. major* enzyme also contains a unique indicator variable, I_{OR}, which shows no correlation in the QSAR for triazine inhibition of DHFR from other species examined.

Triazines appear to inhibit *L. major* promastigote growth via direct inhibition of DHFR as is shown by the good correlation between log $1/K_i$ values for inhibition of the purified enzyme and log $1/EC_{50}$ values for the inhib-

Table II. Parameters Used To Derive Eq 6 and 7 for the Inhibition of Growth of Promastigotes in Culture

no.	X	log $1/EC_{50}$, M obsd[a]	log $1/EC_{50}$, M calcd[b]	$\|\Delta \log 1/EC_{50}\|$	π[c]
11	$3\text{-}(CH_2)_8CH_3$	5.07	4.73	0.34	4.79
12	$3\text{-}(CH_2)_{11}CH_3$	5.10	5.21	0.11	6.41
19	$3\text{-}O(CH_2)_2OC_6H_5\text{-}3'\text{-}CF_3$	3.90	4.08	0.18	2.56
23	$3\text{-}CH_2NHC_6H_3\text{-}3',5'\text{-}(CONH_2)_2$	3.30	2.94	0.36	-1.34
27	$3\text{-}CH_2OC_6H_4\text{-}3'\text{-}OCH_3$	3.51	3.81	0.30	1.64
29	$3\text{-}CH_2OC_6H_4\text{-}3'\text{-}CH_3$	3.66	3.98	0.32	2.22
31	$3\text{-}CH_2OC_6H_4\text{-}3'\text{-}CH(CH_3)_2$	4.24	4.26	0.02	3.19
32	$3\text{-}CH_2OC_6H_4\text{-}3'\text{-}C_6H_5$	4.64	4.41	0.23	3.69
35	$3\text{-}CH_2O$-1-naphthyl	4.29	4.20	0.09	2.98
39	$3\text{-}SCH_2C_6H_4\text{-}4'\text{-}Cl$	4.22	4.21	0.01	3.01
40	$3\text{-}CH_2OC_6H_2\text{-}2',4',5'\text{-}Cl_3$	4.60	4.44	0.16	3.79
41	$3\text{-}CH_2NHC_6H_4\text{-}4'\text{-}Cl$	3.58	3.83	0.25	1.71
46	methotrexate[d]	6.00			

[a] The observed values were all within 5% of the mean. [b] Calculated by using eq 6. [c] Taken from ref 12. [d] Not used in the derivation of eq 6.

ition of cell culture growth (eq 7). Of the triazines tested, lipophilic, straight-chain analogues [i.e., where X of **1a** is $(CH_2)_8CH_3$ or $(CH_2)_{11}CH_3$] are the most toxic to *L. major* cells in culture. Likewise, these compounds are among the most potent inhibitors of *L. major* DHFR in vitro. However, the log $1/K_i$ values of the analogues in Table I are two- to threefold greater than the log $1/EC_{50}$ values of the same compounds (Table II). Presumably, in comparison to purified enzyme in vitro, higher concentrations of drug are required to bind and inhibit DHFR within cells because of barriers imposed by the cell membrane and/or intracellular environment. It may also be that *L. major* DHFR must be much more than 50% inhibited for there to be 50% inhibition of growth. Although not enough analogues were tested against cell culture to correlate the MR variable with inhibitory potency in eq 6, the two bulky lipophilic analogues that were tested (1a, X = 3-$CH_2OC_6H_4$-3'-C_6H_5 and 1a, X = 3-CH_2-1-naphthyl) showed less activity than the in vitro data would predict, suggesting that steric effects are important for triazine binding to *L. major* DHFR in vivo.

With this data in hand, we may begin to rationally design a clinically relevant selective inhibitor of *L. major* DHFR by optimizing the parameters that distinguish QSAR 4 and 7 from eq 10 (i.e., the presence of MR and I_{OR} terms and lack of a σ term). Evidence from crystal structure studies of triazine **1a**, X = 4-OCH_3, binding with chicken DHFR[20] shows that of the 12 amino acid residues interacting directly with the triazine inhibitor, six amino acids are identical and five are conserved when *L. major* DHFR amino acid sequence is compared to the human reductase. Overall, however, *L. major* DHFR sequence homology with human DHFR is only 37%.[1,2] Thus, judiciously placed substituents on parent triazines **1a** and **1b** that possess hydrophobic and steric features that interact favorably at the nonhomologus regions (63%) of *L. major* vs. human DHFR may provide the desired selective inhibition. In this regard, we point out that at least two lipophilic, sterically large analogues in Table I (**1a**, X = $(CH_2)_8CH_3$ and **1a**, X = $(CH_2)_{11}CH_3$) are about 10-fold more inhibitory to *L. major* DHFR than to human DHFR.[15]

Experimental Section

Materials. DHFR from methotrexate-resistant *L. major* promastigotes was purified as described by Meek et al.[5] NADPH was purchased from United States Biochemical Co. All other reagents were obtained from Sigma.

