Azasterols as Inhibitors of Sterol 24-Methyltransferase in *Leishmania* Species and *Trypanosoma cruzi*

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This paper describes the synthesis of some novel azasterols based on (20*R*,22ξ)-5α-pregnan-20-(piperidin-2-yl)-3β,20-diol. These compounds are potential inhibitors of the enzyme sterol 24-methyltransferase (24-SMT), which is a vital enzyme in the biosynthesis of ergosterol and related 24-alkyl sterols. Structure−activity studies were undertaken to understand the important features for activity against the enzyme, with the aim of increasing activity and selectivity. The compounds were evaluated for inhibition of recombinant *Leishmania major* 24-SMT and the effect of compounds on sterol composition and parasite proliferation. Essentially, compounds which showed good activity against the recombinant enzyme had a significant effect on the sterol composition and growth of parasites. The activity of compounds was found to be related to the basicity and stereochemical location of the nitrogen. Also, presence of an unprotected 3β-OH seemed to be important for activity. However, some azasterols which were not good inhibitors of 24-SMT also showed antiproliferative activity, suggesting that there may be other modes of actions of these compounds.

Introduction

The parasites that cause leishmaniasis and Chagas's disease require ergosterol and related 24-alkylated sterols for survival and growth as opposed to vertebrate cells, which require cholesterol. The structural differences between ergosterol and cholesterol are an additional alkyl group at the 24-position and unsubstitution at positions 5 and 7 (Figure 1). Inhibitors of various steps of steroid biosynthesis have been developed both as antifungal drugs and for treatment for raised cholesterol levels.

It has been shown that inhibitors of steroid biosynthesis have potential for the treatment of both Chagas's disease and leishmaniasis.[1] We were particularly interested in developing inhibitors of 24-sterol methyltranferase (24-SMT), an enzyme that methenylates the steroid at the 24 position during ergosterol biosynthesis. There is no comparable enzyme in the biosynthesis of cholesterol, and thus inhibition of this enzyme should be selective for ergosterol biosynthesis and hence the parasites. Studies on the mechanism of action of 24-SMT have been reviewed by Nes.[2]

Background

The starting point for our synthetic program was the azasterol **1** (Figure 1). This compound has been shown to have activity against *T. cruzi* (the causative organism of Chagas's disease)[3−5] and various species of *Leishmania* (the causative organism of leishmaniasis).[6,7] It has been shown to inhibit incorporation of ^{14}C-labeled *S*-adenosylmethionine into sterols in *T. cruzi* epimastigotes (the vector stage) with an IC_{50} of about 640 nM and to cause a reduction in levels of 24-alkylated sterols.[4] The compound appears to inhibit both the $\Delta^{24(25)}$ sterol methyltransferase (the enzyme discussed here) and the $\Delta^{24(24')}$ sterol methyltransferase (which catalyzes methenylation of fecosterol type sterols, in-

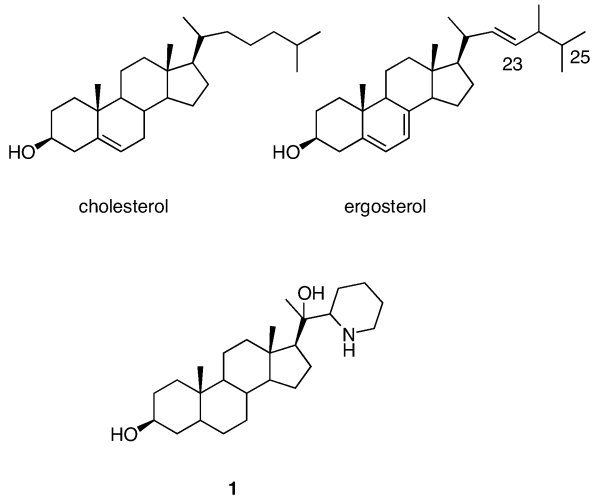

Figure 1. Structures of cholesterol and ergosterol.

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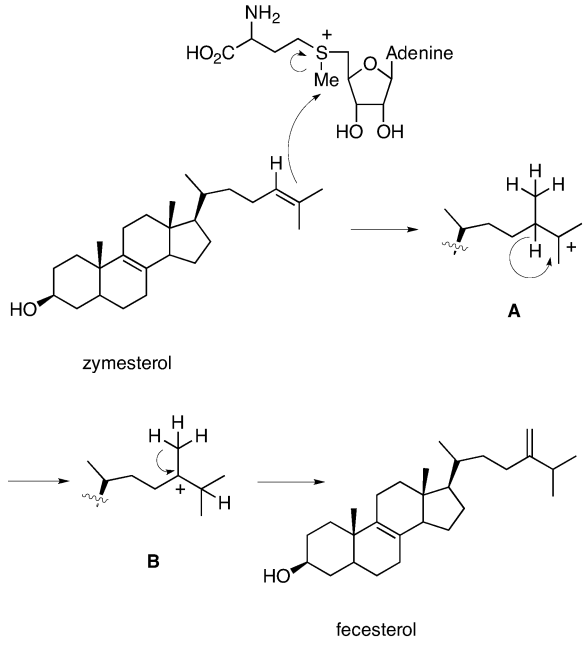
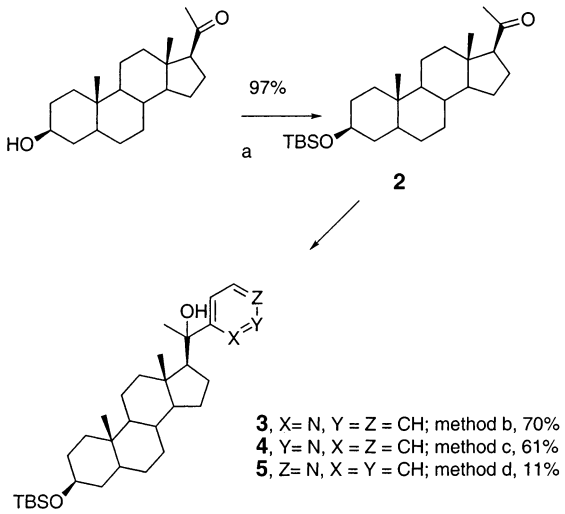
Scheme 1. Proposed Mechanism of 24-SMT



