# Design, Synthesis, and Evaluation of Inhibitors of Trypanosomal and Leishmanial Dihydrofolate Reductase

Shafinaz F. Chowdhury,[†] Victor Bernier Villamor,[‡] Ramon Hurtado Guerrero,[‡] Isabel Leal,[‡] Reto Brun,[§] Simon L. Croft,[▽] Jonathan M. Goodman,[∥] Louis Maes,[⊥] Luis M. Ruiz-Perez,[‡] Dolores Gonzalez Pacanowska,[‡] and Ian H. Gilbert*[,†]

*Welsh School of Pharmacy, Cardiff University, Redwood Building, King Edward VII Avenue, Cardiff CF10 3XF, U.K., Instituto de Parasitologia y Biomedicina, Consejo Superior de Investigaciones Cientificas, C/Ventanilla 11, 18001-Granada, Spain, Swiss Tropical Institute, Socinstrasse 57, CH-4002 Basel, Switzerland, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, U.K., Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, U.K., and Tibotec n.v., Intercity Business Park, Gen. De Wittelaan L11B 3, B-2800 Mechelen, Belgium*

*Received December 14, 1998*

This paper concerns the design, synthesis, and evaluation of inhibitors of leishmanial and trypanosomal dihydrofolate reductase. Initially study was made of the structures of the leishmanial and human enzyme active sites to see if there were significant differences which could be exploited for selective drug design. Then a series of compounds were synthesized based on 5-benzyl-2,4-diaminopyrimidines. These compounds were assayed against the protozoan and human enzymes and showed selectivity for the protozoan enzymes. The structural data was then used to rationalize the enzyme assay data. Compounds were also tested against the clinically relevant forms of the intact parasite. Activity was seen against the trypanosomes for a number of compounds. The compounds were in general less active against *Leishmania*. This latter result may be due to uptake problems. Two of the compounds also showed some in vivo activity in a model of African trypanosomiasis.

## Introduction

The protozoan parasites, which give rise to leishmaniasis and African and South American trypanosomiasis, are major health problems. The current drugs available to treat these diseases are inadequate, suffering from poor clinical efficacy and adverse side effects, and often require hospital admission for administration. Therefore there is an urgent need for the development of new drugs to treat these diseases.

Dihydrofolate reductase (DHFR) is a well-known and successful drug target for anticancer, antibacterial, and antimalarial treatments. The reason for the success of DHFR as a drug target is its pivotal role in nucleotide metabolism and the fact that the structure of the active site varies slightly from species to species allowing design of selective inhibitors. Little has been done to study DHFR as a drug target in leishmaniasis and trypanosomiasis. A few compounds have been assayed against *Leishmania*, but virtually no DHFR inhibitors have been assayed against trypanosomes.[1−6] The data that have been obtained to date suggest that most common DHFR inhibitors are actually selective for the human enzyme rather than the parasite enzyme, which suggests that these compounds are of little therapeutic value for these diseases.[1] The protozoan enzymes differ from most other DHFR enzymes in that they are found as bifunctional enzymes with thymidylate synthase. Structurally the protozoan enzymes are closely related to one another, suggesting that it may be possible to design a compound which simultaneously inhibits the enzymes from all three species.[7] The active sites of the enzymes also show significant differences from the human enzyme, which suggests the possibility of designing compounds which are selective for the protozoan DHFR. These differences are found in the shapes of the active sites and the nature of residues present in the active site.

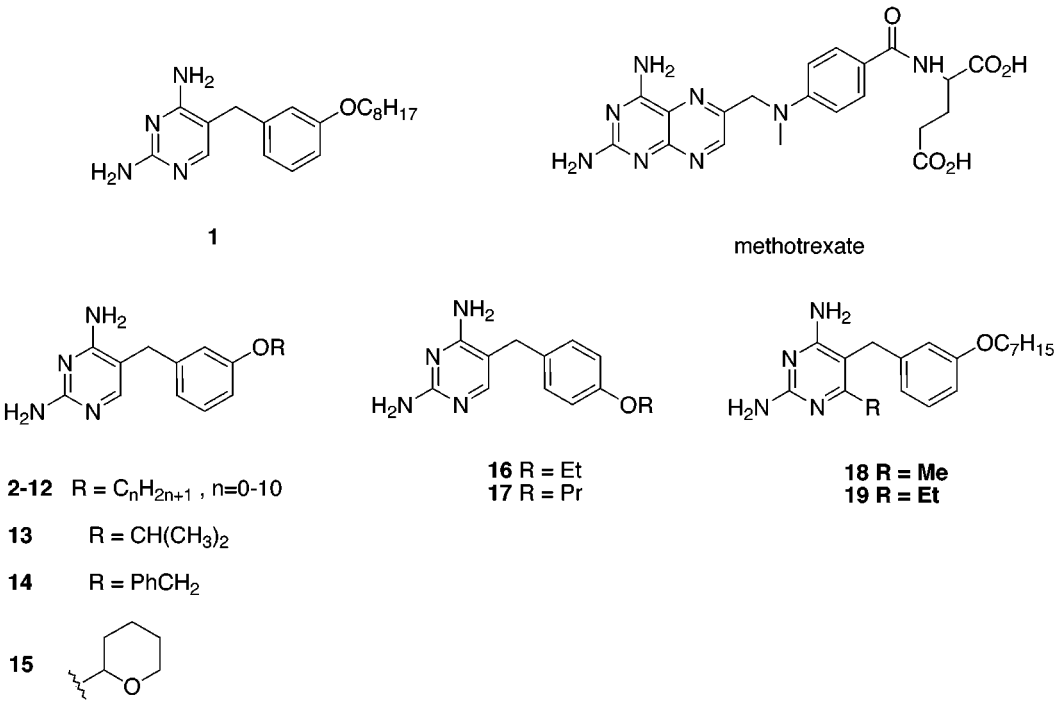
We were particularly interested by a publication from Sirawaraporn et al.[1] in which they describe testing of some 5-benzyl-2,4-diaminopyrimidines against *L. major* DHFR. Some of these compounds showed good selectivity for the leishmanial enzyme and in vitro activity against both *L. major* promastigotes (form found in the insect vector) and *L. donovani* amastigotes (form found in the human host). However they only describe a limited number of compounds of which the most active was the octyloxy derivative **1** (Chart 1).

This paper describes first an exploration of structure−activity relationships of this class of compounds against *L. major*, *T. cruzi*, *T. brucei*, and human DHFR with the aim of developing potential new drugs. Second the activities of these compounds are related to structures of the enzymes to produce a predictive model to guide the synthesis of new inhibitors. Third in vitro studies are reported against the clincally relevant forms of the intact parasites. Fourth in vivo studies in rodent models of disease are reported. The importance of the investigation is that none of these promising lead compounds have been tested against the trypanosomes and only a

* Address for correspondence: Dr. Ian Gilbert. Tel: +44 29 2087 5800. Fax: +44 29 2087 4149. E-mail: gilbertih@cf.ac.uk.
[†] Cardiff University.
[‡] CSIC.
[§] Swiss Tropical Institute.
[▽] London School of Hygiene and Tropical Medicine.
[∥] University of Cambridge.
[⊥] Tibotec n.v.

10.1021/jm981130+ CCC: $18.00 
Published on Web 10/01/1999

**Chart 1**




limited series against *Leishmania*. Further a predictive model is derived, using structural information, to explain the experimental results. This should aid in the future design of more potent and selective inhibitors.

## Comparison of the Structure of Human and Leishmanial DHFR

Structural information is available for the human and *L. major* DHFR. The coordinates of the human structure are deposited in the Brookhaven Protein Database (code 1drf)[8] with the substrate folate in the active site. The *L. major* DHFR structure has been solved by Matthews and co-workers[9] with the inhibitor methotrexate and the cofactor NADPH co-crystallized. These structures were used to investigate differences between leishmanial and human DHFR. It is important to see if there are differences in structure of the active sites as this would indicate whether it is possible to design selective leishmanial (and trypanosomal) inhibitors. The sequence alignments for *T. brucei* and *T. cruzi* DHFR have also been published. We have used these sequences to produce models of these proteins and carried out a detailed comparison of structures.[7]

Initially sequence alignment of the structures was undertaken. Alignments were found as follows: *L. major* and *T. cruzi*, 50%; *L. major* and *T. brucei*, 46%; *T. brucei* and *T. cruzi*, 58%; *L. major* and human, 26%; *T. cruzi* and human, 27%; *T. brucei* and human, 26%.[7] The leishmanial and trypanosomal enzymes showed much higher sequence identity to one another than to the human enzyme. This suggests that structurally the leishmanial and trypanosomal structures are more closely related to one another than to the human structure.

The active site consists of a cavity and a cleft in the surface of the enzyme. The pteridine moiety of the folate substrate binds deep in the active site. Similarly the pteridine moiety of the classical DHFR inhibitor methotrexate binds here. There is a major interaction between the 2,4-diaminopyrimidine moiety and an aspartic acid or glutamic acid residue found in the active site. The glutamate moiety of the substrate then lies in the cleft along the surface of the protein.

Detailed comparison of residues thought to be important in interaction with the ligands methotrexate and folate was then made. The *L. major* structure is co-crystallized with methotrexate (Chart 1) and the human with folate. These ligands occupy similar positions in the active sites. All residues within 6 Å of the ligands were selected and compared. These are shown in Table 1, together with the interactions that can be observed between the leishmanial enzyme and the inhibitor methotrexate. Of the 20 residues in the active sites of the leishmanial and human enzymes, 12 are conserved, 5 show very high homology, and 3 are different. The key differences are Met53, Lys57, and Phe91. These differences are important in rationalizing the selectivity of compounds and also in designing new selective compounds (see below). Met53 is key in the active site, contacting a large part of the inhibitor (pteridine, benzamide, and glutamate moieties). The Lys57 and Phe91 are situated in the glutamate binding site of the protein, indicating significant differences in this region of the binding site (Figure 1).

## Design

The most promising lead compound produced by Sirawaraporn et al.[1] is the 3′-octyloxy-5-benzyl-2,4-diaminopyrimidine (**1**) (Chart 1). This compound is reported to have a 130-fold selectivity for the leishmanial enzyme and activity against both promastigotes and amastigotes. We planned to synthesize a variety of compounds with different substituents on the benzyl ring related to the lead compound (**1**). Straight chain analogues (**2**−**12**) and branched chain analogues (**13**−**15**) were synthesized. Preliminary studies were under-

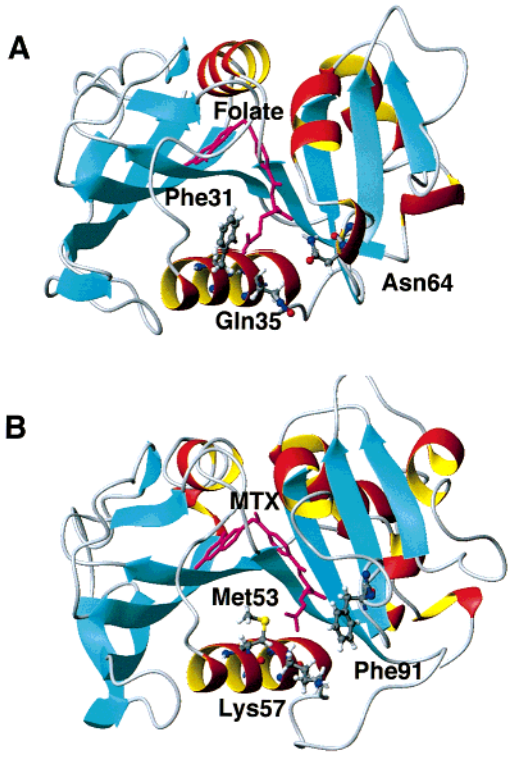



**Figure 1.** Secondary structure of the active site of (A) human and (B) *L. major* DHFR. The human enzyme is co-crystallized with folate in the active site (shown in purple) and the *L. major* enzyme with methotrexate (shown in purple). Residues showing significant variation between the human and protozoan enzymes are also indicated (human Phe31, Gln35, Asn64; *L. major* Met53, Lys57, Phe91).

taken with 4′-substituted benzyl compounds (**16**, **17**). Further analysis of protein structures with the inhibitors minimized in the active site revealed a binding pocket adjacent to the 6-position of the pyrimdine ring suggesting it should be possible to put additional substituents in this position (**18**, **19**).

## Chemistry

The 5-benzyl-2,4-diaminopyrimidines were prepared by a modification of the procedure of Stenbuck and coworkers.[10] Two basic procedures were adopted (Scheme 1). In the first procedure commercially available alkoxybenzaldehyde was condensed with 3-ethoxypropionitrile and then reacted with guanidine.

In the second procedure (Scheme 2), the 3-hydroxybenzaldehyde was protected with a tetrahydropyranyl group, condensed with 3-ethoxypropionitrile, and then reacted with guanidine. The resultant product was then deprotected and alkylated. The compounds were isolated in yields ranging from 35% to 66%. To prove alkylation was selective for the hydroxy group over the amino groups, compound **7** was also made by alkylation of 3-hydroxybenzaldehyde, condensation with 3-ethoxypropionitrile, and condensation with guanidine. This route unambiguously placed the alkyl group on the hydroxyl group. The spectral data of the compound prepared by this method was identical to that obtained using the route in Scheme 2. This second procedure provides a rapid method for synthesizing compounds from a late stage intermediate by a 1-step procedure.

The 6-alkyl-5-benzyl-2,4-diaminopyrimidines (**18**, **19**) were prepared using the method shown in Scheme 3. Ethyl acetoacetate or ethyl propionylacetate was alkylated with the appropriately derivatized benzyl bromide. This was followed by condensation with guanidine, chlorination, and then displacement of the chloride by ammonia.