Enzyme Assays. Solutions of inhibitor were prepared at the time of assay by dissolving the inhibitor in 50 mM 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid (TES) buffer (potassium salt), pH 7.4. Highly lipophilic congeners were first dissolved in a minimal volume of Me_2SO and then diluted with buffer such that the concentration of Me_2SO in the final assay solution was about 1% v/v; it was found that 2% v/v Me_2SO had no effect of the reaction velocity of *L. major* DHFR. All solutions were stored at 4 °C and gave reproducible assays for at least 3 months.

The DHFR assay mixture (1.0 mL) contained 25 μM H_2folate, 0.1 mM NADPH, varying concentrations of inhibitors, 50 mM TES, 75 mM 2-mercaptoethanol, 1 mM EDTA, and 1.0 mg of BSA at pH m.0, 25 °C.[21] The reaction was initiated by addition of 0.5 unit of DHFR, and conversion of NADPH to $NADP^+$ was monitored spectrophotometrically at 340 nm on a Cary-18 instrument. One unit of enzyme activity is defined as that amount of enzyme that produces 1 nmol of product/min.

Calculation of K_i Values. Lineweaver–Burk plots showed the compounds tested to be competitive with respect to H_2folate. The K_i values were determined by using the equation. $K_i = ([I]/i - [I])/(1 + [S]/K_m)$, where [I] is the concentration of inhibitor used, [S] is the concentration of H_2 folate used (25 μM), K_m is the Michaelis constant of H_2folate (1.5 μM), and i is 1 − (initial velocity in presence of inhibitor/initial velocity in absence of inhibitor, V_i/V_0).[22] The reported K_i for each inhibitor is the mean value of 8–12 spectrophotometric assays using four to six concentrations of inhibitor, varied above and below the I_{50}. I_{50} is that concentration of drug that inhibits the enzyme by 50%.

Cell Culture. *L. major* promastigotes (clone POJ of Iran strain, 252, obtained from B. Ullman) were grown at 26 °C in room air supplemented to 9% CO_2 with a defined medium consisting of Medium 199 (Gibco) containing Earle's buffered saline salts, 20% fetal calf serum, 25 mM Hepes (pH 7.4), and 50 μg/mL gentamicin. Stock cultures were maintained by reseeding into fresh medium at 3–4-day intervals. Growth inhibition studies were performed in standard 24-well assay plates (area 2.0 cm^2/well, flow Laboratories) with 2.0 mL of growth medium containing four to six different concentrations (spanning the I_{50}) of inhibitor. Wells were seeded at 5×10^6 cells/mL and counted daily for 5 days, or until they were out of log phase growth, with a Coulter Counter ZBI. The EC_{50} was calculated from a plot of log dose of inhibitor vs. percent cell growth.

Synthesis. Melting points (Büchi capillary apparatus) and boiling points are uncorrected. Microanalyses were performed by C. F. Geiger (Ontario, CA) and are within ±0.4% of the theoretical values. Thin-layer chromatography (Analtech precoated silica gel or alumina glass plates with fluorescent indicator) was used to check the purity of the synthetic intermediates. Ultraviolet

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Table III. 4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-(3-substituted-phenyl)-*s*-triazines

no.	X	mp, °C (solvent)	yield, %	formula[a]
17	3-O$(CH_2)_{10}CH_3$	181–182 (H_2O)	71	$C_{22}H_{37}N_5O{\cdot}HCl$
18	3-O$(CH_2)_{13}CH_3$	173–175 (H_2O)	75	$C_{25}H_{43}N_5O{\cdot}HCl$
22	3-OCH_2-1-adamantyl	194–196 (H_2O)	40	$C_{22}H_{31}N_5O{\cdot}HCl{\cdot}H_2O$
35	3-CH_2O-1-naphthyl	178–180 (ethanol)	56	$C_{22}H_{23}N_5O{\cdot}HCl$
41	3-CH_2NH-C_6H_4-4′-Cl	183–185 (ethanol)	4	$C_{18}H_{21}ClN_6{\cdot}HCl$

[a] Analyzed for carbon, hydrogen.