troducing an ethyl group at the 24-position).[4] This azasterol displayed antiproliferative activity in vitro against both the extracellular epimastigotes (complete growth inhibition at 10 μM) and the clinically relevant intracellular amastigotes forms cultured in Vero cells (parasites eradicated at 100 nM).[5] In addition, **1** was effective in a rodent model of Chagas's disease, increasing survival and reducing parasitemia of infected mice by 90%.[5] In a similar fashion, *L. donovani* promastigotes, were inhibited by compound **1**, with an IC_{50} of 12 μM, followed by cytotoxic effects at higher concentrations. At lower concentrations, a reduction in percentage of 24-alkylated sterols has been observed, commensurate with inhibition of 24-SMT.[7] In a recent study,[6] we showed that this compound was active against *L. amazonensis* promastigotes (IC_{50} = 0.1 μM) and that axenic amastigotes were more sensitive to inhibition than promastigotes. Steroid analysis of the promastigotes again showed a marked reduction in 24-alkylated sterols, which correlated with growth inhibition. Furthermore, we showed that morphological changes occurred in both promastigotes and axenic amastigotes. Initially changes were observed in the mitochondrion which exhibited intense swelling and several alterations in the inner mitochondrial membrane. These alterations were followed by changes in the flagellar pocket, increase in the number of acidocalcisomes and megasomes, increase in the number of lipid inclusions, and appearance of myelin-like figures in the cytoplasm.[6] Similar ultrastructural effects were observed in *T. cruzi*.[3]

In compound **1**, the nitrogen atom is found at the equivalent of the 23-position of the side chain. These compounds are probably not in the optimal position to mimic the high-energy intermediates, **A** and **B** (Scheme 1). Therefore, we decided to prepare analogues in which the nitrogen is located at different positions around the piperidine ring, which should correspond to the high energy intermediates with the nitrogen atom located at position 24 (intermediate **B**) or at position 25 (intermediate **A**). We also wanted to investigate the effect of having a pyridine ring instead of a piperidine ring and the effect of substitution on the 3β-OH. The aim of these studies was to design more active and selective compounds.

Scheme 2[a]



[a] Reagents and conditions: (a) TBSCl, imidazole, DMF; (b) 2-bromopyridine, BuLi, Et_2O, -78°C; (c) 3-bromopyridine, BuLi, Et_20, -78 °C; (d) 4-bromopyridine hydrochloride, BuLi, Et_2O, -78 °C.

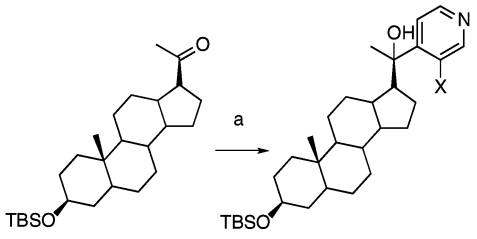
Chemistry

The azasterols were prepared as follows (Scheme 2). The commercially available 5α-pregnan-3β-ol-20-one was protected at the 3-position with the *tert*-butyldimethylsilyl protecting group. This was then reacted with a carbanion, generated from either 2-, 3-, or 4-bromopyridine and butyllithium. This led to production of the required intermediate pyridyl adducts (**3**−**5**). These intermediates are mildly basic, and may mimic the carbocationic high energy intermediates **A** and **B**. Reactions of both 2-bromopyridine and 3-bromopyridine gave the products (**3** and **4**) in good yields (70% and 61%, respectively). However, in the case of 4-bromopyridine, there was a much lower yield of product **5** (11%). This is probably due to the instability of both the 4-bromopyridine and the resultant carbanion. 4-Bromopyridine was supplied as a hydrochloride salt. Attempts to generate the free base by partitioning between an aqueous base and organic solvent led to a product that decomposed rapidly. Use of 2 equiv of strong base (BuLi) with the hydrochloride salt to generate the 4-lithiated pyridine also led to these low yields. While we cannot be certain of the stereochemistry of the condensation reaction, only one diastereomer was produced. According to the Felkin−Anh model, this is most likely to be the *R* stereochemistry at the 20-position.

To circumvent the problems of the 4-bromopyridine, an alternate strategy was adopted. It is noted that treatment of 3-chloropyridine or 3-fluoropyridine with LDA generated the 4-lithiated species. This was successfully done for both 3-chloro- and 3-fluoropyridine, and the resultant carbanion condensed with the silylated starting material (**2**) to give required pyridines **6** and **7** in 31% and 65% yield, respectively (Scheme 3).