**Table 1.** Active Site Residues in *L. major* and Human DHFR[a]

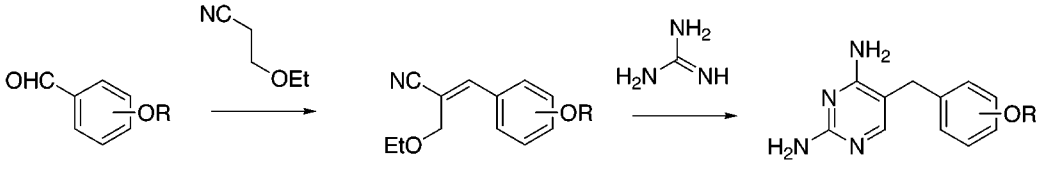
| *L. major* | human | *L. major* interaction with methotrexate |
|---|---|---|
| Val30 | Ile7 | hydrophobic sc contact with pteridine ring |
| | | polar mc contact with pteridine |
| Val31 | Val8 | polar mc contact with pteridine |
| Ala32 | Ala9 | hydrophobic sc contact with pteridine ring |
| Ile45 | Leu22 | hydrophobic sc contact with N-Me |
| Asp52 | Glu30 | electrostatic sc contact with pteridine |
| Met53 | Phe31 | hydrophobic sc contact with pteridine ring |
| | | hydrophobic sc contact with benzamide |
| | | hydrophobic sc contact with glutamate |
| Phe55 | Tyr33 | hydrophobic sc contact with pteridine ring |
| Phe56 | Phe34 | hydrophobic sc contact with pteridine ring |
| | | hydrophobic sc contact with benzamide |
| Lys57 | Gln35 | hydrophobic sc contact with glutamate |
| | | electrostatic sc contact with glutamate |
| Thr60 | Thr38 | |
| Thr61 | Thr39 | |
| Ser86 | Ser59 | hydrophobic sc contact with N-Me |
| Val87 | Ile60 | hydrophobic sc contact with benzamide |
| Pro88 | Pro61 | hydrophobic sc contact with benzamide |
| Phe91 | Asn64 | hydrophobic sc contact with glutamate |
| Leu94 | Leu67 | hydrophobic sc contact with benzamide |
| | | hydrophobic sc contact with glutamate |
| Arg97 | Arg70 | electrostatic sc contact with glutamate |
| Val156 | Val115 | hydrophobic sc contact with pteridine ring |
| | | polar mc contact with pteridine |
| Thr180 | Thr136 | polar sc contact with pteridine |

[a] Residues selected are those within 6 Å of the MTX in the active site of *L. major* DHFR and the corresponding residues in the human structure. Principal interactions of MTX with the leishmanial enzyme active site are also shown. sc = side chain; mc = main chain

## Enzyme Assays

Compounds were assayed against recombinant DHFR from *L. major*, *T. brucei*, *T. cruzi*, and human. A selection of compounds was assayed at a variety of substrate concentrations to establish that inhibition was competitive. As an example the Lineweaver−Burk plot

**Scheme 1**

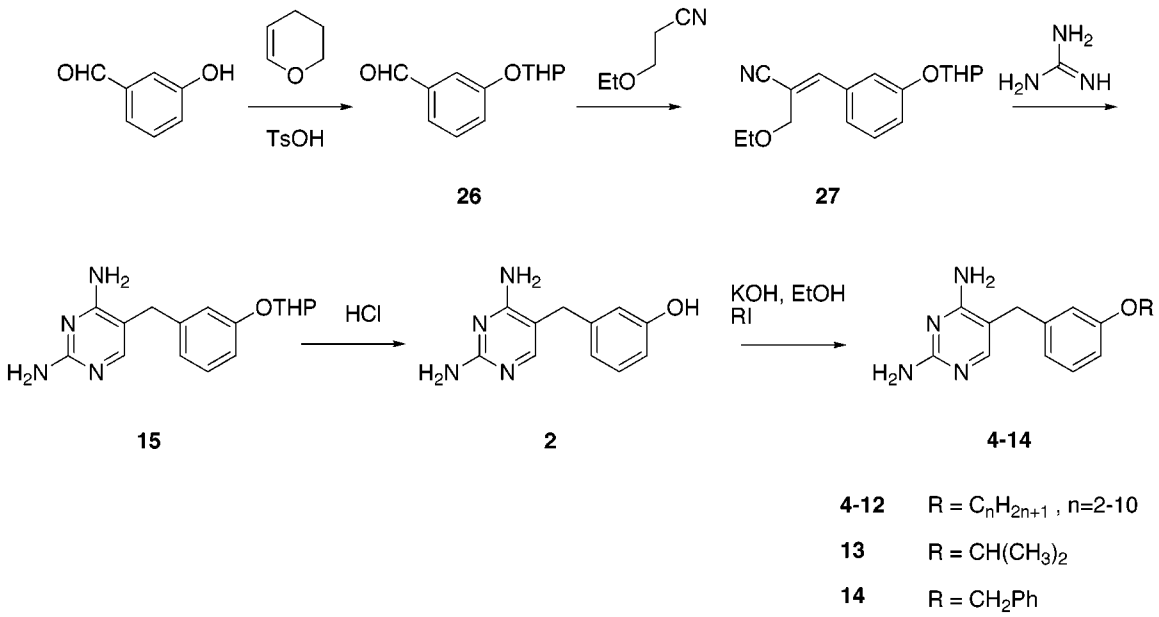



**20** R = 3-OMe
**21** R = 4-OEt
**22** R = 5-OPr

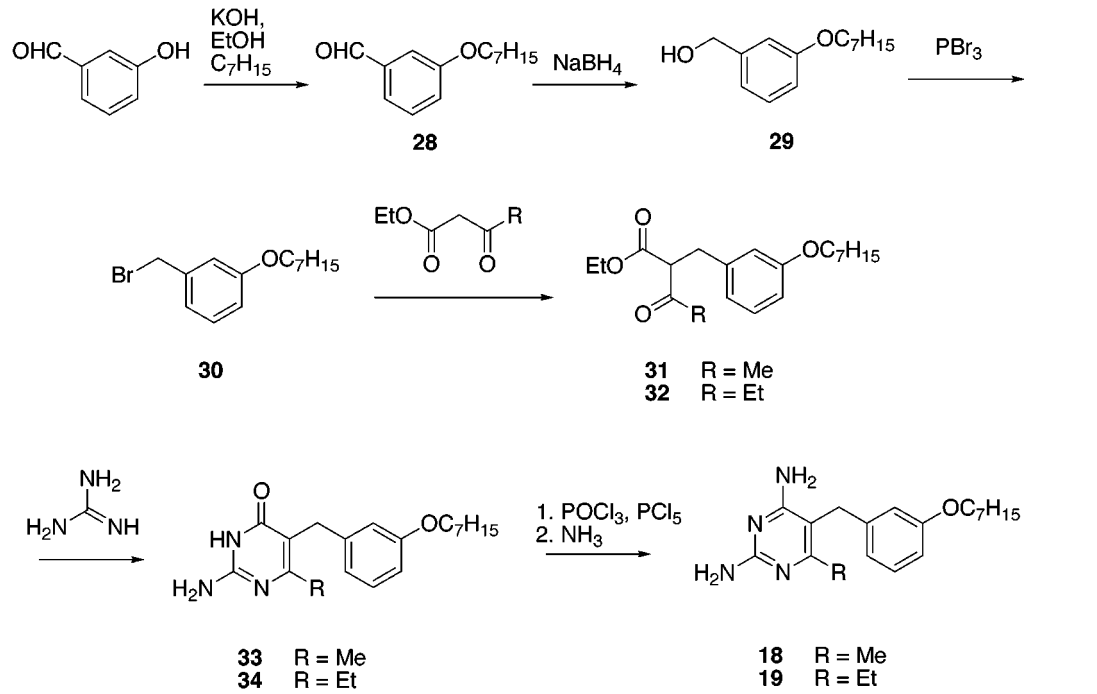
**23**
**24**
**25**

**3**
**16**
**17**

**Scheme 2**




**Scheme 3**




for compound **4** against *T. cruzi* DHFR is shown in Figure 2. Enzyme inhibition and selectivity data are presented in Table 2. Several broad conclusions can be drawn. In general the compounds are selective for the parasite DHFR over human DHFR. This is encouraging from the perspective of designing selective compounds. Compounds are significantly more active against *T. brucei* DHFR than any other enzyme. This difference is somewhat surprising given the high structural similarity between the *T. cruzi* and *T. brucei* enzymes.[7] For the 3-substituted compounds, maximum activity against all the parasitic DHFRs occurs for chain length of 2−6 (compounds **4**−**8**). As the chain length increases further the activity decreases. The branched chain substituents, isopropyl (**13**), benzyl (**14**), and THP (**15**), also show good activity. Similarly the 4-substituted compounds (**16**, **17**) show good activity. In the case of the 6-substituted pyrimidines, the methyl substituent (**18**) has little effect on the enzyme activity, if anything causing a slight decrease. However with the ethyl substituent (**19**) there is a marked increase in activity.

Selectivity data for the compounds is also shown in Table 2. For the straight chain 3′-alkoxy-substituted compounds (**2**−**12**) there is a slight decrease in selectivity as the chain length is increased, particularly in the case of the *T. brucei* and *T. cruzi* enzymes. The branched chain substituted (**13**−**15**) and the 4′-substituted (**16**, **17**) compounds also show good selectivity. The 6-methyl (**18**) and 6-ethyl (**19**) pyrimidines show slightly better selectivity than the corresponding 6-hydro compound (**9**).

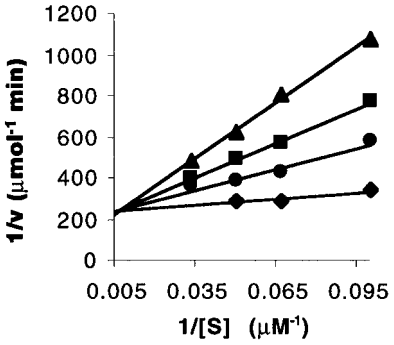



**Figure 2.** Lineweaver−Burk plot for the inhibition of *T. cruzi* DHFR by compound **4**. The reciprocal of the reaction velocity is plotted against the reciprocal of the concentration of substrate (dihydrofolate) in the presence of varying concentrations of inhibitor. Concentrations of inhibitor: ▲, 8 $\mu$M; ■, 4 $\mu$M; ●, 2 $\mu$M; ◆, 0 $\mu$M.

## Modeling

We were interested to see if we could explain the activity and selectivity of compounds on a qualitative manner by comparing the crystal structures of the human[8] and leishmanial[9] enzymes.

Compounds **3**−**12** were built into the active site of the leishmanial enzyme as follows. The crystal structure of the methotrexate bound to the leishmanial enzyme was modified to form unsubstituted 5-benzyl-2,4-diaminopyrimidine in the active site. 5-Benzyl-2,4-diaminopyrimidine was minimized in the active site together with all residues within 8 Å of the ligand, while the other residues were constrained. The Amber force field as implemented in Macromodel[11] was used for the minimizations. Monte Carlo conformational searching was used to explore the conformations of the ligand within the active site. This procedure moved the flexible torsion angles of the ligand and moved it within the active site. This structure was then used as the basis for exploration of the conformational space of related molecules. An alkoxy chain was grown onto the 3′-position of the ligand, one carbon atom at a time. At each step, the possible orientations of the new bond were explored in isolation, so that the conformational preferences of the new molecules were found very rapidly.

The validity of this stepwise approach was tested by performing unrestricted conformational search on the side chain of butyl derivative **6**. This found the same conformation as the more rapid method. The structure of the inhibitor obtained by this method was superimposed on that obtained by the first method with root-mean-square deviation of 0.079 Å.

The results of the Monte Carlo conformational searching for the inhibitors in the leishmanial enzyme are shown in Figure 3A. The 2,4-diaminopyrimidine moiety interacts with the enzyme through a charge-mediated hydrogen bond to the Asp52 through N1 and the 2-amino group. The 2-amino group also donates a hydrogen bond to Thr180. Further hydrogen bonds are from the 4-amino group of the pyrimidine to the carbonyls of Val30 and Val156. The phenyl ring of the inhibitors interacts with Met53 and Phe56. The hydrophobic side chain lies in a cleft principally bound by Met53, Leu94, and Val87. In the case of the leishmanial enzyme the hydrocarbon side chain also interacts with Phe91, which presumably is a favorable hydrophobic interaction. The distance from the end of the side chain to the phenyl group of Phe91 is shown in Table 3. The closest interaction is about 3.5 Å and occurs when the side chain is 4−6 carbons in length (compounds **6**−**8**). This corresponds to the optimum activity against the *L. major* enzyme. As the hydrocarbon chain becomes longer, it moves slightly away from Phe91.

The minimization experiments were also conducted with the compounds in the active site of the human enzyme. The corresponding results for the human enzyme are shown in Figure 3B. In this case the Phe91 has been replaced by Asn64. The hydrocarbon side chain no longer is found in this location, presumably because the side chain cannot undergo favorable hydrophobic interaction with Asn64. The lack of interaction may explain the selectivity of compounds for the leishmanial enzyme.

## In Vitro Assays

Compounds were assayed against the clinically relevant stage of the parasites: *L. infantum* amastigotes cultured in mouse peritoneal macrophages; *T. cruzi* amastigotes cultured in mouse peritoneal macrophages; *T. brucei rhodesiense*, the trypomastigote form cultured axenically. A cytotoxicity study was also undertaken.

***L. infantum.*** Data for *L. infantum* is presented in Table 4. Only a few compounds showed in vitro activity. Essentially only the hexyl (**8**) and heptyl (**9**) derivatives showed marginal activity which was retained in the 6-methyl (**18**) and 6-ethyl (**19**) pyrimidines. Surprisingly no in vitro activity was found for compound **10** which was reported to have an $EC_{50}$ of 4.6 $\mu$M by Sirawaraporn and co-workers against *L. donovani* amastigotes.[1] The assay for compound **10** was repeated against *L. donvani* amastigotes and showed no inhibition at 30 $\mu$M. Similarly the compound did not give the reported selectivity in our enzyme assay experiments.

***T. cruzi.*** In vitro data against *T. cruzi* is shown in Table 5. Activity was seen for a number of compounds. For the 3′-alkoxy-substituted series there is good activity for the longer length compounds. In particular the hexyl (**8**), heptyl (**9**), octyl (**10**), nonyl (**11**), and decyl (**12**) substituted compounds show good activity. This activity is marginally lowered in the 6-methyl (**18**) and 6-ethyl (**19**) substituted pyrimidines. With the branched chain derivatives, the benzyl substituent (**14**) has good activity, but the isopropyl has low activity. Activity is poor in the case of the 4-substituted compounds (**16**, **17**).

***T. brucei rhodesiense.*** In vitro activity against *T. brucei rhodesiense* is shown in Table 6. The results parallel those obtained with *T. cruzi*, with good activity being obtained with the long chain alkyl derivatives. The butyl to decyl derivatives (**6**−**12**) all show good activity, with the nonyl (**11**) and decyl (**12**) derivatives being the most active. This activity is retained in the 6-methylpyrimidine (**18**). The benzyl derivative shows reasonable activity. Some of the compounds, particularly **10**−**12**, show some cytotoxicity against L-6 cells.

## In Vivo Assays

Lead compounds were then assessed in rodent models of *L. infantum*, *T. brucei*, and *T. cruzi* infection.