(water), NMR, and IR spectra of the triazines were consistent with the assigned structures. H_2folate was synthesized according to the method of Plante et al.[23] The syntheses of most of the triazines have been previously reported as follows: triazines **1–9**, **13**, **16**, **19**, **20**, **21**, **23**, **24**, **42**, **43**, and **45**,[17] triazines **25–31**, **33**, **34**,[16] triazine **40**,[18] triazine **36**,[24] and triazines **10–13**, **15**, **32**, **37**, **38**, **39**, **44**.[11] The synthesis of triazines **17**, **18**, **22**, **35**, and **41** are reported here.

General Method of Synthesis of 1,2-Dihydro-(3-substituted-phenyl)-*s*-triazines. The general procedure of Modest was used.[25] A mixture of 0.1 mol of the substituted aniline hydrochloride (or 0.1 mol of the aniline and 1 equiv of concentrated hydrochloric acid), 0.107 mol of dicyandiamide, and 50 mL of acetone was refluxed with stirring for 24 h. Generally, the reaction mixture becomes a clear amber solution from which crystals begin to separate in 10 min to 1 h. In those instances in which crystals were not deposited during the reaction, the product was obtained when the reaction mixture was cooled. On completion of the reaction, the product was collected, washed with acetone, and dried. Yields were between 30% and 90% and were augmented by the separation of further material when the mother liquor was concentrated. Further purification was achieved by crystallization from water or ethanol, with an average recovery of 70%. The acid employed, in general, may be one of several strong inorganic or organic acids, including nitric and sulfuric acids.

3-(Undecanyloxy)- and 3-(Tetradecanyloxy)aniline Hydrochloride. A mixture of *m*-acetamidophenol (16.6 g, 100 mmol), the appropriate alkyl bromide (100 mmol), and anhydrous potassium carbonate (15.2 g, 110 mmol) in acetone (100 mL) was refluxed with stirring for 40 h. The resulting suspension was poured into 10% NaOH (1000 mL), stirred, and filtered. The white solid was washed, dried, and recrystallized from 95% ethanol. The following 3-substituted acetanilides were obtained: 3-O$(CH_2)_{10}CH_3$ (26 g, 85% yield, mp 68–69.5 °C), 3-0$(CH_2)_{13}CH_3$ (33 g, 94% yield, mp 78–79 °C).

The acetanilides were suspended in a mixture of ethanol (200 mL) and 12 N HCl (200 mL) and heated for 1 h to effect hydrolysis. After cooling in an ice bath, the solids were collected and recrystallized from ethanol to yield the substituted aniline hydrochlorides: 3-0$(CH_2)_{10}CH_3$ (12 g, 90%, mp 118–119 °C), 3-0$(CH_2)_{13}CH_3$ (30 g, 95%, mp 118–120 °C). Triazines **17** and **18** (Table III) were then synthesized by using the general procedure.

3-[(1-Adamantyl)methoxy]aniline. $LiAlH_4$ (2.2 g, 55 mmol) was suspended in THF (100 mL) under nitrogen at 0 °C. 1-Adamantanecarboxylic acid (10 g, 55 mmol) in THF (100 mL) was added dropwise. After continuous stirring for 16 h, the excess $LiAlH_4$ was decomposed by slow addition of water and the resulting suspension was filtered over Celite. The filtrate was washed with ether, and after careful drying over $MgSO_4$, the solvent was removed to yield 1-adamantanemethanol (8.0 g, 48 mmol), mp 114–115 °C (lit.[26] mp 115 °C). The 1-adamantanemethanol (8.0 g, 48 mmol) was then reacted with *p*-toluenesulfonyl chloride (10 g, 52 mmol) in pyridine to yield 1-[(*p*-tolylsulfonyl)methoxy]adamantane (12 g, 38 mmol), mp 77–79 °C (lit.[26] mp 76 °C).