According to Nes,[2] the presence of a 3β-hydroxy group is essential for activity against 24-SMT. Therefore, the

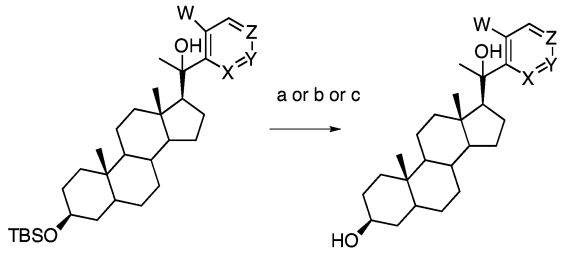
Scheme 3[a]



2 **6,** X = Cl, 31%
7, X = F, 65%

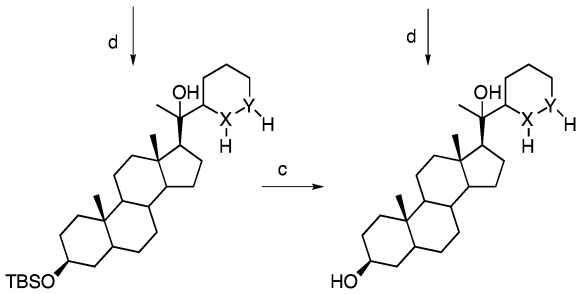
[a] Reagents and conditions: (a) 3-chloropyridine or 3-fluoropyridine, LDA, THF, −78 °C.

Scheme 4[a]



3, W=H, X=N, Y=CH, Z=CH
4, W=H, X=CH, Y=N, Z=CH
5, W=H, X=CH, Y=CH, Z=N
6, W=Cl, X=CH, Y=CH, Z=N

8, W=H, X=N, Y=CH, Z=CH
9, W=H, X=CH, Y=N, Z=CH, 35% (method a)
10, W=H, X=CH, Y=CH, Z=N 65% (method b)
11, W=Cl, X=CH, Y=CH, Z=N 78% (method c)



12, X=N, Y=CH, 88%
13, X=CH, Y=N, 89%

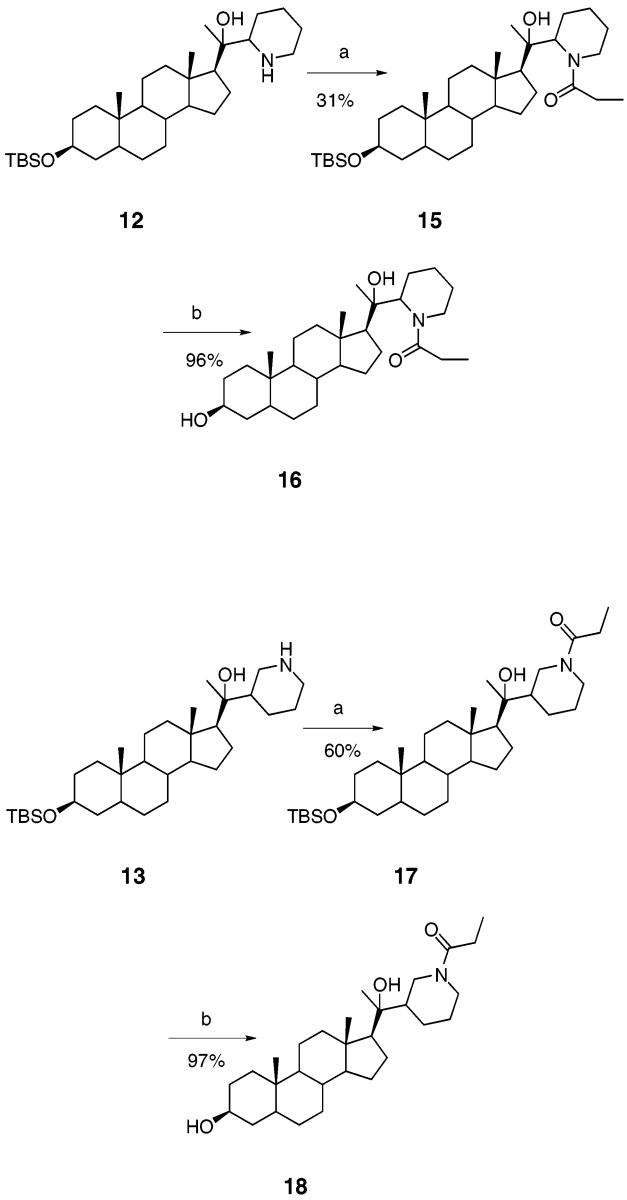
1, X=N, Y=CH, 75%, method d
14, X=CH, Y=N, 34%, method c

[a] Reagents and conditions: (a) AcOH:THF:water (3:1:1), 75 °C; (b) Dowex H^+, MeOH, $CHCl_3$; (c) HCl, MeOH; (d) H_2, PtO_2, AcOH.

silyl protecting group was removed from the pyridine intermediates. This was achieved by the use of acetic acid in THF/water (to give compound **9**), or cationic resins (to give compound **10**), or hydrochloric acid in methanol (to give compound **11**) (Scheme 4). Compound **8**, the 2-pyridyl derivative, was actually formed in 68% yield by condensing 3 equiv of anion generated from 2-bromopyridine and butyllithium with the commercially available 3β-yl-5α-pregnan-20-one acetate.