**Table 2.** Inhibition of DHFR: $K_i$ (nM)[a]

| compd | substituent | *L. major* | | *T. cruzi* | | *T. brucei* | | human |
|---|---|---|---|---|---|---|---|---|
| **2** | 3′-OH | 750 | (4.4) | 990 | (3.4) | ND | (ND) | 3300 |
| **3** | 3′-OMe | 300 | (7.8) | 1000 | (2.3) | 9.6 | (245) | 2300 |
| **4** | 3′-OEt | 156 | (10) | 430 | (3.7) | 8.6 | (184) | 1600 |
| **5** | 3′-OPr | 130 | (19) | 510 | (4.8) | 10.5 | (242) | 2400 |
| **6** | 3′-OBu | 150 | (6.2) | 220 | (4.2) | 3.6 | (257) | 930 |
| **7** | 3′-OPent | 140 | (7.7) | 210 | (5.2) | 7.1 | (154) | 1100 |
| **8** | 3′-OHex | 171 | (11) | 600 | (3.2) | 9.8 | (197) | 1900 |
| **9** | 3′-OHept | 221 | (2.8) | 1330 | (0.47) | 131 | (4.7) | 620 |
| **10** | 3′-OOct | 97 | (25) | 1130 | (2.1) | 24 | (100) | 2400 |
| **11** | 3′-ONon | 360 | (2.7) | 990 | (1.0) | 36 | (28) | 990 |
| **12** | 3′-ODec | 750 | (3.0) | 3110 | (0.72) | 52 | (43) | 2200 |
| **13** | 3′-OiPr | 160 | (8.5) | 23 | (60) | 8.8 | (156) | 1400 |
| **14** | 3′-OBn | 65 | (15) | 200 | (4.9) | 11 | (88) | 1000 |
| **15** | 3′-OTHP | 93 | (14) | 360 | (3.6) | 13 | (101) | 1300 |
| **16** | 4′-OEt | 130 | (47) | 210 | (29) | 9.8 | (610) | 6000 |
| **17** | 4′-OPr | 330 | (8.7) | 230 | (13) | 6.4 | (442) | 2900 |
| **18** | | 370 | (2.7) | 1500 | (0.66) | 75 | (13) | 1000 |
| **19** | | 48 | (8.3) | 710 | (0.57) | 19 | (21) | 400 |
| TMP | | 120 | (12) | 1000 | (1.3) | 10 | (134) | 1380 |
| PYR | | 250 | (0.49) | 98 | (1.2) | 11 | (11) | 120 |

[a] TMP = trimethoprim; PYR = pyrimethamine. The selectivity is shown in parentheses where selectivity is defined as: $K_i$(human)/$K_i$(parasite).

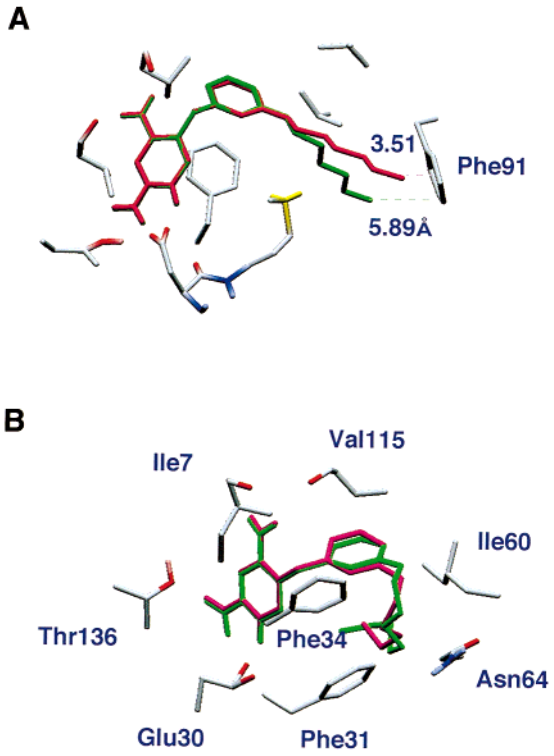



**Figure 3.** Inhibitors **20** (purple) and **23** (green) modeled into the active site of (A) *L. major* and (B) human DHFR showing interaction of the side chains with Phe91 in the case of the leishmanial enzyme.

***T. brucei.*** Compounds **10** and **11** were assessed at a dose of 100 mg/ kg and were found to be toxic at this level, causing death faster than that assessed in a model of *T. brucei* infection. However at lower dosing **10** and **11** caused a limited in vivo activity. The mean survival time at a dose of 10 mg/kg was extended from 7.5 to 11.0 days (Table 7).

***T. cruzi.*** Compounds **6**, **8**, **12**, **14**, and **18** were assayed in a rodent model of *T. cruzi* infection at 40 mg/ kg and were found to be inactive. Compounds **7**, **10**, and **11** were toxic at 40 mg/kg and inactive at 20 mg/kg, and **21** was inactive at 20 mg/kg.

**Table 3.** Closest Distance from the End of the Hydrocarbon Chain to the Phenyl Group of Phe91 in *L. major* DHFR

| compd | substituent | distance (Å) | inhibn of *L. major* DHFR $K_i$ (nM) |
|---|---|---|---|
| **3** | 3′-OMe | 5.9 | 300 |
| **4** | 3′-OEt | 5.2 | 156 |
| **5** | 3′-OPr | 4.8 | 130 |
| **6** | 3′-OBu | 3.5 | 150 |
| **7** | 3′-OPent | 3.6 | 140 |
| **8** | 3′-OHex | 3.5 | 171 |
| **9** | 3′-OHept | 4.3 | 221 |
| **10** | 3′-OOct | 4.4 | 97 |
| **11** | 3′-ONon | 5.9 | 360 |
| **12** | 3′-ODec | 6.2 | 750 |

**Table 4.** Percentage Inhibition of *L. infantum* Amastigotes Growth Obtained at Various Concentrations of Inhibitors[a]

| compd | substituent | 12.5 $\mu$M | 6.25 $\mu$M |
|---|---|---|---|
| **2** | 3′-OH | 0 | |
| **3** | 3′-OMe | 0 | |
| **4** | 3′-OEt | 0 | |
| **5** | 3′-OPr | 0 | |
| **6** | 3′-OBu | 0 | |
| **7** | 3′-OPent | 40 | 0 |
| **8** | 3′-OHex | 99 | 55 |
| **9** | 3′-OHept | 80 | 55 |
| **10** | 3′-OOct | 0 | |
| **11** | 3′-ONon | 0 | |
| **12** | 3′-ODec | 0 | |
| **13** | 3′-OiPr | 0 | |
| **14** | 3′-OBn | 0 | |
| **15** | 3′-OTHP | ND | ND |
| **16** | 4′-OEt | 0 | |
| **17** | 4′-OPr | 0 | |
| **18** | | 99 | 68 |
| **19** | | 90 | 0 |

[a] Cytotoxicity was determined in uninfected macrophages (MRC-5) at 25 $\mu$M. No toxicity was observed for any compound except compound **15** which was not assayed.

***L. infantum.*** Compound **34** was inactive at 40 mg/ kg against *L. infantum* infected mice.

## Discussion

Comparison of the structures of human and *L. major* DHFR active sites shows significant differences that may be possible to exploit for selective drug design.

**Table 5.** Percentage Inhibition of *T. cruzi* Amastigotes Growth at Various Concentrations of Inhibitors[a]

| compd | substituent | 12.5 μM | 6.25 μM | 3.13 μM | 1.56 μM | cytotoxicity |
|---|---|---|---|---|---|---|
| **2** | 3′-OH | 0 | | 0 | | 0 |
| **3** | 3′-OMe | 0 | | 0 | | 0 |
| **4** | 3′-OEt | 40 | 0 | 0 | 0 | 0 |
| **5** | 3′-OPr | 80 | 30 | 0 | 0 | 0 |
| **6** | 3′-OBu | 99 | 92 | 65 | 20 | 0 |
| **7** | 3′-OPent | 100 | 97 | 93 | 70 | 0 |
| **8** | 3′-OHex | 100 | 100 | 100 | 97 | 0 |
| **9** | 3′-OHept | 100 | 100 | 100 | 95 | 0 |
| **10** | 3′-OOct | 100 | 100 | 100 | 99 | 0 |
| **11** | 3′-ONon | 100 | 98 | 97 | 90 | 0 |
| **12** | 3′-ODec | 100 | 100 | 99 | 90 | 0 |
| **13** | 3′-OiPr | 40 | 0 | 0 | 0 | 0 |
| **14** | 3′-OBn | 97 | 95 | 80 | 30 | 0 |
| **15** | 3′-OTHP | ND | ND | ND | ND | ND |
| **16** | 4′-OEt | 0 | | 0 | | 0 |
| **17** | 4′-OPr | 75 | 40 | 10 | 0 | 0 |
| **18** | | 99 | 97 | 95 | 80 | 0 |
| **19** | | 98 | 99 | 90 | 80 | 0 |

[a] Cytotoxicity was determined in uninfected macrophages (MRC-5) at 25 μM. No toxicity was observed for any compound except compound **15** which was not assayed.

**Table 6.** Inhibition of *T. brucei rhodesiense* Growth[a]

| compd | substituent | $IC_{50}$ (μM) | toxicity (μM) |
|---|---|---|---|
| **2** | 3′-OH | 78 | >463 |
| **3** | 3′-OMe | 204 | >434 |
| **4** | 3′-OEt | 21 | >410 |
| **5** | 3′-OPr | 13 | 388 |
| **6** | 3′-OBu | 5 | 121 |
| **7** | 3′-OPent | 4 | 115 |
| **8** | 3′-OHex | 2 | 110 |
| **9** | 3′-OHept | 5 | ND |
| **10** | 3′-OOct | 2 | <34 |
| **11** | 3′-ONon | 1 | <32 |
| **12** | 3′-ODec | 1 | <31 |
| **13** | 3′-OiPr | 14 | >388 |
| **14** | 3′-OBn | 5 | 327 |
| **15** | 3′-OTHP | ND | ND |
| **16** | 4′-OEt | 24 | >410 |
| **17** | 4′-OPr | 10 | 388 |
| **18** | | 1 | <34 |
| **19** | | ND | ND |
| TMP | | 148 | ND |
| PYR | | 7 | ND |

[a] TMP = trimethoprim; PYR = pyrimethamine. Toxicity was determined against L-6 cells and is quoted as MIC.

**Table 7.** In Vivo Activity against a Rodent Model of *T. brucei*

| compd | dose (mg/kg) | route | no. of days dosing | mean no. of days post-infection to death |
|---|---|---|---|---|
| **10** | 25 | ip | 4 | 9.5 |
| **11** | 25 | ip | 4 | 9.5 |
| celacol | 0.25% | oral | 4 | 9.0 |
| Mel B | 5 | ip | 1 | all survived |
| **10** | 10 | ip | 4 | 11.0 |
| **11** | 10 | ip | 4 | 11.0 |
| celacol | 0.25% | oral | 4 | 7.5 |
| Mel B | 5 | ip | 1 | all survived |

[a] Celacol is the inoculum medium; Mel B is melarsoprol; ip = intraperitoneal; 5 mice in each group

These differences are mirrored in the case of the *T. brucei* and *T. cruzi* enzymes.[7]

We have prepared a number of compounds that show good inhibition of the protozoan DHFRs and also show selectivity. All the protozoan enzymes follow similar trends. For the 3′-substituted compounds (**2**−**12**), optimum activity against the enzyme appears to occur with a chain length of about 3−6 carbon atoms (**5**−**8**) (Table 2). This also corresponds to maximum selectivity against the human enzyme (Table 2) for the 3′-substituted series. Addition of the 6-ethyl (**19**) group seemed to increase potency against the enzyme and the selectivity. Branched chain analogues (**13**−**15**) showed good enzyme activity and selectivity. Also the 4-substituted compounds (**16**, **17**) showed good enzyme activity and were the most selective.

Some in vitro activity against the parasite was also observed in the case of *T. cruzi* and *T. brucei rhodesiense* for some of the compounds (Tables 5 and 6). Among the 3′-alkyl-substituted compounds, maximum in vitro activity occurred with the longer chain lengths of 6−10 carbons atoms (**8**−**12**). The maximum in vitro activity does not correspond to maximum activity against the enzyme. The reason for this may be due to other factors such as cellular uptake—the more lipophilic compounds show greater activity. Addition of the 6-methyl (**18**) or 6-ethyl (**19**) group had a marginal effect on activity. Of the 3′-branched compounds, the benzyl (**14**) derivative showed better in vitro activity than the isopropyl (**13**); this may be due to the greater lipophilicity of the former compound.

In vitro activity against *L. infantum* amastigotes was poorer (Table 4), with marginal activity only observed for compounds **8**, **9**, **18**, and **19**, compounds with long chain alkyl substituents. This is in contrast to activity seen against *L. major* promastigotes (data not shown), where most of the compounds showed $EC_{50}$ values in the range 3−14 μM. Again uptake of compounds to the amastigotes may well be the problem. This is especially problematic for leishmanial amastigotes that are in a vacuole inside a cell: therefore, compounds will have to traverse the macrophage in which they are cultured and then the vacuole before reaching the parasite. An additional problem is that the vacuole is acidic, so it may trap these compounds which are slightly basic. In contrast the promastigotes are cultured directly in solution and are freely accessible to potential inhibitors.

Some of the compounds were also tested against rodent models of the infection. Some activity was seen in the case of *T. brucei*, increasing the life span of infected mice. Unfortunately these results were not found in the rodent models of Chagas' disease and leishmaniasis. Problems were found with toxicity in some cases. Therefore there is need for more potent and selective inhibitors.

Factors other than strength of inhibition of DHFR may be important in in vitro activity such as uptake into the parasite. There is also the possibility of activity against other enzymatic targets involved in folate metabolism, which may complicate the issue.[6] For example, the pterin reductase, *ptr1*, may well also be a molecular target.

Overexpression of *ptr1* has been postulated to be a potential method of resistance to antifolates in *L. major* promastigotes.[6] However it is unlikely to be the cause of the low in vitro activity against *L. infantum* in this study as the compounds were active against *L. major* promastigotes, suggesting that cellular uptake is the reason for poor activity. *ptr1* has also been observed in *T. cruzi*[12] but is only expressed in the epimastigote (vector form) and not in the amastigote form.