Crude 1-[(*p*-tolylsulfonyl)methoxy]adamantane (8.0 g, 25 mmol), *m*-nitrophenol (3.5 g, 25 mmol), and K_2CO_3 (3.5 g, 25 mmol) were heated at reflux in DMF (100 mL) for 40 h.[27] The dark solution was poured into water (600 mL) and the resulting oil was extracted with hexane (2 × 400 mL) and ether (200 mL). The organic layers were combined and washed by 1 N NaOH (400 mL) and water (400 mL). The organic layer was then dried over $MgSO_4$ and the solvent removed to yield an oil, which crystallized on standing. The crude 3-[(1-adamantyl)methoxy]nitrobenzene (5 g, 18 mmol) was used without further purification, in the next step.

3-[(1-Adamantyl)methoxy]nitrobenzene (4.0 g, 14 mmol) in ethanol was hydrogenated under pressure with 5% palladium/carbon (0.5 g) as the catalyst. The yield of 3-[(1-adamantyl)-methoxy]aniline was essentially quantitative (3.5 g, 14 mmol), mp 79–81 °C. Triazine **22** (Table III) was then obtained by following the general procedure.

3-(Naphthoxymethyl)aniline Hydrochloride. A suspension of *m*-nitrobenzyl chloride (8.6 g, 50 mmol), α-naphthol (7.7 g, 50 mmol), and anhydrous potassium carbonate (7 g, 50 mmol) in acetone (100 mL) was refluxed for 48 h with continuous stirring. On cooling, the inorganic precipitate was removed by filtration and the filtrate was evaporated to dryness. The oil was dissolved in chloroform, washed with 5% NaOH (2 × 100 ml) and H_2O (2 × 100 mL), and dried over $MgSO_4$. After removal of the drying agent and evaporation of the chloroform, a solid was obtained. The crude 3-nitrobenzyl α-napthyl ether (12 g, 43 mmol) was recrystallized twice from 1-propanol (mp 79–80 °C).

To a solution of 3-nitrobenzyl α-napthyl ether (3.9 g, 14 mmol) in ethyl acetate (100 mL) was added 5% Pd/C (1 g) and the resulting suspension was hydrogenated under pressure (50 psi). After filtration and evaporation, the oil was dissolved in anhydrous ethyl ether and dry HCl was passed through the solution. The 3-(naphthoxymethyl)aniline hydrochloride (4.0 g, 14 mmol) was collected, recrystallized from ethanol, and dried (mp 236–238 °C). The general procedure was then followed to synthesize triazine **35** (Table III).

***N*-(*m*-Aminobenzyl)-*p*-chloroaniline.** A solution of *m*-nitrobenzaldehyde (7.6 g, 50 mmol) and *p*-chloroaniline (6.4 g, 50 mmol) in benzene (100 mL) was stirred at reflux temperature overnight. The solvent was removed under reduced pressure and the residue was recrystallized from absolute ethanol to yield *N*-(*m*-nitrobenzylidene)-*p*-chloroaniline (11 g, 39 mmol), mp 84–58 °C (lit.[28] mp 81 °C).

N-(*m*-Nitrobenzylidene)-*p*-chloroaniline (2.8 g, 10 mmol) was dissolved in ethanol (100 mL) and $NaBH_4$ (1.1 g, 30 mmol) was added to it. After addition, the solution was stirred for 1 h longer and then diluted with water. The resulting orange solid was collected and recrystallized from dilute ethanol yield orange plates of *N*-(*m*-nitrobenzyl)-*p*-chloroaniline (1.6 g, 6 mmol), mp 80–81 °C.

A suspension of *N*-(*m*-nitrobenzyl)-*p*-chloroaniline (2.0 g, 7 mmol), iron powder (7 g), acetic acid (0.1 mL), and water (50 mL) was stirred for 6 h at 80–90 °C. The mixture was filtered and the iron residue was carefully washed with hot $CHCl_3$ (3 × 50 mL). The aqueous layer was also washed with hot $CHCl_3$. The $CHCl_3$ fractions were combined and dried over $MgSO_4$. The $CHCl_3$ was removed under reduced pressure to yield *N*-(*m*-aminobenzyl)-*p*-chloroaniline (1.5 g, 6 mmol), which was used immediately in the

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synthesis of triazine 41 (Table III) by the general procedure.

Physicochemical Constants. The π constants of all of these substituents have been well documented[12] while others have been calculated.[19] MR values are scaled by 0.1 to keep them roughly equiscalar with π values. Indicator variables are defined as follows: I_{OR} = 1 for alkoxy derivatives, e.g., 3-OCH_3, and 0 for all others.