The next stage was to hydrogenate the pyridines to give the corresponding piperidines. The piperidines should be more basic than the pyridines, and hence a higher proportion should be protonated at physiological pH. The silylated pyridine compound **3** was reduced using hydrogen in the presence of platinum dioxide to give the required piperidine **12** in 88% without loss of

Scheme 5[a]



[a] Reagents and conditions: (a) $CH_3CH_2CO_2H$, DIC, HOBt, THF; (b) HCl, MeOH.

the silyl group. The reaction appeared to be highly diastereoselective, with only one compound being detected. The high degree of selectivity could be attributed to an intramolecular hydrogen bond between the nitrogen (which is probably protonated under the reaction conditions) and the tertiary hydroxyl group at position 20. Similarly, hydrogenation of compound **4** led to compound **13**. Unfortunately, the 3-chloro (**6**) and 3-fluoro (**7**) derivatives were too insoluble in solvents commonly used for hydrogenation to undergo hydrogenation.

Hydrogenation was also carried out on the corresponding compounds with the 3β-OH group deprotected. Thus, the lead compound **1** was produced in 75% yield. The analogue with the nitrogen in the 24-position, **14**, was produced in 34% yield (Scheme 4) by deprotection of the silyl derivative **13**.

In addition to investigation of the role of the amino group, capping with an amide was accomplished (Scheme 5). This was achieved for compounds **12** and **13** as

Table 1. In Vitro Activity (IC_{50}, μM) of Azasterols against *L. major* 24- SMT, *L. amazonensis*, *L. donovani*, *T. cruzi*, and Vertebrate Cells[a]

no.	recombinant L. major 24-SMT	*L. amazonesis* promastigotes	*L. amazonesis* intracellular amastigotes	*L. donovani* intracellular amastigotes	*T. cruzi* epimastigotes	*T. cruzi* intracellular amastigotes	toxicity to KB cells
1	0.028 ± 0.009	0.10	0.01	8.9	0.94	7.4	11.9
3	>100	1.88	1.0	>59	0.68	>59	>586
4	>100	1.75	1.5	13.9	1.2	51.2	>586
5	>100	3.21	2.0	15.0	0.67	6.1	574
6	>100			>55		>55	234
8	N.D.	1.14	1.0	14.6	1.03	32.9	11.3
9	38.54 ± 6.19	4.24	3.0	23.4	0.62	13.3	>755
10	>100	3.41	3.0	11.3	0.68	35.0	30.7
11	>100			>69		50.0	<0.7
12	12.2 ± 4.8	1.09	0.02	>58	0.6	50.0	>579
13	8 ± 1.05	1.40	1.0	6.4	1.01	42.1	2.5
14	0.10 ± 0.1			4.8		18	29.2
15	>100	10.28	8.0	>52	1.0	>52	>520
16	12.87 ± 5.9			23.1		22.8	11.1
17	>100	1.21	1.0	>52	0.6	>52	>520
18	54 ± 5.5			37.9		64.6	336

[a] ED_{50} values of standard control drugs: *Leishmania* spp. Sodium stibogluconate (Sb^V) 16.6 μg Sb^V/mL; *T. cruzi*, benznidazole 5.3 μg/mL; toxicity, podophyllotoxin 0.0017 μg/mL. N.D. = not determined.

follows. Acylation was carried out using diispropylcarbodiimide, hydrobenzotriazole, and proprionic acid. With compound **12** the reaction was much slower than with compound **13**, presumably due to steric hindrance. The silyl protecting group was removed from the 3-position using hydrochloric acid and methanol, to give compounds **16** and **18**.

Biology

Enzyme Assays. A number of assays were carried out with the synthesized compounds. The 24-SMT gene from *L. major* was cloned in pET28 and overexpressed in *E. coli* BL21(DE3)pLysS cells (D. Gonzalez Pacanowska et al. unpublished data). The enzyme was expressed at low levels; however, it was possible to conduct enzyme assays using *E. coli* cell-free extracts containing soluble protein as the enzyme source. The *L. major* enzyme was chosen for cloning, as this is a commonly cloned species of *Leishmania*, and there is generally very high sequence homology for the enzymes from different species of *Leishmania*.

The IC_{50} values are presented in Table 1. The lead azasterol, compound **1**, showed the greatest activity with an IC_{50} of 0.028 μM. Similarly the "24" analogue, **14**, showed good inhibition of the enzyme (IC_{50} = 0.1 μM). Compounds **3**−**6**, in which the 3β-OH is blocked as a silyl ether and there is a pyridine ring, showed no inhibition of the enzyme at 100 μM. This is in line with Nes's model,[2] in which the 3β-OH is involved in a hydrogen bond in the active site. Removal of the 3β-OH blocking group gave increased inhibition of the enzyme (compound **9** compared to compound **4**). According to Nes, the 3β-OH can now interact with the enzyme active site. However the 25-analogue (compound **10**, IC_{50} >100 μM)) showed no measurable inhibition of the enzyme. Presumably, the rigidity of the molecule places the nitrogen in a position in which it does not interact strongly with residues in the enzyme active site. Reduction of compound **9** (IC_{50} = 38.5 μM) to give the fully saturated analogue **14** (IC_{50} = 0.10 μM) caused a marked increase in inhibitory activity. The nitrogen now is more basic and hence has a higher degree of protonation and stronger interaction with the enzyme.

Interestingly, compounds **12** and **13**, in which the 3-OH group is blocked, showed moderate activity against the enzyme (IC_{50} of 12.2 and 8 μM, respectively). The blockage of the 3β-OH position may in part be compensated by the stronger interaction of the charged aza group in the piperidine compounds compared to the pyridine derivatives **3** and **4**.