Modeling studies were undertaken to try and rationalize the activities and selectivities observed against the enzyme. In the case of the leishmanial enzyme, the alkyl chains of the inhibitors were located close to Phe91 found in the active site of the enzyme. This presumably gives rise to a hydrophobic interaction. The optimum interaction was seen where the chain length was around 4−6 carbon atoms (**6**−**8**). When the chain length is shorter, the chain does not reach the Phe, and when it is longer, it is too large for the active site. This may explain the optimum enzyme activity being found with a chain length of 3−6 carbons (**5**−**8**). In the case of the human enzyme, the corresponding residue is Asn64. The alkyl chain of the inhibitors is not located near Asn64, presumably as there is no possibility of a hydrophobic interaction. This may explain the selectivity of compounds for the leishmanial enzyme over the human enzyme.

## Conclusion

We have synthesized a number of compounds that show good activity and selectivity for protozoan DHFR, and we have produced a structure-based model to try and rationalize the data that we obtained. This is the first time, to our knowledge, that such compounds have been tested against all three species (*T. brucei*, *T. cruzi*, and *Leishmania*) of protozoan. Prior to this report no such compounds had been evaluated against *T. brucei* or *T. cruzi* and only the lead compound had been tested against *Leishmania*.

Some of the compounds prepared show in vitro activity, particularly against *T. cruzi* and *T. brucei*. Fewer of the compounds were active against *L. infantum*. We were unable to reproduce the in vitro activity reported for the lead compound **1** against *Leishmania*. However, we report other compounds that have greater in vitro activity against *L. infantum*. In general terms, optimum in vitro activity is seen for the more lipophilic compounds. Greatest enzyme activity and selectivity is seen for compounds with medium length chains and with 4′-alkyl substituents. By increasing activity and selectivity at the enzyme, and having a good lipophilicity, it should be possible to produce compounds with enhanced activity—further work should address these issues.

## General Experimental Details

Where applicable all glassware was oven-dried overnight and all reactions were carried out under an atmosphere of nitrogen. Ether refers at all times to diethyl ether. Dry solvents (EtOH, MeOH, $CH_2Cl_2$, DMF, and pyridine) were purchased from Aldrich and Fluka in Sure Seal bottles. Reactions were monitored by TLC using silica gel 60 $F_{254}$ plates (Merck). Infrared spectra were recorded on a Perkin-Elmer 1600 series FTIR spectrometer as films on sodium chloride disks or as solids via a diffuse reflectance accessory using a potassium bromide matrix. $^1$H and $^{13}$C spectra were recorded on a Bruker Avance DPX 300-MHz spectrometer at 300 and 75 MHz, respectively. Elemental analyses were determined on a Perkin-Elmer 240C elemental analyzer. Low-resolution mass spectra were recorded on a Fison VG Platform II spectrometer using the electrospray (ES) ionization and atmospheric pressure chemical ionization (APCI) techniques. High-resolution mass spectra (HRMS) were determined by the EPSRC Mass Spectroscopy Centre, Swansea, U.K.

**Preparation of Compounds 3, 16, and 17: Scheme 1. General Procedure for Synthesis of Compounds 23−25.** Ethoxypropionitrile (1 equiv) was added dropwise to a stirred solution of the appropriate benzaldehyde (3-methoxybenzaldehyde (**20**), 4-ethoxybenzaldehyde (**21**), or 4-propoxybenzaldehyde (**22**)) (1 equiv) in dry ethanol (4 mL/mmol) under an atmosphere of nitrogen. Sodium ethoxide was added (1.24 equiv) and the reaction was heated to reflux. Water was removed by azeotropic distillation with dry ethanol. The mixture was refluxed for a further 3 h and the solvent was evaporated in vacuo. The reaction mixture was poured into water (100 mL), extracted with $Et_2O$ (4 × 100 mL), dried with $MgSO_4$, and concentrated in vacuo to yield the crude product as an oil.

**2-(Ethoxymethyl)-3-(3-methoxyphenyl)-2-propenenitrile (23).** The crude product was purified by flash column chromatography on silica gel using a gradient an elution of 5−15% EtOAc in hexane to yield the title compound **23** as a brown oil (0.56 g, 55%): TLC $R_f$ 0.3 (10% EtOAc in hexane); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 1.3 (3H, t, $J$ = 6.9 Hz, $OCH_2CH_3$), 3.6 (2H, q, $J$ = 6.8 Hz, $OCH_2CH_3$), 3.9 (3H, s $OCH_3$), 4.28 (2H, s, $OCH_2C$), 7.0 (2H, d, $J$ = 8.1 Hz, Ar−H), 7.2 (1H, s, NCC=C*H*), 7.4 (2H, m, Ar−H); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 15.5 ($CH_3$), 55.8 ($OCH_3$), 67.2 ($OCH_2$), 72.1 ($OCH_2$), 109.1, 113.6, 117.4, 119.5, 122.4, 130.2, 134.7, 144.8, 160.7; $\upsilon_{max}$(liquid film) 2959 (CH aromatic), 2821 (CH aliphatic), 2214 (CN), 1642 (C=C) cm$^{-1}$; MS (ES, +ve) *m/e* 218 (M + H$^+$, 100%); HRMS calcd for $C_{13}H_{15}NO_2$ (M$^+$) 217.1103, found 217.1099.

**2-(Ethoxymethyl)-3-(4-ethoxybenzyl)-2-propenenitrile (24).** The crude product was purified by flash column chromatography on silica gel using a gradient elution of 5−15% EtOAc in hexane to yield the title compound **24** as a brown oil (1.53 g, 51%): TLC $R_f$ 0.3 (10% EtOAc in hexane); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 1.2 (3H, t, $J$ = 7.1 Hz, $OCH_2CH_3$), 1.4 (3H, t, $J$ = 7.3 Hz, $OCH_2CH_3$), 3.6 (2H, q, $J$ = 7.4 Hz, $OCH_2$-$CH_3$), 3.9 (2H, q, $J$ = 7.0 Hz, $OCH_2CH_3$), 4.1 (s, 2H, $OCH_2$), 6.9 (2H, d, $J$ = 8.2 Hz, Ar−H), 7.2 (1H, s, CNC=*H*), 7.8 (2H, d, $J$ = 8.3 Hz, Ar−H); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 14.4 ($CH_3$), 15.3 ($CH_3$), 63.9 ($OCH_2$), 67.4 ($OCH_2$), 72.3 ($OCH_2$), 105.3, 114.1, 118.5, 126.1, 131.9, 144.8, 160.7; $\upsilon_{max}$(liquid film) 3059 (CH aromatic), 2821 (CH aliphatic), 2246 (CN), 1642 (C=C) cm$^{-1}$; MS (ES, +ve) *m/e* 232 (M + H$^+$, 100%); HRMS calcd for $C_{14}H_{17}NO_2$ (M$^+$) 231.1259, found 231.1267.

**2-(Ethoxymethyl)-3-(4-propoxybenzyl)-2-propenenitrile (25)**. The crude product was purified by flash column chromatography on silica gel using a gradient elution of 5−15% EtOAc in hexane to yield the title compound **25** as a brown oil (0.62 g, 56%): TLC $R_f$ 0.31 (10% EtOAc in hexane); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 1.0 (3H, t, $J$ = 6.8 Hz, $(CH_2)_2CH_3$), 1.2 (3H, t, $J$ = 6.9 Hz, $OCH_2CH_3$), 1.8 (2H, sextuplet, $J$ = 6.8 Hz, $OCH_2CH_2CH_3$), 3.6 (2H, q, $J$ = 7.0 Hz, $OCH_2CH_3$), 3.9 (2H, t, $J$ = 6.7 Hz, $OCH_2CH_2CH_3$), 4.2 (2H, s, $OCH_2$), 7.2 (1H, s, NCC=C*H*), 6.9−7.9 (4*H*, m, Ar-*H*).

**General Procedure for the Synthesis of Compounds 3, 16, and 17.** Solutions of guanidine hydrochloride (6 equiv) in warm anhydrous ethanol (0.3 mL/mmol of guanidine hydrochloride) and sodium ethoxide (6 equiv) in warm anhydrous ethanol (0.3 mL/mmol of sodium ethoxide) were prepared separately and allowed to cool to room temperature. The guanidine solution was added to the stirred solution of sodium ethoxide under an atmosphere of nitrogen. The mixture was stirred for 5 min, filtered, and added to **23**, **24**, or **25** (1 equiv). The reaction mixture was heated to reflux for 40 h, cooled, concentrated in vacuo, and purified by flash column chromatography on silica gel using a gradient elution of 2−10% MeOH in EtOAc to yield the title compounds.

**5-(3-Methoxybenzyl)-2,4-pyrimidinediamine (3).** Compound **3** was isolated as a pale yellow powder (0.356 g, 60%): mp 190.6−192.3 °C; TLC $R_f$ 0.21 (10% MeOH in EtOAc); $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 3.52 (2H, s, Ph-C$H_2$−Ph), 3.70 (3H, s, $OCH_3$), 5.82 (2H, s, $NH_2$), 6.20 (2H, s, $NH_2$), 6.68 (3H, m, Ar-*H*), 7.2 (1H, t, $J$ = 7.8 Hz, Ar-*H*), 7.5 (1H, s, H-pyrimidine); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 30.9 ($CH_2$), 53.4 ($OCH_3$), 104.3, 109.5, 112.7, 119.0, 123.9, 127.8, 140.1, 153.9, 157.6, 160.3; $\upsilon_{max}$ (KBr) 3500 and 3314 ($NH_2$), 3123 (aromatic C−H), 2925 (aliphatic C−H), 1602 (aromatic C=C) cm$^{-1}$; MS

(ES, +ve) *m/e* 231 (M + $H^+$, 100%); HRMS calcd for $C_{12}H_{15}N_4O$ (M + $H^+$) 231.1246, found 231.1246. Anal. ($C_{12}H_{14}N_4O$) C, H, N.

**5-(4-Ethoxybenzyl)-2,4-pyrimidinediamine (16).** Compound **16** was isolated as a pale yellow powder (0.83 g, 39%): mp 209−211 °C; TLC $R_f$ 0.22 (10% MeOH in EtOAc); $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 1.2 (3H, t, $J$ = 6.9 Hz, $CH_2CH_3$), 3.6 (2H, s, $CH_2$Ph), 3.9 (2H, q, $J$ = 6.9 Hz, $OCH_2CH_3$), 5.7 (2H, s, $NH_2$), 6.1 (2H, s, $NH_2$), 6.8 (2H, d, $J$ = 8.3 Hz, Ar-*H*), 7.1 (2H, d, $J$ = 8.4 Hz, Ar-*H*), 7.5 (1H, s, *H*-pyrimidine); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 15.5 ($CH_3$), 31.5 ($CH_2$), 63.6 ($OCH_2$), 107.0, 114.9, 126.2, 130.2, 132.7, 156.3, 157.5, 163.0; $\upsilon_{max}$ (KBr) 3480 and 3314 ($NH_2$), 3163 (aromatic C−H), 2975 (aliphatic C−H), 1628 (aromatic C=C) cm$^{-1}$ ; MS (ES, +ve) *m/e* 245.04 (M + $H^+$, 100%); HRMS calcd for $C_{13}H_{17}N_4O$ (M + $H^+$) 245.1402, found 245.1402. Anal. ($C_{13}H_{16}N_4O$) C, H, N.

**5-(4-Propoxybenzyl)-2,4-pyrimidinediamine (17).** Compound **17** was isolated as a white powder (0.4 g, 40%): mp 150.2−151.3 °C; TLC $R_f$ 0.25 (10% MeOH in EtOAc); $^1$H NMR (300 MHz, MeOH-$d_4$) $\delta$ 0.9 (3H, t, $J$ = 7.3 Hz, $CH_2CH_3$), 1.7 (2H, sextuplet, $J$ = 7 Hz, $OCH_2CH_2CH_3$), 3.5 (2H, s, Ph-$CH_2$−Ph), 3.8 (2H, t, $J$ = 6.5 Hz, $OCH_2CH_2CH_3$), 6.9 (2H, d, $J$ = 8.5 Hz, Ar-*H*), 7.2 (2H, d, $J$ = 8.6 Hz, Ar-*H*), 7.5 (1H, s, *H*-pyrimidine); $^{13}$C NMR (75 MHz, MeOH-$d_4$) $\delta$ 11.4 ($CH_3$), 24.2 ($CH_2$), 33.9 ($CH_2$), 71.0 ($OCH_2$), 109.3, 116.2, 128.8, 132.1, 155.1, 159.8, 162.3, 165.0; $\upsilon_{max}$ (KBr) 3480 and 3312 ($NH_2$), 3179 (aromatic C−H), 2975 (aliphatic C−H), 1628 (aromatic C=C) cm$^{-1}$; MS (ES, +ve) 259 (M + $H^+$, 100%); HRMS calcd for $C_{14}H_{19}N_4O$ (M + $H^+$) 259.1558, found 259.1559. Anal. ($C_{14}H_{19}N_4O$) C, H, N.

**Preparation of Compounds 2 and 4−15: Scheme 2. 3-(Tetrahydro-2*H*-2-pyranyloxy)benzaldehyde (26).** To a solution of 3-hydroxybenzaldehyde (8 g, 65.51 mmol, 1 equiv) and dihydropyran (30 mL, 327.55 mmol, 5 equiv) in dry dichloromethane (260 mL) at 0 °C was added *p*-toluenesulfonic acid monohydrate (0.01 mL, 0.655 mmol, 0.01 equiv) and the mixture was maintained at 0 °C for 2 h. The solution was diluted with ether (150 mL) and successively washed with satd $NaHCO_3$ (450 mL), brine (180 mL), and water (180 mL). The organic extracts were combined and dried over $MgSO_4$ to yield the crude product as yellow oil. Purification by flash column chromatography using a gradient elution of 5−20% EtOAc in hexane yielded the title compound as a pale yellow oil **26** (9 g, 66%): TLC $R_f$ 0.48 (20% EtOAc in hexane); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 1.5−2.1 (6H, m, $(CH_2)_3$), 3.6−3.9 (2H, m, $OCH_2$), 5.4 (1H, t, $J$ = 3.5 Hz, OC*H*O), 7.2−7.6 (4H, m, Ar-*H*), 10.0 (s, 1H, C*H*O); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 16.7 ($CH_2$), 23.4 ($CH_2$), 28.7 ($CH_2$), 60.9 ($OCH_2$), 94.4 (OCHO), 114.5, 120.2, 121.4, 128.0, 135.7, 155.6, 190.1 (*C*HO); $\upsilon_{max}$ (liquid film) 2944 (aliphatic C−H), 2850 (aldehyde C−H), 1699 (C=O) cm$^{-1}$; MS (APCI, +ve) 207 (M + $H^+$, 100%).