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Novel Renin Inhibitors Containing Analogues of Statine Retro-Inverted at the C-Termini: Specificity at the P_2 Histidine Site[1,2]

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Substituted 1,3- and 1,4-diamines were prepared from epoxides derived from Boc-leucine or Boc-cyclohexylalanine. These diamines were incorporated into renin inhibitors (IC_{50} = 4–1500 nM) replacing the Leu-Val scissile bond in small peptide analogues of angiotensinogen. Replacement of the P_2 histidine imidazole with other heterocycles maintained or enhanced binding while changing the overall basicity of the inhibitor. Finally, substitution of *O*-methyltyrosine for the P_3 phenylalanine suppressed chymotrypsin cleavage of the P_3–P_2 bond.

Renin is a proteolytic enzyme that selectively cleaves its substrate angiotensinogen to begin the cascade that produces the potent pressor octapeptide angiotensin II. The basic strategy behind the design of renin inhibitors has been to modify the Leu-Val scissile bond in an analogue of the natural substrate angiotensinogen in order to maintain binding while preventing proteolytic cleavage. One approach has been to replace this bond with a hydroxyethylene isostere.[3] This modification not only suppresses proteolysis but also enhances binding by mimicking the presumed transition state for cleavage.[4,5] It has been discovered that the unusual γ-amino acid statine is a suitable replacement for the Leu-Val hydroxyethylene isostere even though it is one atom shorter and lacks the valine side chain.[6]

In this paper we describe novel renin inhibitors based upon statine and the one carbon extended analogue homostatine that have been retro-inverted at the C-terminus. We also have examined several amino acids as replacements for the P_2 histidine and have determined some of the stereoelectronic requirements of this site. Finally, a modification that prevents chymotrypsin cleavage between the P_2 and P_3 residues is disclosed.

(1) Presented in part at the 191st National Meeting April 1986.
(2) Abbreviations follow IUPAC–IUB Joint Commission on Biochemical Nomenclature for amino acids and peptides (*Eur. J. Biochem.* 1984, *158*, 9–31). Additional abbreviations used are as follows: THF, tetrahydrofuran; DMF, dimethylformamide; TFA, trifluoroacetic acid; Boc, *tert*-butyloxycarbonyl; Cbz, benzyloxycarbonyl; Me_2SO, dimethyl sulfoxide; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride.

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Results

Synthesis: Diamines and Amino Nitriles Syntheses of the monoprotected diamines 3a and **3b**, which are precursors to structures related to statine retro-inverted at the C-terminus, are shown in Scheme I. Treatment of epoxide 1a (96% ee, 15:1 2*R*:2*S*)[7] with sodium azide and isolation of the major product afforded (2*S*,3*S*)-azido alcohol **2a**, which was hydrogenated over palladium on carbon to provide 3a. Similarly, epoxide **1b** (94% ee, >10:1 2*R*:2*S*)[7] afforded the monoprotected diamine **3b**.

The synthesis of the homologous (3*S*,4*S*)-1-amino-3-hydroxy-4-[(*tert*-butyloxycarbonyl)amino]-5-cyclohexylpentane (6) began with Boc-cyclohexylalanal (4) as shown in Scheme I.

Condensation of this aldehyde with the lithium enolate of acetonitrile afforded a readily separable mixture of the (3*S*,4*S*)- and (3*R*,4*S*)-hydroxynitriles **5** in a 3:2 ratio. Alternately, epoxide **1b**[7] could be opened with cyanide to provide as the major product the 3*S* isomer **5a**, thereby establishing the stereochemistry of the products derived from the enolate condensation. Hydrogenation of nitrile **5a** over Raney Ni provided the monoprotected diamine **6**.

The optical purity of nitrile **5b** was established by the preparation of diastereomeric derivatives. Deprotection of **5b** with 4 M HCl/dioxane followed by coupling with both (+)- and (−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride[8] provided the diastereomeric amides. Analysis by proton NMR indicated that each diastereomer was contaminated with 3.0% of the other, demonstrating that the material prepared by the acetonitrile enolate condensation had 94% ee.

Synthesis: Extension at N-1. Monoprotected diamines **3a**,**b** and **6** were acylated with short-chain aliphatic acids designed to mimic the P_2' isoleucine side chain of human angiotensinogen (Scheme II). Amine **6** was also reductively alkylated with isovaleraldehyde and epoxide **1b** was opened with isoamylamine to provide examples of reduced amides in these series. The secondary amines

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