Compounds **16** and **18**, which have the nitrogen blocked as an amide, gave moderate inhibition of the enzymes (IC_{50} 12.9 μM and 54 μM, respectively). While the amide group will prevent the amine being protonated, the amide will have a small partial positive charge on the nitrogen due to delocalization of the amide bond, which could account for some residual activity, although there could be other explanations for this residual activity. Blocking the 3β-OH of these compounds to give **15** and **17** led to a loss of activity.

Sterol Composition. Sterols of *L. mexicana amazonensis* promastigotes incubated in the presence or absence of the experimental compounds were analyzed to see their effect on de novo synthesis by whole cells. These data provided information on the biochemical basis of the antiproliferative effects of the azasterols. A reduction in the proportion of 24-alkylated sterols and a corresponding increase in the proportion of nonalkylated steroids indicated that the compounds were indeed inhibiting sterol 24-methyltransferase. The results from these assays are shown in Table 2. A breakdown of the percentages of 24-alkylated and nonalkylated sterols is given in Table 3.

For the lead compound, **1**, there was a decrease in the proportion of 24-alkylated steroids (ergosterol, 5-dehydroepisterol, and episterol) and an increase in proportion of nonalkylated steroids (cholesta-5,7,24-trien-3β-ol and cholesta-7,24-dien-3β-ol) at concentrations of 0.1 μM and 1.0 μM. This is in line with inhibition of 24-SMT. A similar effect was seen for compound **14**, the "24"-position analogue, which showed an effect of a similar magnitude.

The pyridine analogues, **8**−**11**, were also evaluated. In compound **8**, the nitrogen is in the "23"-position and showed a small effect at 0.1 μM, which increased at 1.0 μM. However, it was a significantly smaller effect than

Table 2. Effects of Inhibitors on the Free Sterol Composition of *Leishmania mexicana amazonensis* Promastigotes: The Percentage Composition of Steroid of the Parasites at Different Concentrations of Inhibitor (0.01, 0.1, 1.0 μM) Is Given

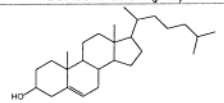
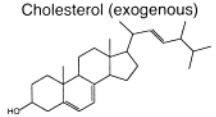
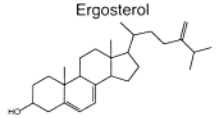
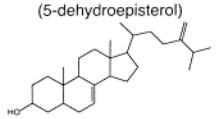
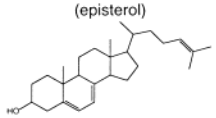
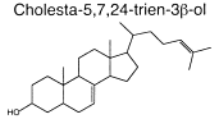
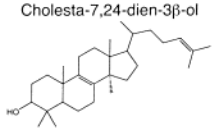
No	1			4		8			9				10		11		12			14			16			18	
Concentration (μM)	0	0.1	1.0	0	1.0	0	0.1	1.0	0	0.01	0.1	1.0	0	1.0	0	1.0	0	0.1	1.0	0	0.1	1.0	0	0.1	1.0	0.1	1.0
 Cholesterol (exogenous)	9.8	11.1	9.7	7.9	7.2	24.4	15.5	17.3	12.1	10.2	12.4	12.4	7.9	7.9	9.8	11.3	10.2	8.5	34.8	9.8	13.1	17.3	9.8	17.7	10.6	11.7	9.7
 Ergosterol	2.8	5.1	3.9	2.5	4.7	4.9	7.9	5.6	2.6	2.8	3.1	2.3	2.5	1.8	2.8	<1	3.2	2.2	<1	2.8	4.0	<1	2.8	<1	<1	<1	<1
 Ergosta-5,7,24(24^1)-trien-3β-ol (5-dehydroepisterol)	76.9	6.0	4.1	81.3	81.3	64.6	57.0	47.6	64.9	64.3	24.3	9.1	81.3	84.0	76.9	56.8	78.8	81.8	12.9	76.9	6.6	11.2	76.9	64.7	29.9	74.2	13.8
 Ergosta-7,24(24^1)-dien-3β-ol (episterol)	10.5	<1	<1	8.3	6.6	12.0	12.6	10.2	10.2	8.5	4.1	<1	8.3	6.3	10.5	17.5	7.8	7.5	2.8	10.5	<1	<1	10.5	13.2	<1	14.1	6.6
 Cholesta-5,7,24-trien-3β-ol	<1	64.2	65.4			<1	1.6	10.2	2.3	4.7	41.9	54.8			<1	5.5	<1	<1	32.3	<1	63.7	57.6	<1	4.4	44.3	<1	49.7
 Cholesta-7,24-dien-3β-ol	<1	13.6	14.1			<1	2.3	6.3	3.2	5.1	11.1	12.2			<1	5.0	<1	<1	17.2	<1	12.6	13.9	<1	<1	14.3	<1	20.2
 Lanosterol						2.7	3.1	2.8	4.7	2.9	3.1	7.4			<1	3.9											