**2-(Ethoxymethyl)-3-(3-(tetrahydro-2*H*-2-pyranyloxy)-phenyl)-2-propenenitrile (27).** Ethoxypropionitrile (4.75 mL, 43.63 mmol, 1 equiv) in dry EtOH (100 mL) was added dropwise to a solution of **12** (9 g, 43.63 mmol, 1 equiv) under an atmosphere of nitrogen. Sodium ethoxide (3.56 g, 52.37 mmol, 1.24 equiv) was added and the reaction was heated to reflux. Water was removed by azeotropical distillation with dry ethanol. The mixture was heated at reflux for a further 3 h and the solvent was evaporated in vacuo. The reaction mixture was poured into water (300 mL), extracted with $Et_2O$ (4 × 300 mL), dried with $MgSO_4$, and concentrated in vacuo to yield the crude product as a dark yellow oil. The crude product was purified by flash column chromatography on silica gel using an elution of 10% EtOAc in hexane to yield the title compound **27** as a brown oil (6.8 g, 54%): TLC $R_f$ 0.31 (10% EtOAc in hexane); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 1.2 (3H, t, $J$ = 6 Hz, $OCH_2CH_3$), 1.6−2 (6H, m, $(CH_2)_3$), 3.7 (2H, q, $J$ = 6 Hz, $OCH_2CH_3$), 4.0 (2H, t, $J$ = 6.5 Hz, $OCH_2$), 4.2 (2H, s, $OCH_2C$=), 5.4 (1H, t, $J$ = 3.5 Hz, OC*H*O), 7.2 (1H, s, NCC=C*H*), 7−7.4 (4H, m, Ar-*H*); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 15.4 ($CH_3$), 19.2 ($CH_2$), 25.5 ($CH_2$), 30.7 ($CH_2$), 62.7 ($OCH_2$), 66.7 ($OCH_2$), 72.1 ($OCH_2$), 97.0 (OCHO), 109.0, 115.5, 116.8, 117.6, 119.4, 122.6, 130.2, 134.6, 144.8, 157.7; $\upsilon_{max}$ (liquid film) 2942 (aromatic C−H), 2871 (aliphatic CH), 2214 (CN), 1642 (C=C) cm$^{-1}$; MS (ES, +ve) *m/e* 310 (M + $Na^+$, 75%); HRMS calcd for $C_{17}H_{25}N_2O_3$ (M + $NH_4^+$) 305.1865, found 305.1865.

**5-(3-(Tetrahydro-2*H*-2-pyranyloxy)benzyl)-2,4-pyrimidinediamine (15).** Solutions of guanidine hydrochloride (5.33 g, 55.76 mmol, 6 equiv) in warm anhydrous ethanol (100 mL) and sodium ethoxide (3.79 g, 55.76 mmol, 6 equiv) in warm anhydrous ethanol (100 mL) were prepared separately and allowed to cool to room temperature. The guanidine solution was added to the stirred solution of sodium ethoxide under an atmosphere of nitrogen. The mixture was stirred for 5 min, filtered, and added to **27** (2 g, 6.97 mmol, 1 equiv). The reaction mixture was heated to reflux for 24 h, cooled, concentrated in vacuo, and purified by flash column chromatography on silica gel using a gradient elution of 2−10% MeOH in EtOAc to yield the title compound **15** as a yellow-white powder (0.92 g, 45%): mp 122.2−123.3 °C; TLC $R_f$ 0.22 (10% MeOH in EtOAc); $^1$H NMR (300 MHz, MeOH-$d_4$) $\delta$ 1.5−2.0 (6H, m, $(CH_2)_3$), 3.5 and 3.85 (2H, m, $OCH_2$), 3.65 (2H, s, $CH_2$ Ph), 5.37 (1H, t, $J$ = 6.3 Hz, OC*H*O), 6.8 (3H, m, Ar-*H*), 7.2 (1H, t, $J$ = 7.6 Hz, Ar-*H*), 7.5 (1H, s, H-pyrimidine); $^{13}$C NMR (75 MHz, MeOH-$d_4$) $\delta$ 19.0 ($CH_2$), 25.3 ($CH_2$), 30.4 ($CH_2$), 33.1 ($CH_2$), 62.1 ($OCH_2$), 96.7 (OCHO), 106.9, 114.4, 116.7, 121.8, 126.2, 129.4, 140.6, 154.6, 157.7, 160.6; $\upsilon_{max}$ (KBr) 3324 and 3130 ($NH_2$), 2932 (aromatic C−H), 2889 (aliphatic C−H), 1622 (C=C) cm$^{-1}$; MS (ES, +ve) *m/e* 301 (M + $H^+$, 100%); HRMS calcd for $C_{16}H_{20}N_4O_2$ ($M^+$) 300.1583, found 300.1586.

**3-((2,4-Diamino-5-pyrimidinyl)methyl)phenol (2).** A catalytic amount of concentrated HCl (2 drops) was added to a solution of **14** (0.924 g, 3.08 mmol) in dry MeOH and the reaction mixture was stirred for 24 h at room temperature. The reaction mixture was reduced in vacuo and purified by flash column chromatography on silica gel using a gradient elution of 2−5% MeOH in EtOAc to yield the compound **2** as a gray-white powder (0.851 g, 99%): mp 228.4−230.6 °C; TLC $R_f$ 0.25 (10% MeOH in EtOAc); $^1$H NMR (300 MHz, MeOH-$d_4$) $\delta$ 3.7 (2H, s, $CH_2$Ph), 6.7 (3H, m, Ar−H), 7.2 (1H, t, $J$ = 7.7 Hz, Ar−H), 7.3 (1H, s, H-pyrimidine); $^{13}$C NMR (75 MHz, MeOH-$d_4$) $\delta$ 33.0 ($CH_2$Ph), 110.4, 114.4, 116.0, 120.4, 130.3, 138.6, 139.7, 165.7; $\upsilon_{max}$ (KBr) 3484 and 3420 (NH2), 3341 (OH), 3187 (aromatic CH) 1632 (aromatic C=C) cm$^{-1}$; MS (ES, +ve) *m/e* 217 (M + $H^+$, 100%); HRMS calcd for $C_{11}H_{13}N_4O$ (M + $H^+$) 217.1089, found 217.1089.

**General Preparation of 5-(3-(Alkyloxy)benzyl)-2,4-pyrimidinediamines 4−14.** Potassium hydroxide (1.5 equiv) was added to a solution of **15** (1 equiv) in dry ethanol (10 mL) and the reaction mixture was heated to reflux for 0.5 h. Then alkyl iodide (1 equiv) was added to the mixture and refluxed for another 1/1/2 to 4 h. The reaction mixture was diluted with water, extracted with ether, dried over $MgSO_4$, and reduced in vacuo to yield the crude product as a pale yellow powder. The crude product was purified by flash column chromatography on silica gel using 5% MeOH in EtOAc ($R_f$ of compounds in the range 0.23−0.26 in 10% MeOH in EtOAc) and yielded the products as gray-white powders.

**5-(3-Ethoxybenzyl)-2,4-pyrimidinediamine (4).** The compound was isolated as a yellow-white solid (0.039 g, 35%): mp 200.6−202.3 °C; $^1$H NMR (300 MHz, MeOH-$d_4$) $\delta$ 1.2 (3H, t, $J$ = 6.9 Hz, $CH_3$), 3.5 (2H, s, $CH_2$Ph), 4.0 (2H, q, $J$ = 6.9 Hz, $OCH_2$), 6.8 (3H, m, Ar-*H*), 7.1 (1H, t, $J$ = 7.8 Hz, Ar-*H*), 7.2 (1H, s, H-pyrimidine); $^{13}$C NMR (75 MHz, MeOH-$d_4$) $\delta$ 14.1 ($CH_3$), 32.6 ($CH_2$), 63.4 ($OCH_2$), 109.8, 112.9, 115.2, 120.9, 124.6, 129.9, 138.3, 139.8, 159.8; $\upsilon_{max}$ (KBr) 3420 and 3314 ($NH_2$), 2934 (aromatic C−H), 2851 (aliphatic C−H), 1631 (C=C) cm$^{-1}$; MS (ES, +ve) *m/e* 245 (M + $H^+$, 100%); HRMS calcd for $C_{13}H_{17}N_4O$ (M + $H^+$) 245.1402, found 245.1402.

**5-(3-Propoxybenzyl)-2,4-pyrimidinediamine (5).** The compound was isolated as a pale yellow powder (0.044 g, 37%): $^1$H NMR (300 MHz, MeOH-$d_4$) $\delta$ 0.8 (3H, t, $J$ = 7.3 Hz, $CH_3$), 1.7 (2H, sextuplet, $J$ = 7.0 Hz $CH_2CH_3$), 3.7 (2H, s, $CH_2$-Ph), 3.9 (2H, t, $J$ = 6.4 Hz, $OCH_2$), 6.9 (3H, m, Ar-*H*), 7.2 (1H, t, $J$ = 7.9 Hz, Ar-*H*), 7.5 (1H, s, *H*-pyrimidine); $^{13}$C NMR (75 MHz, MeOH-$d_4$) $\delta$ 9.8 ($CH_3$), 22.6 ($CH_2$), 33.1 ($CH_2$), 69.3 ($OCH_2$), 107.0, 112.4, 114.7, 120.6, 129.5, 140.6, 154.6, 159.8,

162.0, 163.4; $\upsilon_{max}$ (Nujol) 3500 and 3314 ($NH_2$), 3123 (aromatic C−H), 2925 (aliphatic C−H), 1602 (C═C) $cm^{-1}$; MS (ES, +ve) *m/e* 259 (M + $H^+$, 100%). Anal. ($C_{14}$ $H_{18}N_4O$) C, H, N.

**5-(3-Butoxybenzyl)-2,4-pyrimidinediamine (6).** The compound was isolated as a white powder (0.043 g, 35%): mp 166.6−168.3 °C; $^1$H NMR (300 MHz, MeOH-$d_4$) $\delta$ 0.8 (3H, t, C$H_3$, $J$ = 6.4 Hz), 1.4 (2H, sextuplet, $J$ = 6.5 Hz, $CH_2CH_3$), 1.6 (2H, quintet, $J$ = 6.5 Hz, $OCH_2CH_2$), 3.5 (2H, s, C$H_2$Ph), 3.8 (2H, t, $J$ = 6.4 Hz, O$CH_2$), 6.7 (3H, m, Ar-*H*), 7.0 (1H, t, $J$ = 6.2 Hz, Ar-*H*), 7.35 (1H, s, *H*-pyrimidine); $^{13}$C NMR (75 MHz, MeOH-$d_4$) $\delta$ 11.2 (*C*$H_3$), 17.2 (*C*$H_2$), 29.4 (*C*$H_2$), 31.1 (*C*$H_2$). 65.4 (O*C*$H_2$), 104.9, 110.3, 112.6, 118.6, 127.4, 138.6, 152.7, 157.7, 160.0, 161.3; $\upsilon_{max}$ (KBr) 3454 and 3420 ($NH_2$), 2958 (aromatic C-H), 2856 (aliphatic C−H), 1602 (C═C) $cm^{-1}$; MS (ES, +ve) *m/e* 273 (M + $H^+$, 100%). Anal. ($C_{15}H_{20}N_4O$) C, H, N.

**5-(3-(Pentyloxy)benzyl)-2,4-pyrimidinediamine (7).** The compound was isolated as a yellow powder (0.065 g, 49%): $^1$H NMR (300 MHz, MeOH-$d_4$) $\delta$ 0.8 (3H, t, $J$ = 7.0 Hz, C$H_3$), 1.3 (4H, m, C$H_2$C$H_2$CH$_3$), 1.6 (2H, quintet, $J$ = 7.0 Hz, $OCH_2CH_2$), 3.5 (2H, s, C$H_2$Ph), 3.8 (2H, t, $J$ = 7.1 Hz, O$CH_2$), 6.7 (3H, m, Ar-*H*), 7.0 (1H, t, $J$ = 7.9 Hz, Ar-*H*), 7.3 (1H, s, *H*-pyrimidine); $^{13}$C NMR (75 MHz, MeOH-$d_4$) $\delta$ 13.3 ($CH_3$), 22.5 ($CH_2$), 28.3 ($CH_2$), 29.0 ($CH_2$), 33.1 ($CH_2$), 67.8 ($OCH_2$), 107.0, 112.4, 114.7, 120.6, 129.5, 140.6, 154.7, 159.8, 162.1, 163.3; $\upsilon_{max}$ (KBr) 3445 and 3307 ($NH_2$), 2949 (aromatic C−H), 1655 (C═C) $cm^{-1}$; MS (ES, +ve) *m/e* 287 (M + $H^+$, 100%). Anal. ($C_{16}H_{22}N_4O$) C, H, N.

**5-(3-(Hexyloxy)benzyl)-2,4-pyrimidinediamine (8).** The compound was isolated as a yellow white powder (0.056 g, 57%): $^1$H NMR (300 MHz, MeOH-$d_4$) $\delta$ 0.8 (3H, t, $J$ = 6.5 Hz, C$H_3$), 1.2 (6H, m, $(CH_2)_3CH_3$), 1.8 (2H, quintet, $J$ = 6.65 Hz, $OCH_2CH_2$), 3.5 (2H, s, C$H_2$Ph), 3.8 (2H, t, $J$ = 6.4 Hz, O$CH_2$), 6.7 (3H, m, Ar−*H*), 7.1 (1H, t, $J$ = 7.0 Hz, Ar−*H*), 7.3 (1H, s, *H*−pyrimidine); $^{13}$C NMR (75 MHz, MeOH-$d_4$) $\delta$ 13.4 (*C*$H_3$), 22.7 (*C*$H_2$), 26.1 (*C*$H_2$), 29.4 (*C*$H_2$), 31.9 (*C*$H_2$), 33.2 (*C*$H_2$), 67.8 (O*C*$H_2$), 107.1, 112.5, 114.7, 120.7, 129. 6, 140.7, 154.7, 159.9, 162.1, 163.4; $\upsilon_{max}$ (KBr) 3489 and 3413 ($NH_2$), 2987 (aromatic C−H), 2825 (aliphatic C−H), 1602 (C═C) $cm^{-1}$; MS (ES, +ve) *m/e* 301 (M + $H^+$, 100%). Anal. ($C_{17}H_{24}N_4O$) C, H, N.