Table 3. Relative Proportions of 24-Alkylated and 24-Nonalkylated Sterols Isolated from *L. mexicana amazonensis* Promastigotes Treated with Azasterols

compound		**1**			**4**			**8**			**9**			**10**		**11**
concentration (μM)	0	0.1	1	0	1	0	0.1	1	0	0.01	0.1	1	0	1	0	1
% 24-alkylated	90.2	11.1	8	92.1	92.6	81.5	77.5	63.4	77.7	75.6	31.5	11.4	92.1	92.1	90.2	74.3
% 24-nonalkylated	0	77.8	79.5	0	0	2.7	7	19.3	10.2	12.7	56.1	74.4	0	0	0	14.4
compound		**12**			**14**			**16**			**18**					
concentration (μM)	0	0.1	1	0	0.1	1	0	0.1	1	0.1	1.0					
% 24-alkylated	89.8	91.5	15.7	90.2	10.6	11.2	90.2	77.9	29.9	88.3	20.4					
% 24-nonalkylated	0	0	49.5	0	76.3	71.5	0	4.4	58.6	0	69.9					

with the reduced lead compound **1**, in line with the effects against the enzyme. The "24"-position analogue, **9**, showed a more significant effect, which was almost comparable to amine analogues **1** and **14**. This suggests that having the carbocation mimic in the "24"-position is more favorable for interaction with the enzyme active site. The "25"-position analogue, **10**, showed little effect on steroid composition, even at 1.0 μM. Although compound **11**, in which the carbocation is in the "25"-position and there is also a chlorine substituent, showed higher activity.

Blocking of the 3β-OH, compound **12**, showed a reduced inhibition of ergosterol biosynthesis compared to the parent compound, **1**, although some activity was still retained. Similarly acylating the nitrogen, compounds **16** and **18**, caused lower activity, compared to **1**. As already discussed, the amides show weaker interaction with 24-SMT, possibly due to only a small partial postive charge on the nitrogen. Finally, compound **4** in which the 3β-OH is blocked, and there is a pyridine in position 23 showed no effects on the sterol composition, which is in line with the results from enzyme assays.

Antiproliferative Activity. The compounds were assayed against *L. amazonesis* promastigotes (Table 1). The lead compound, **1**, showed good activity against the promastigotes with an IC_{50} of 0.1 μM. The remaining compounds assayed showed about 10-fold lower activity. There does not appear to be a direct relationship between activity against the parasite and activity against 24-SMT. This lack of correlation may be due to differences of compound uptake or additional mode(s) of action.

The compounds were also assayed against the intracellular amastigote form of the parasites *L. amazonesis* and *L. donovani* cultured in mouse macrophages. In general they were more active against the *L. amazonesis* model than the *L. donovani* model. In the case of *L. amazonesis*, the activity against the amastigotes mirrored the activity against the promastigotes: compound **1** was the most potent and **15** the least potent. The only exception to this was compound **12**, which is very potent against amastigotes (IC_{50} 0.02 μM) but much less active against the promastigotes (IC_{50} 1.09 μM).

Against the amastigote form of *L. donovani*, the most active compounds were **1**, **13**, and **14**. The higher activities of **1** and **14** were expected on the basis of their effect against the enzyme, sterol composition, and promastigote viability. However, the activity of **13** was surprising owing to its relatively small effect on the enzyme.

Differences in activity of compounds between species of *Leishmania* are probably due to slight differences in protocols, mammalian cells used, and differences between species of *Leishmania*. Indeed azoles, which inhibit ergosterol biosynthesis at different biosynthetic step show variation in activity between different species of *Leishmania*.[8]

Compounds were also evaluated against *T. cruzi*. In the case of the epimastigotes, all of the compounds showed similar activity, with an IC_{50} in the range of 0.6−1.2 μM. However significant variations were observed against the intracellular amastigote stage. The most active compounds were **1** (IC_{50} = 7.4 μM) and **5** (IC_{50} = 6.1 μM), followed by **9** (IC_{50} = 13.3 μM) and **14** (IC_{50} = 18 μM). The activity of **5** was unexpected, as this compound has a blocking group of the 3β-OH group and is a pyridine rather than a piperidine, both factors which tend to reduce activity against 24-SMT. The antiparasitic activity of compound **5** is almost certainly due to action against an alternate molecular target. It is not due to general toxicity, as the compound shows high selectivity with respect to vertebrate (KB) cells. Interestingly, the pyridine **9**, which only slightly reduced 24-alkylation compared to **1** and **14** (Table 2), showed similar activity against *T. cruzi*.

Compounds displayed a variety of toxicities against vertebrate cells. These assays were conducted to give a preliminary indication of the toxicity of the compounds and a measure of where the compounds were acting selectively or through general toxicity.

Ultrastructural Effects. Profound alterations were observed when promastigotes were treated with compound **12**. Figure 2 shows a control parasite presenting a normal morphology. The presence of a mitochondrial profile (M), kinetoplast (K), nucleus (N), flagellar pocket (FP), and flagellum (F) are indicated. The first alteration observed in treated cells was in the inner mitochondrion membrane, which acquired a concentric array, forming complex and elaborated structures (Figures 3 and 4, arrows and arrowheads). Myelin-like figures were also observed in the mitochondrial matrix (data not shown). Other alterations in the mitochondrion include an intense mitochondrial swelling where the matrix became much less electrondense (Figure 5).

Another alteration observed in the parasites treated with compound **12** was the presence of myelin-like figures in the cytoplasm (Figure 6, arrows). We also observed the presence of protrusions of the cell body toward the flagellar pocket (Figure 7, arrows).