**5-(3-(Heptyloxy)benzyl)-2,4-pyrimidinediamine (9).** The compound was isolated as a pale yellow powder (0.065 g, 45%): $^1$H NMR (300 MHz, MeOH-$d_4$) $\delta$ 0.8 (3H, t, $J$ = 6.7 Hz, C$H_3$), 1.3 (8H, m, $(CH_2)_4CH_3$), 1.8 (2H, quintet, $J$ = 6.4 Hz, $OCH_2CH_2$), 3.5 (2H, s, C$H_2$Ph), 3.8 (2H, t, $J$ = 6.4 Hz, O$CH_2$), 6.7 (3H, m, Ar-*H*), 7.0 (1H, $J$ = 7.8 Hz, Ar-*H*), 7.5 (1H, s, *H*-pyrimidine); $^{13}$C NMR (75 MHz, MeOH-$d_4$) $\delta$ 13.4 (*C*$H_3$), 22.6 (*C*$H_2$), 26.1 (*C*$H_2$), 29.2 (*C*$H_2$), 29.4 (*C*$H_2$), 31.9 (*C*$H_2$), 33.2 (*C*$H_2$), 67.8 (O*C*$H_2$), 107.0, 112.4, 114.7, 120.7, 129.5, 140.6, 154.7, 159.8, 162.0, 163.4; $\upsilon_{max}$ (KBr) 3474 and 3305 ($NH_2$), 2926 (aromatic C-H), 2868 (aliphatic C−H), 1604 (aromatic C═C) $cm^{-1}$; MS (ES, +ve) *m/e* 315.0 (M + $H^+$, 100%). Anal. ($C_{18}H_{26}N_4O$) C, H, N.

**5-(3-(Octyloxy)benzyl)-2,4-pyrimidinediamine (10).** The compound was isolated as a white powder (0.2 g, 66%): $^1$H NMR (300 MHz, MeOH-$d_4$) $\delta$ 0.8 (3H, t, $J$ = 6.4 Hz, C$H_3$), 1.2 (10H, m, $(CH_2)_5CH_3$), 1.7 (2H, quintet, $J$ = 6.5 Hz, $OCH_2CH_2$), 3.7 (2H, s, C$H_2$Ph), 3.9 (2H, t, $J$ = 6.4 Hz, O$CH_2$), 6.8 (3H, m, Ar-*H*), 7.1 (1H, t, $J$ = 7.8 Hz, Ar-*H*), 7.5 (1H, s, *H*-pyrimidine); $^{13}$C NMR (75 MHz, MeOH-$d_4$) $\delta$ 13.4 (*C*$H_3$), 22.6 (*C*$H_2$), 26.1 (*C*$H_2$), 29.2 (*C*$H_2$), 29.4 (*C*$H_2$), 31.9 (*C*$H_2$), 33.2 (*C*$H_2$), 33.4 (*C*$H_2$), 67.8 (O*C*$H_2$), 107.0, 112.4, 114.7, 120.7, 129.5, 140.6, 154.7, 159.8, 162.0, 163.4; $\upsilon_{max}$ (KBr) 3314 and 3123 ($NH_2$), 2925 (aromatic C−H), 2875 (aliphatic C−H), 1611 (aromatic C═C) $cm^{-1}$; MS (ES, +ve) *m/e* 328.9 (M + $H^+$, 100%). Anal. ($C_{19}H_{28}N_4O$) C, H, N.

**5-(3-Nonyloxy)benzyl)-2,4-pyrimidinediamine (11).** The compound was isolated as a white powder (0.088 g, 57%): $^1$H NMR (300 MHz, MeOH-$d_4$) $\delta$ 0.8 (3H, t, $J$ = 6.8 Hz, C$H_3$), 1.2 (12H, m, $(CH_2)_7$ $CH_3$), 1.7 (2H, quintet, $J$ = 7.0 Hz, $OCH_2CH_2$), 3.7 (2H, s, C$H_2$Ph), 3.9 (2H, t, $J$ = 6.5 Hz, O$CH_2$), 6.8 (3H, m, Ar-*H*), 7.2 (1H, t, $J$ = 7.9 Hz, Ar-*H*), 7.5 (1H, s, *H*-pyrimidine); $^{13}$C NMR (75 MHz, MeOH-$d_4$) $\delta$ 14.1 (*C*$H_3$), 23.4 (*C*$H_2$), 26.8 (*C*$H_2$), 30.1 (*C*$H_2$), 30.2 (*C*$H_2$), 30.3 (*C*$H_2$), 32.7 (*C*$H_2$), 33.8 (*C*$H_2$), 68.5 (O*C*$H_2$), 107.7, 113.1, 115.4, 121.3, 126.9, 130.2, 141.3, 160.5, 162.8, 164.0; $\upsilon_{max}$ (KBr) 3350 and 3343 ($NH_2$), 2923 (aromatic C−H), 2854 (aliphatic C−H), 1633 (aromatic C═C) $cm^{-1}$; MS (ES, +ve) *m/e* 343 (M + $H^+$, 100%); HRMS calcd for $C_{20}H_{31}N_4O$ (M + $H^+$) 343.2498, found 343.2498.

**5-(3-(Decyloxy)benzyl)-2,4-pyrimidinediamine (12).** The compound was isolated as a white powder (0.096 g, 59%): $^1$H NMR (300 MHz, MeOH-$d_4$) $\delta$ 0.8 (3H, t, $J$ = 6.5 Hz, C$H_3$), 1.2 (14H, m, $(CH_2)_7CH_3$), 1.7 (2H, quintet, $J$ = 6.6 Hz, $OCH_2CH_2$), 3.7 (2H, s, C$H_2$Ph), 3.9 (2H, t, $J$ = 6.5 Hz, O$CH_2$), 6.8 (3H, m, Ar-*H*), 7.0 (1H, t, $J$ = 7.4 Hz, Ar-*H*), 7.5 (1H, s, *H*-pyrimidine); $^{13}$C NMR (75 MHz, MeOH-$d_4$) $\delta$ 14.0 ($CH_3$), 23.3 (*C*$H_2$), 26.8 (*C*$H_2$), 30.0 (*C*$H_2$), 30.1 (*C*$H_2$), 30.2 (*C*$H_2$), 30.3 (*C*$H_2$), 30.4 (*C*$H_2$). 32.6(*C*$H_2$), 33.8 ($CH_2$), 68.4 ($OCH_2$), 107.6, 113.0, 115.3, 121.3, 130.1, 141.3, 155.4, 160.4, 162.7, 164.0; $\upsilon_{max}$ (KBr) 3388 and 3325 ($NH_2$), 2926 (aromatic C−H), 2845 (aliphatic C−H), 1626 (C═C) $cm^{-1}$; MS (ES, +ve) *m/e* 358.09 (M + $H^+$, 100%). Anal. ($C_{21}H_{32}N_4O$) C, H, N.

**5-(3-Propoxybenzyl)-2,4-pyrimidinediamine (13).** The compound was isolated as a pale yellow powder (0.073 g, 60%): $^1$H NMR (300 MHz, MeOH-$d_4$) $\delta$ 1.2 (6H, d, $J$ = 6.1 Hz, 2x$CH_3$), 3.6 (2H, s, C$H_2$Ph), 4.6 (1H, m, O*CH*), 6.7 (3H, m, Ar-*H*), 7.2 (1H, t, $J$ = 7.4 Hz, Ar-*H*), 7.4 (1H, s, *H*-pyrimidine); $^{13}$C NMR (75 MHz, MeOH-$d_4$) $\delta$ 20.3 (*C*$H_3$), 32.1 (*C*$H_2$), 68.7 (O*C*$H_2$), 106.1, 115.2, 117.6, 126.0, 130.5, 139.6, 153.4, 157.5, 160.9, 162.4; $\upsilon_{max}$ (KBr) 3318 and 3122 ($NH_2$), 2972 (aromatic C−H), 1621 (C═C) $cm^{-1}$; MS (ES, +ve) *m/e* 259 (M + $H^+$, 100%). Anal. ($C_{14}H_{19}N_4O\cdot 0.06H_2O$) C, H, N.

**5-(3-(Benzyloxy)benzyl)-2,4-pyrimidinediamine (14).** The compound was isolated as a yellow white powder (0.058 g, 41%): $^1$H NMR (300 MHz, MeOH-$d_4$) $\delta$ 3.7 (2H, s, C$H_2$Ph), 5.1 (2H, s, O$CH_2$Ph), 6.7−7.4 (10H, m, Ar-*H*); $^{13}$C NMR (75 MHz, MeOH-$d_4$) $\delta$ 33.1 (*C*$H_3$), 69.8 (O*C*$H_2$), 107.0, 113.7, 115.8, 121.8, 128.3, 128.5, 129.2, 130.3, 138.4, 141.5, 155.5, 160.2, 161.4, 164.1; $\upsilon_{max}$ (KBr) 3452 ($NH_2$), 3140 (aromatic C−H), 1652 (aromatic C═C) $cm^{-1}$; MS (ES, +ve) *m/e* 307.9 (M + $H^+$, 100%). Anal. ($C_{18}H_{18}N_4O$) C, H, N.

**Preparation of Compounds 18 and 19: Scheme 3. 3-(Heptyloxy)benzaldehyde (28).** To a solution of 3-hydroxybenzaldehyde (5 g, 40 mmol, 1 equiv) in dry EtOH (250 mL) was added potassium hydroxide (4.49 g, 80 mmol, 1.2 equiv). Iodoheptane (1.08 mL, 7.36 mmol, 1.2 equiv) was added to the reaction mixture dropwise after 10 min. The mixture was heated to reflux for 5 h and was allowed to cool to room temperature. The reaction mixture was extracted with $Et_2O$ (1000 mL), dried over $MgSO_4$, and reduced in vacuo to yield the crude product as a brown oil. The crude product was purified by flash column chromatography on silica gel using gradient elution of 5−15% EtOAc in hexane (10% EtOAc in hexane,) to yield the title compound as a pale yellow oil **28** (4.18 g, 50%): TLC $R_f$ 0.35 (10% EtOAc in hexane); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 0.9 (3H, t, $J$ = 6.7 Hz, C$H_3$), 1.3−1.6 (8H, m, C$H_2$), 1.8 (2H, quintet, $J$ = 6.6 Hz, $OCH_2CH_3$), 4 (2H, t, $J$ = 6.5 Hz, O$CH_2$), 7.1−7.5 (4H, m, Ar-*H*), 10 (IH, s, C*H*O); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 15.5 (*C*$H_3$), 24.1 ($CH_2$), 27.6 ($CH_2$), 30.5 ($CH_2$), 30.8 ($CH_2$), 32.2 ($CH_2$), 69.7 ($OCH_2$), 114.1, 123.3, 124.2, 131.4, 139.1, 160.1, 193.5 (*C*HO); $\upsilon_{max}$ (liquid film), 2930 (aromatic C−H), 2856 (aliphatic C−H), 1699.0 (CHO) $cm^{-1}$; MS (APCI, +ve) 221 (M + $H^+$, 100%); HRMS calcd for $C_{14}H_{20}O_2$ ($M^+$) 220.3143, found 220.1463.

**3-(Heptyloxy)phenylmethanol (29).** To an ethanolic solution of **28** (4 g, 18 mmol) was added sodium borohydride (0.34 g, 9 mmol) and the reaction was left at room temperature for 20 min. The reaction mixture was reduced in vacuo, extracted with EtOAc, and dried over $MgSO_4$ to yield the title compound **29** as a clear oil (3.16 g, 79%): $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 0.8 (3H, t, $J$ = 6.5 Hz, C$H_3$), 1.3−1.6 (8H, m, C$H_2$), 1.7 (2H, quintet, $J$ = 6.5 Hz, C$H_2$), 1.71 (1H, s, $CH_2$O*H*), 3.8 (2H, t, $J$ = 6.8 Hz, O$CH_2$), 4.65 (2H, s, C$H_2$Ph), 7.1−7.2 (3H, m, Ar-*H*), 7.24 (1H, t, $J$ = 8.0 Hz, Ar-*H*); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 14.5 (*C*$H_3$), 23.0 (*C*$H_2$), 26.4 (*C*$H_2$), 29.5 (*C*$H_2$), 29.7 (*C*$H_2$), 32.2 (*C*$H_2$), 65.7 (O*C*$H_2$), 68.3 (O*C*$H_2$), 113.2, 114.2, 119.3, 129.9, 142.8, 159.8; $\upsilon_{max}$ (liquid film) 3412.0 (O−H), 2926 (aromatic C−H), 2855 (alipahtic C−H), 1602 (C═C) $cm^{-1}$; MS (APCI,

+ve) *m/e* 223.02 (M + $H^+$, 100%); HRMS calcd for $C_{14}H_{26}NO_2$ (M + $NH_4^+$) 240.1964, found 240.1963.

**1-(3-(Bromomethyl)phenoxy)heptane (30).** $PBr_3$ (4.47 mL, 47.08 mmol, 5.5 equiv) was added dropwise to a solution of **29** (1.9 g, 8.56 mmol, 1 equiv) in dry dioxane (80 mL) under an atmosphere of nitrogen and the reaction mixture was stirred overnight at room temperature. The reaction mixture was partitioned with water and $Et_2O$ and was washed with $NaHCO_3$ to yield the title compound **30** as a light yellow oil (1.194 g, 50%): $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 0.9 (3H, t, $J$ = 6.5 Hz, C$H_3$), 1.2−1.6 (8H, m, 4xC$H_2$), 1.8 (2H, quintet, $J$ = 6.5 Hz, OCH$_2$C$H_2$), 3.9 (2H, t, $J$ = 6.4 Hz, OC$H_2$), 4.5 (2H, s, C$H_2$Ph), 6.8−7.0 (3H, m, Ar-*H*), 7.2 (1H, t, $J$ = 7.8 Hz, Ar-*H*); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 9.9 (*C*H$_3$), 21.9 (*C*H$_2$), 24.9 (*C*H$_2$), 28.2 (*C*H$_2$), 28.6 (*C*H$_2$), 30.7 (*C*H$_2$), 32.5 (*C*H$_2$), 66.9 (O*C*H$_2$), 113.6, 113.9, 120.0, 128.7, 138.0, 158.2; MS (APCI, +ve) *m/e* 284.9 (M + H, 100%), 286.9 (M + 2 + H, 90%); HRMS calcd for $C_{14}H_{21}BrO$ ($M^+$) 284.0776, found 284.0776.