Discussion

A series of azasterols was successfully prepared. From the results of the enzyme assays, compounds with an unblocked 3β-OH and an amine in the 23 or 24 position showed the strongest activity (**1** and **14**). Replacement of the amine with a pyridine (**9**, **10**, **11**), converting the amine to an amide (**16, 18**) or blocking the 3β-OH (**12**, **13**) reduced activity against the enzyme. Combinations

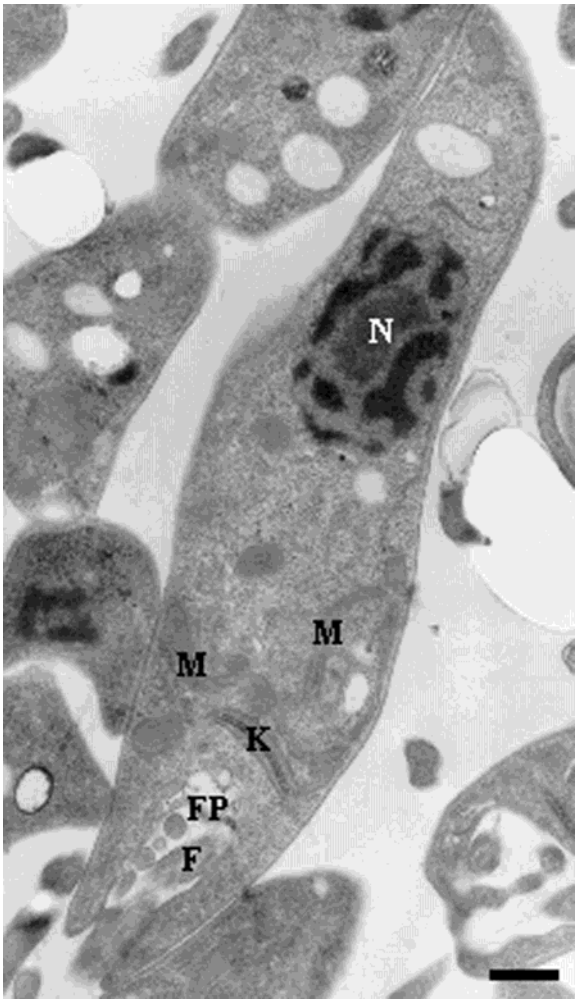


Figure 2. This image shows the morphology of a control promastigote form, where mitochondrion profiles (M), nucleus (N), kinetoplast (K), flagellar pocket (FP), and flagellum (F) can be observed. Bar, 1 μm.

of these factors led to inactive compounds (**3**−**6**, **15**, **17**). This is a slight modification of the Nes model,[2] which implies that both interactions are essential for activity. Interestingly compound **10**, in which the nitrogen is conformationally constrained in the 25-position, shows greatly reduced activity compared to the analogue **9** in which the nitrogen is in the 24-position. Similarly, the 24-analogue **13** may show a slightly higher activity against the enzyme (IC_{50} = 8 μM) compared to the 23-analogue **12** (IC_{50} = 12.2 μM). These data suggest that having the nitrogen in the 24-position may be optimum for activity in this series of compounds. These conclusions are supported by the results of the sterol composition analysis for compounds **1** and **14** and **8**−**11** (Table 2).

Analysis of the effect of azasterols on the sterol composition demonstrates whether the compounds are inhibiting ergosterol biosynthesis in the cell. There is a good correlation between inhibition of the recombinant 24-SMT and effects of sterol composition, implying that inhibition of the enzyme does have a measurable physiological response. Thus compounds **1**, **9**, **12**, (**13**), **14**, **16**, **18** show activity against the enzyme, and the same compounds show effect on steroid composition, with compounds **1** and **14** having the greatest effect in both assays. (The correlation is not completely direct, because other factors are important such as uptake of compounds into cells.) Conversely, compounds which show no effect on the enzyme show no effect on the steroid composition. Comparing compounds **8**−**11** shows the sensitivity of inhibition of the enzyme to the position of the nitrogen in these conformationally constrained

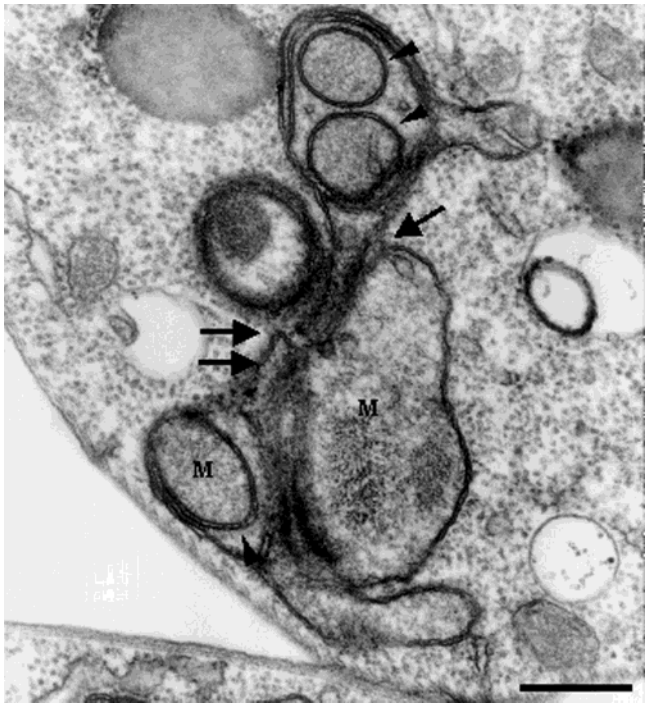


Figure 3. Electron micrograph of promastigote forms treated with 1.0 μM compound **12** for 24 h showing the alteration in the mitochondrion that presented some invaginations of its inner membrane (arrows) and the presence of concentric membranes in the matrix (arrowheads). Bar, 0.5 μm.