**2-Ethyl 2-(Heptoxybenzyl)-3-oxobutanoate (31).** Sodium ethoxide (0.368 g, 5.41 mmol, 1 equiv) was added to a solution of ethyl acetoacetate (0.441 mL, 3.16 mmol, 1 equiv) in dry ethanol (20 mL) and compound **30** (0.9 g, 3.16 mmol, 1 equiv) was added to the reaction mixture after 20 min. The reaction mixture was heated to reflux for 4 h. The reaction mixture was extraction with $Et_2O$, dried over $MgSO_4$, and reduced in vacuo. The crude product was purified by flash column chromatography using an elution of 5% EtOAc in hexane to yield the title compound as a light yellow oil **31** (0.686 g, 68.6%): TLC $R_f$ 0.35 (10% EtOAc in hexane); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 0.9 (3H, t, $J$ = 6.4 Hz, C$H_3$), 1.1 (3H, t, $J$ = 7.1 Hz, C$H_3$), 1.2−1.4 (8H, m, C$H_2$), 1.7 (2H, quintet, $J$ = 6.5 Hz, OCH$_2$C$H_2$), 2.1 (3H, s, C$H_3$), 3.0 (2H, d, $J$ = 7.5 Hz, C$H_2$Ph), 3.7 (1H, t, $J$ = 7.5 Hz, C*H*CH$_2$Ph), 3.8 (2H, t, $J$ = 6.5 Hz, OC$H_2$CH$_2$), 4.1 (2H, q, $J$ = 7.1 Hz, OC$H_2$ CH$_3$), 6.7 (3H, m, Ar-*H*), 7.1 (1H, t, $J$ = 7.6 Hz, Ar-*H*); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 14.4 (*C*H$_3$), 14.5 (*C*H$_3$), 23.0 (*C*H$_2$), 26.4 (*C*H$_2$), 29.4 (*C*H$_2$), 29.6 (*C*H$_2$), 30.1 (*C*H$_2$), 32.2 (*C*H$_2$), 34.4 (*C*H$_3$), 61.6 (COCHCO), 61.9 (O*C*H$_2$), 68.2 (O*C*H$_2$), 113.0, 115.4, 121.2, 129.9, 140.0, 159.6, 169.5 (CO), 202.9 (CO); MS (ES, +ve) *m/e* 356.9 (M + $Na^+$, 100%); HRMS calcd for $C_{20}H_{31}O_4$ (M + $H^+$) 334.2144, found 334.2144.

**Ethyl 2-(3-Heptoxybenzyl)-3-oxopentonoate (32).** The method for compound **31** was used. The crude product was purified by flash column chromatography using an elution of 5% EtOAc in hexane to yield the title compound **32** as a clear oil (1.107 g, 82%): TLC $R_f$ 0.35 (10% EtOAc in hexane); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 0.9 (3H, t, $J$ = 7.2 Hz, C$H_3$), 1.0 (3H, t, $J$ = 7.2 Hz, C$H_3$), 1.2 (3H, t, $J$ = 7.1 Hz, C$H_3$), 1.3−1.6 (8H, m, 4xC$H_2$), 1.8 (2H, quintet, $J$ = 7.0 Hz, OC$H_2$CH$_2$), 2.3 (2H, q, $J$ = 5.6 Hz, C$H_2$CH$_3$), 3.1 (2H, d, C$H_2$Ph), 3.8 (1H, t, $J$ = 7.3 Hz, C*H*CH$_2$Ph), 3.9 (2H, t, $J$ = 6.5 Hz, OC$H_2$CH$_2$), 4.1 (2H, q, $J$ = 6.3 Hz, OC$H_2$ CH$_3$), 6.8 (3H, m, Ar-*H*), 7.2, (1H, t, $J$ = 7.6 Hz, Ar-*H*); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 9.9 (*C*H$_3$), 16.5 (*C*H$_3$), 23.4 (*C*H$_2$), 25.0 (*C*H$_2$), 28.7 (*C*H$_2$), 31.5 (*C*H$_2$), 31.7 (*C*H$_2$), 34.2 (*C*H$_2$), 36.6 (*C*H$_2$), 38.6 (*C*H$_2$), 62.6 (CO*C*HCO), 63.8 (O*C*H$_2$), 70.3 (O*C*H$_2$), 115.0, 117.4, 123.3, 131.9, 142.2, 161.6, 171.6 (*C*=O), 207.8 (*C*=O); MS (ES, +ve) *m/e* 366 (M + $NH_4^+$, 100%), 371 (M + $Na^+$, 100%), 387 (M + $K^+$, 100%); HRMS calcd for $C_{21}H_{32}O_4$ ($M^+$) 348.2301, found 348.2301.

**2-Amino-4-hydroxy-5-benzyl-3-heptoxy-6-methylpyrimidine (33).** Solutions of guanidine hydrochloride (1 g, 10.46 mmol, 5 equiv) in warm anhydrous ethanol (25 mL) and sodium ethoxide (0.712 g, 10.46 mmol, 5 equiv) in warm anhydrous ethanol (25 mL) were prepared separately and allowed to cool to room temperature. The guanidine solution was added to the stirred solution of sodium ethoxide. The mixture was stirred for 5 min, filtered, and added to **31** (0.686 g, 2.09 mmol, 1 equiv). The reaction mixture was heated to reflux for 20 h and allowed to cool to room temperature. The reaction mixture was diluted with 500 mL of water and concentrated HCl was added until pH became 6.5. The solid from the reaction mixture was separated by vacuum filtration and washed with cold water, acetone, and $Et_2O$ to yield the title compound **33** as a white powder (0.530 g, 62.4%): $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 0.8 (3H, br m, C$H_3$), 1.2−1.4 (8H, m, 4xC$H_2$), 1.6 (2H, m, OCH$_2$C$H_2$), 2.0 (3H, s, ArC$H_3$), 3.7 (2H, s, C$H_2$Ph), 3.9 (2H, m, OC$H_2$CH$_2$), 6.3 (2H, s, N$H_2$), 6.8 (3H, m, Ar-*H*), 7.0 (1H, t, $J$ = 8.1 Hz, Ar-*H*), 10.9 (1H, s, O*H*); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 14.7 (*C*H$_3$), 22.8 (*C*H$_3$), 26.3 (*C*H$_2$), 29.3 (*C*H$_2$), 29.5 (*C*H$_2$), 30.6 (*C*H$_2$), 30.8 (*C*H$_2$), 32.0 (*C*H$_2$), 67.9 (O*C*H$_2$), 111.0, 111.9, 115.1, 120.8, 126.2, 129.8, 143.6, 154.1, 159.4, 162.1; MS (ES, +ve) *m/e* 330.0 (M + $H^+$, 100%); HRMS calcd for $C_{19}H_{28}N_3O_2$ (M + $H^+$) 330.2181, found 330.2182.

**2-Amino-4-hydroxy-5-benzyl-3-heptoxy-6-ethylpyrimidine (34).** The same method as for compound **33** was used to yield the title compound **34** as a white powder (0.624 g, 62.4%): $^1$H NMR (300 MHz, DMSO−$d_6$) $\delta$ 0.8 (3H, t, $J$ = 6.5 Hz, C$H_3$), 0.9 (3H, t, $J$ = 7.5 Hz, C$H_3$), 1.2−1.5 (8H, m, C$H_2$), 1.7 (2H, quintet, $J$ = 6.4 Hz, OCH$_2$C$H_2$), 2.8 (2H, q, $J$ = 7.4 Hz, C$H_2$CH$_3$), 3.8 (2H, s, C$H_2$Ar), 3.9 (2H, t, $J$ = 6.4 Hz, OC$H_2$-CH$_2$), 6.2 (2H, s, NH2), 6.7 (3H, m, Ar−*H*), 7.0 (1H, t, $J$ = 8.1 Hz, Ar−*H*), 10.9 (1H, s, OH); $^{13}$C NMR (75 MHz, DMSO−$d_6$) $\delta$ 13.2 (*C*H$_3$), 14.7 (*C*H$_3$), 22.8 (*C*H$_2$), 26.3 (*C*H$_2$), 29.8 (*C*H$_2$), 30.1 (*C*H$_2$), 30.8 (*C*H$_2$), 32.6 (*C*H$_2$), 43.6 (*C*H$_2$), 67.9 (OCH$_2$), 111.8, 112.5, 113.1, 115.6, 120.2, 121.4, 130.5, 144.6, 160.0, 161.4; MS (ES, +ve) *m/e* 344.1 (M + $H^+$), 366 (M + $Na^+$), 382 (M + $K^+$); HRMS calcd for $C_{20}H_{30}N_3O_2$ (M + $H^+$) 344.2338, found 344.2338.

**2,4-Diamino-5-benzyl-3-heptoxy-6-methylpyrimidine (18).** A solution of **33** (0.530 g, 1.61 mmol, 1 equiv), $POCl_3$ (0.755 mL, 8.05 mmol, 5 equiv), and $PCl_5$ (0.34 g, 1.61 mmol, 1 equiv) was refluxed for 1 h at 105 °C. The reaction mixture was allowed to cool to room temperature and slowly poured on to ice (200 mL) with vigorous stirring to give an oil which gradually solidified upon standing. The product, 2-amino-4-chloro-5-benzyl-3-heptoxy-6-methylpyrimidine, was isolated by filtration as a light yellow powder (0.52 g, 81.25%): $^1$H NMR (300 MHz, MeOH-$d_4$) $\delta$ 0.8 (3H, t, $J$ = 6.5 Hz, C$H_3$), 1.1 (3H, s, C$H_3$), 1.2−1.5 (8H, m, C$H_2$), 1.7 (2H, quintet, $J$ = 6.6 Hz, OCH$_2$C$H_2$), 3.8 (2H, t, $J$ = 6.4 Hz, OC$H_2$CH$_2$), 4.0 (2H, s, C$H_2$-Ph), 6.6 (3H, m, Ar-*H*), 7.1 (1H, t, $J$ = 7.8 Hz, Ar-*H*); $^{13}$C NMR (75 MHz, MeOH-$d_4$) $\delta$ 13.4 (*C*H$_3$), 17.3 (*C*H$_3$), 24.2 (*C*H$_2$), 26.1 (*C*H$_2$), 28.9 (*C*H$_2$), 29.2 (*C*H$_2$), 29.3 (*C*H$_2$), 29.8 (*C*H$_2$), 67.9 (O*C*H$_2$), 112.5, 114.5, 119.8, 122.6, 129.6, 129.8, 159.4, 160.8, 162.0, 163.8; MS (ES, +ve) *m/e* 348.0 (M + $H^+$, 100%), 350.0 (M + 2 + $H^+$), HRMS calcd for $C_{19}H_{27}ClN_3O$ (M + $H^+$) 348.1843, found 348.1842.

$NH_3$ (g) was bubbled into a solution of 2-amino-4-chloro-5-benzyl-3-heptoxy-6-methylpyrimidine (0.643 g, 1.96 mmol) in absolute EtOH (100 mL) and cooled to 0 °C for 15 min. The reaction mixture was heated to 150 °C for 24 h under an atmosphere of nitrogen. The reaction mixture was allowed to cool and reduced in vacuo. The crude product was purified by flash column chromatography on silica gel using an elution of 5% MeOH in EtOAc and yielded the title product **18** (0.190 g, 33%): TLC $R_f$ 0.25 (10% MeOH in EtOAc); $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 0.8 (3H, t, $J$ = 6.6 Hz, C$H_3$), 1.1−1.6 (8H, m, C$H_2$), 1.7 (2H, quintet, $J$ = 6.5 Hz, OCH$_2$C$H_2$), 2.0 (3H, s, C$H_3$), 3.7 (2H, s, C$H_2$Ph), 3.8 (2H, t, $J$ = 6.7 Hz, OC$H_2$), 5.6 (2H, s, N$H_2$), 6.0 (2H, s, N$H_2$), 6.7 (3H, m, Ar-*H*), 7.1 (1H, t, $J$ = 8.1 Hz, Ar-*H*); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 14.7 (*C*H$_3$), 22.1 (*C*H$_2$), 22.8 (*C*H$_2$), 26.3 (*C*H$_2$), 29.2 (*C*H$_2$), 29.5 (*C*H$_2$), 31.1 (*C*H$_3$), 32.0 (Ph*C*H$_2$), 67.9 (O*C*H$_2$), 103.1, 112.0, 115.1, 120.7, 129.9, 142.9, 159.4, 160.9, 163.8, 163.9; $\upsilon_{max}$ (KBr) 3334 and 3152 (N$H_2$), 2926 (aromatic C−H), 2872 (aliphatic C−H), 1614 (C=C) cm$^{-1}$; MS (ES +ve) *m/e* 329 (M + $H^+$, 100%). Anal. ($C_{19}$ $H_{28}N_4O$) C, H, N.

**2,4-Diamino-5-benzyl-3-heptoxy-6-ethylpyrimidine (19).** A solution of **34** (0.5 g, 1.45 mmol, 1 equiv), $POCl_3$ (0.681 mL, 7.3 mmol, 4 equiv), and $PCl_5$ (0.3 g, 1.45 mmol, 1 equiv) was heated to refluxed for 1 h. The reaction mixture was allowed to cool to room temperature and slowly poured on to ice (200 mL) with vigorous stirring to give an oil which gradually solidified upon standing. The product, 2-amino-4-chloro-5-benzyl-3-heptoxy-6-ethylpyrimidine, was isolated by filtration as a white powder (0.5 g, 95%): $^1$H NMR (300 MHz, MeOH-$d_4$) $\delta$ 0.8 (3H, t, $J$ = 6.5 Hz, C$H_3$), 1.1 (3H, t, $J$ = 7.4 Hz, C$H_3$), 1.2−1.5 (8H, m, C$H_2$), 1.7 (2H, quintet, $J$ = 6.5 Hz, OCH$_2$C$H_2$),

2.8 (2H, q, $J = 7.5$ Hz, $CH_2CH_3$), 3.8 (2H, t, $J = 6.4$ Hz, $OCH_2$-$CH_2$), 4.1 (2H, s, $CH_2Ar$), 6.7 (3H, m, Ar-*H*), 7.1 (1H, t, $J = 6.9$ Hz, Ar-*H*); $^{13}$C NMR (75 MHz, MeOH-$d_4$) $\delta$ 8.4 ($CH_3$), 11.5 ($CH_3$), 20.7 ($CH_2$), 23.1 ($CH_2$), 24.4 ($CH_2$), 27.4 ($CH_2$), 30.0 ($CH_2$), 30.6 ($CH_2$), 65.9 ($OCH_2$), 110.3, 112.5, 113.1, 115.6, 136.9, 137.1, 151.8, 158.0, 161.8, 164.3; MS (ES, +ve) *m/e* 362.10 ([M + H]$^+$), 364 ([M + 2 + H]$^+$); HRMS calcd for $C_{20}H_{28}ClN_3O$ ($M^+$) 361.1921, found 361.1921.

$NH_3$ (g) was bubbled into a solution of 2-amino-4-chloro-5-benzyl-3-heptoxy-6-ethylpyrimidine (0.5 g, 1.38 mmol, 1 equiv) in absolute EtOH (100 mL) and cooled to 0 °C for 15 min. The reaction mixture was heated to 150 °C for 24 h under an atmosphere of nitrogen. The reaction mixture was allowed to cool and reduced in vacuo. The crude product was purified by flash column chromatography on silica gel using an elution of 5% MeOH in EtOAc and yielded the title product **19** as a white solid (0.123 g, 29%): TLC $R_f$ 0.22 (10% MeOH in EtOAc); $^1$H NMR (300 MHz, MeOH-$d_4$) $\delta$ 0.9 (3H, t, $J = 6.5$ Hz, $CH_3$), 1.1 (3H, t, $J = 7.4$ Hz, $CH_3$), 1.3−1.5 (8H, m, $CH_2$), 1.8 (2H, quintet, $J = 6.5$ Hz, $OCH_2CH_2$), 2.5 (2H, q, $J = 7.5$ Hz, $CH_2$-$CH_3$), 3.8 (2H, s, $CH_2Ar$), 3.9 (2H, t, $J = 6.6$ Hz, $OCH_2CH_2$), 6.9 (3H, m, Ar-*H*), 7.2 (1H, t, $J = 8.1$ Hz, Ar-*H*); $^{13}$C NMR (75 MHz, MeOH-$d_4$) $\delta$ 12.5 ($CH_3$), 13.4 ($CH_3$), 22.6 ($CH_2$), 23.0 ($CH_2$), 25.9 ($CH_2$), 26.1 ($CH_2$), 29.2 ($CH_2$), 29.9 ($CH_2$), 31.9 ($CH_2$), 67.8 ($OCH_2$), 103.3, 112.2, 114.1, 119.9, 122.6, 129.6, 140.6, 159.4, 160.7, 164.0; $\upsilon_{max}$ (KBr) 3415 and 3314 ($NH_2$), 2946 (aromatic C-H), 2887 (aliphatic C−H), 1602 (C=C) cm$^{-1}$; MS (ES, +ve) *m/e* 343.0 (M + H$^+$, 100%); HRMS calcd for ($C_{20}H_{31}N_4O$) (M + H$^+$) 343.2498, found 343.2498.

**Modeling.** Modeling was performed on Silicon Graphics O2 and Indigo 2 workstations using MACROMODEL 4.5 and 6.0. Conformational searching was carried out on the minimized structure using the Monte Carlo method as documented in the Macromodel software package, using the AMBER force field. For the Monte Carlo calculations, only the inhibitor and a shell of residues surrounding the active site were subject to minimization. Residues within 8 Å of the inhibitor were selected. The initial inhibitor (5-benzyl-2,4-diaminopyrimidine) was unconstrained during the conformational search. All active site polar hydroxyl groups and amino hydrogens were also unconstrained to allow reorientation during the conformational search. All other atoms of the active site were constrained as follows: 0−2 Å from inhibitor, 100 kJ mol$^{-1}$ Å$^{-1}$; 3−5 Å from inhibitor, 200 kJ mol$^{-1}$ Å$^{-1}$; 6−8 Å from inhibitor, 400 kJ mol$^{-1}$ Å$^{-1}$; 1000 steps of the conformational search were undertaken.

A methoxy group was then introduced into the 3-position of the phenyl ring to give compound **3**. This was again subject to Monte Carlo simulation with only the new bonds allowed to rotate. Subsequently addition of extra methylene groups was added to generate compounds **4**−**12**. Each time conformational searching was carried out, allowing only the new bond to rotate.

**Enzyme Assays.** Recombinant DHFR from *L. major*, *T. cruzi*, and *T. brucei* was purified according to the method of Meek and co-workers.[13] Human DHFR was a gift of Dr. R. Ridley, Roche Pharmaceuticals.

The standard DHFR assay[1] was carried out at 28 °C by measuring the decrease in absorbance due to NADPH at 340 nm in a Hewlett-Packard model 8543 spectrophotometer. Standard reactant concentrations were for *L. major* DHFR: enzyme 2.9 units (2.7 nM), dihydrofolate DHF 20 $\mu$M, NADPH 100 $\mu$M, bovine serum albumin (BSA) (1 mg/mL), variable inhibitor concentration (0.4−2.4 $\mu$M); for *T.cruzi* DHFR: enzyme 3.5 units (1.8 nM), DHF 30 $\mu$M, NADPH 100 $\mu$M, BSA (1 mg/mL), variable inhibitor concentration (0.4−2.4 $\mu$M); for *T. brucei* DHFR: enzyme 3.5 units (5.9 nM), DHF 25 $\mu$M, NADPH 100 $\mu$M, BSA (1 mg/mL), variable inhibitor concentration (0.4−2.4 $\mu$M); for human DHFR: enzyme 5 units (0.01 mg/mL), DHF 38 $\mu$M, NADPH 100 $\mu$M, BSA (1 mg/mL), variable inhibitor concentration (5−60 $\mu$M). Typically eight different concentrations of inhibitor were used. In each case a control was carried out which contained all reagents except the inhibitor. The DHFR assay was performed by adding the above-mentioned reagents to a quartz cuvette with 500 $\mu$L of 2×TES buffer, pH 7.0 (100 mM TES, 150 mM $\beta$-mercaptoethanol, 2 mM EDTA). DHF was prepared from folic acid by reduction with sodium dithionite according to the method of Fulterman.[14] Reaction mixtures containing enzyme plus all standard assay components except the initiating substrate were first monitored to determine any background activity. Reactions were then initiated by adding the measured amount of substrate and were assayed by measuring the rate in decrease of NADPH absorbance at 340 nm.

To establish that the inhibition was competitive, compounds **4**, **10**, **14**, and **16** were assayed at four substrate and three to five inhibitor concentrations against the *T. cruzi* DHFR and compounds **4** and **10** were similarly assayed against the human DHFR. Compounds **4**, **10**, **14**, and **16** were selected on the basis of being representative compounds. Lineweaver−Burk plots established that inhibition was competitive.

$K_i$ values were calculated from Dixon plots. The Dixon plot uses the following equation. Essentially the reciprocal of the reaction velocity is plotted against the concentration of the inhibitor, and the value of $K_i$ is determined from the slope of the plot and the intercept on the *y*-axis. No extrapolations were required for calculating $K_i$.

$$\frac{1}{v} = \frac{K_m}{k_i[S]V_{max}}[I] + \frac{1}{V_{max}}\left(1 + \frac{K_m}{[S]}\right)$$

where $v$ = reaction velocity, $V_{max}$ = limiting value of the velocity of the reaction obtained at high substrate concentrations, $K_m$ = Michaelis constant, [S] = substrate concentration (held constant), [I] = inhibitor concentration, $K_i$ = inhibition constant. One unit of enzyme is the amount of enzyme required to produce 1 nmol of NADP in 1 min at 28 °C. The following $K_m$ values were used (as reported in the literature and as supplied by Roche): *L. major* DHFR, 1.3 $\mu$M; *T. cruzi* DHFR, 1.2 $\mu$M; *T. brucei* DHFR, 0.6 $\mu$M; human DHFR, 0.72 $\mu$M.

**In Vitro Assays. *T. cruzi*:** Primary mouse peritoneal macrophages were seeded in 96 well microplates at 30 000 cells/well. After 24 h, about 100 000 trypomastigotes of *T. cruzi* (strain Tulaheun CL2) were added per well together with 2-fold dilutions of the drug. The cultures were incubated at 37 °C in 5% $CO_2$ for 4 days. To cope with the problem of aspecific toxicity, a cytotoxicity assay on MRC-5 cells or uninfected macrophage cultures were treated likewise to determine a selectivity index. Drug activity was semiquantitatively scored as percentage reduction of the total parasite load (free trypomastigotes and intracellular amastigotes) compared to untreated control cultures. Scoring was performed microscopically, and $ED_{50}$ values were extrapolated.

***L. infantum*:** Primary mouse peritoneal macrophages were seeded in 96-well microplates at 30 000 cells/well. After 24 h, amastigotes of *L. infantum* (derived from the spleen of an infected donor animal) were added at an infection ratio of 10/1 together with 2-fold dilutions of the drug. The cultures was incubated in 5% $CO_2$ for 7 days. Treatment of uninfected control cultures was also included to determine a selectivity index. Drug activity is semiquantitatively scored as percentage reduction of the total parasite load in Wright-stained preparations. Scoring was performed microscopically, and $ED_{50}$ values were extrapolated.

***T. brucei rhodesiense*:** MEM medium (50 $\mu$L), according to Baltz et al.,[15] with 2-mercaptoethanol and 10% inactivated horse serum was added to each well of a Costar 96-well microtiter plate. Serial drug dilutions were added to the wells. Then 50 $\mu$L of trypanosome (*T. brucei rhodesiense* STIB 900) suspension was added to each well and the plate incubated at 37 °C under a 5% $CO_2$ atmosphere for 72 h. Alamar Blue (10 $\mu$L) was then added to each well and incubation continued for a further 2 h. The plate was then read with a Millipore Cytofluor 2300 using an excitation wavelength of 530 nm and emission wavelength of 590 nm.[16] Fluorescence development was expressed as percentage of the control, and $IC_{50}$ values were determined.

**In Vivo Assays.** CD1 mice were dosed intraperitoneally with 0.2 mL of an inoculum of $2.5 \times 10^5$ trypomastigotes of *T. brucei* S427 on day 0. Drugging was commenced on day 1 and continued daily until and including day 4. There were 5 mice in each group. The mean survival time was calculated for each group. Controls were dosed with the inoculum medium (celacol) or melarsoprol.

**Acknowledgment.** We thank the British Council and Overseas Development Agency and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases for financial support (R.B., S.F.C., S.L.C., L.M.); J. Johal and C. Y. Nang for technical assistance; Prof. Peter Edwards of the Department of Chemistry, Cardiff University, for access to his high-pressure facilities; Dr. Robert Ridley of Roche Pharmaceuticals for a supply of recombinant human DHFR; the Daresbury facility of the EPSRC for access to the United Kingdom Chemical Database Service; and the EPSRC National Mass Spectrometry Service Centre, Swansea.

## References

(1) Sirawaraporn, W.; Sertsrivanich, R.; Booth, R. G.; Hansch, C.; Neal, R. A.; Santi, D. V. Selective Inhibition of *Leishmania* dihydrofolate reductase and *Leishmania* growth by 5-benzyl-2,4-diamionopyrimidines. *Mol. Biochem. Parasitol.* **1988**, *31*, 79−86.

(2) Jaffe, J. J.; McCormack, J. J. Dihydrofolate Reductase from Trypanosoma equiperdum 1.Isolation, Partial Purification, and Properties. *Mol. Pharmacol.* **1967**, *3*, 359. Ferone, *J. Mol. Pharmacol.* **1969**, *5*, 49.

(3) Scott, D. A.; Coombs, G. H.; Sanderson, B. E. Effects of methotrexate and other anti-folates on the growth and dihydrofolate reductase activity of *Leishmania* promastigotes. *Biochem. Pharmacol.* **1987**, *36*, 2043−2045.

(4) Berman, J. D.; King, M.; Edwards, N. Antileishmanial activities of 2,4-diaminoquinazolines putative dihydrofolate reductase inhibitors. *Antimicrob. Agents Chemother.* **1989**, *33*, 1860−1863.

(5) Booth, R. G.; Selassie, C. D.; Hansch, C.; Santi, D. V. Quantitative Structure−Activity Relationship of Triazine-Antifolate Inhibition of Leishmanial Dihydrofolate Reductase and Cell Growth. *J. Med. Chem.* **1987**, *30*, 1218−1224. Hathaway, B. A.; Guo, Z.; Hansch, C.; Delcamp, T. J.; Susten, S. S.; Freisheim, J. H. Inhibition of Human Dihydrofolate Reductase by 4,6-Diamino-1,2-dihydrol-2,2-dimethyl-1-(substituted-phenyl)-s-triazines. A Quantitative Structure−Activity Relationship Analysis. *J. Med. Chem.* **1984**, *27*, 144−149.

(6) Hardy, L. W.; Matthews, W.; Nare, B.; Beverley, S. M. Biochemical and Genetic Tests for inhibitors of *Leishmania* Pteridine Pathways. *Exp. Parasitol.* **1997**, *87*, 157−169.

(7) Zuccotto, F.; Martin, A. C. R.; Laskowski, J. M.; Thornton, J. M.; Gilbert, I. H. Dihydrofolate Reductase: a Potential Drug Target in Trypanosomes and *Leishmania*. *J. Computer-Aided Mol. Des.* **1998**, *12*, 241−257

(8) Fletcher, D. A.; McMeeking, R. F.; Parkin, D. The United Kingdom Chemical Database Service. *J. Chem. Inf. Comput. Sci.* **1996**, *36*, 746−749.

(9) Knighton, D. R.; Kan, C.-C.; Howland, E.; Janson, C. A.; Hostomska, Z.; Welsh, K. M.; Matthews, D. A. Structure of and kinetic channelling in bifunctional dihydrofolate reductase-thymidylate synthase. *Nature Struct. Biol.* **1994**, *1*, 186−194.

(10) Stenbuck, P.; Baltzly, R.; Hood, H. M.; A New Synthesis of 5-Benzylpyrimidines. *J. Med. Chem.* **1963**, *28*, 1983−1985.

(11) Macromodel, Columbia University.

(12) Robello, C.; Navarro, P.; Castanys, S.; Gamarro, F. A pteridine reductase gene ptr1 contiguous to a P-glycoprotein confers resistance to antifolates in *Trypanosoma cruzi*. *Mol. Biochem. Parasitol.* **1997**, *90*, 525−535.

(13) Meek, T. D.; Garvey, E. P.; Santi, D. Purification and Characterisation of the Bifunctional Thymidyate Synthetase − Dihydrofolate Reductase from Methotrexate-Resistant Leishmania tropica. *Biochemistry* **1985**, *24*, 678−686.

(14) Futterman, S. Enzymatic reduction of folic acid and dihydrofolic acid to tetrahydrofolic acid. *J. Biol. Chem.* **1957**, *28*, 1031−1039.

(15) Baltz, T.; Baltz, D.; Giroud, C.; Crockett, J. Cultivation in a semidefined medium of animal infective forms of *Trypanosoma brucei*, *T. equiperdum*, *T. evansi*, *T. rhodesiense* and *T. gambiense*. *EMBO J.* **1985**, *4*, 1273−1277.

(16) Raz, B.; Iten, M.; Grether-Buhler, Y.; Kaminsky, R.; Brun, B. The Alamar Blue assay to determine drug sensitivity of African trypanosomes (*T. b. rhodesiense* and *T. b. gambiense*) in vitro. *Acta Tropica* **1997**, *68*, 139−147.

JM981130+