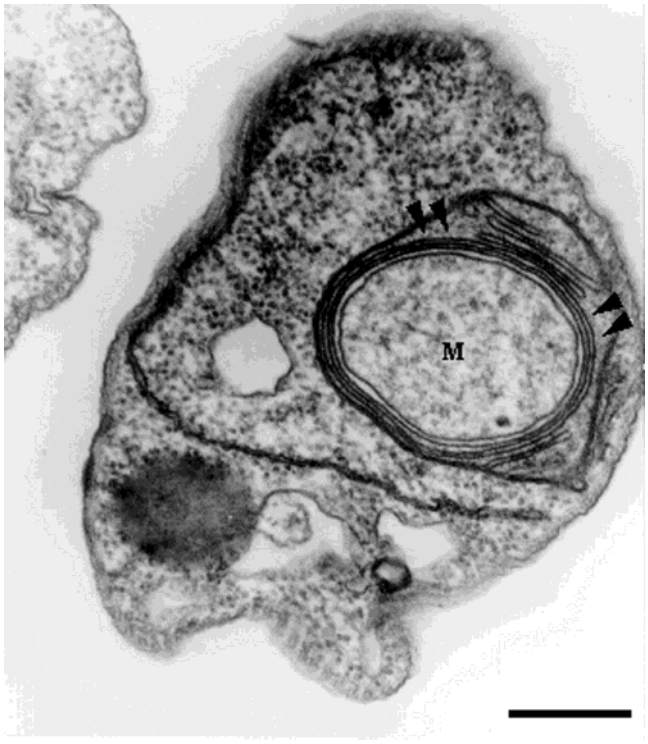


Figure 4. Promastigote form treated with 1.0 μM compound **12** for 48 h. The mitochondrion presented some concentric membranes in the matrix (arrowheads). Bar, 0.5 μm.

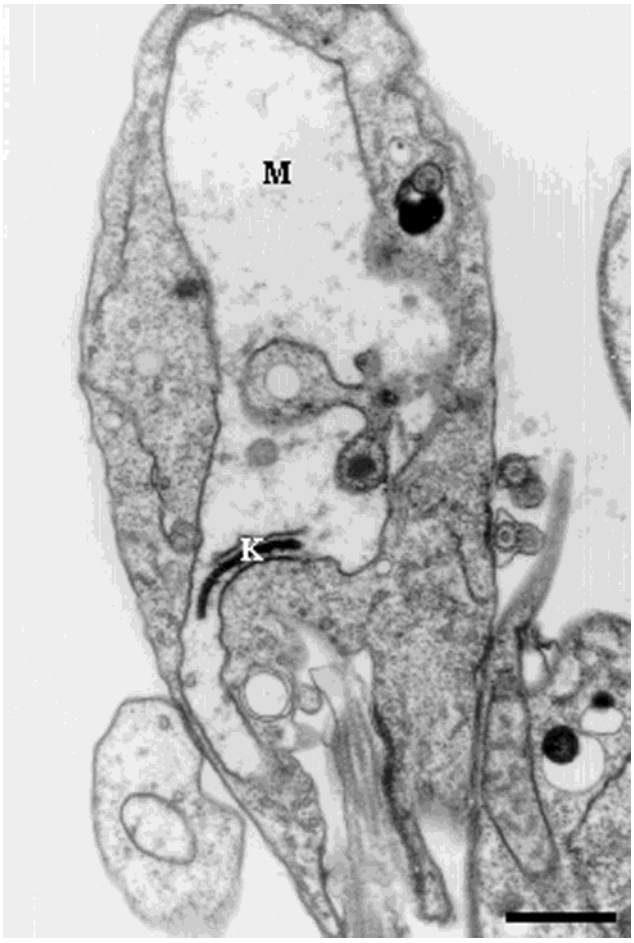


Figure 5. Parasite treated with 5.0 μM compound **12** for 48 h, showing an intense mitochondrial swelling where the matrix became much less electrondense. K, kinetoplast; M, mitochondrion. Bar, 1 μm.

molecules, with the 24-position analogue (**9**) showing better inhibition than the 23- (**8**) or 25- (**10**, **11**) position analogues.

The correlation between antiproliferative effects and activity against the enzyme was poor, suggesting permeability barriers and alternative mechanisms of action. However, the two compounds which showed the greatest activity against the enzyme (**1** and **14**), also showed activity against the whole parasites. Some of the compounds are inactive against the enzyme, but show activity against the whole organisms, which implies there may be another molecular target for these compounds within the cell, or perhaps they are "dissolving" in the plasma membrane and affecting the structure of the membrane. Interestingly, activity against *L. donovani* amastigotes showed a closer correlation to the inhibition of the enzyme than *L. amazonensis*, with compounds **1**, **13**, and **14** being the most active against both the enzyme and *L. donovani*.

The *T. cruzi* enzyme is likely to be very similar to the *Leishmania* enzyme, structurally and functionally, as there is a high degree of similarity between the *Leishmania* and *T. cruzi* enzyme sequences and both organisms have ergosta-type steroids as the principle membrane steroids. (Preliminary analysis of a partial sequence of the *T. cruzi* enzyme suggests an identity of about 64% with the *L. major* enzyme.) Therefore, compounds which inhibit ergosterol biosynthesis in

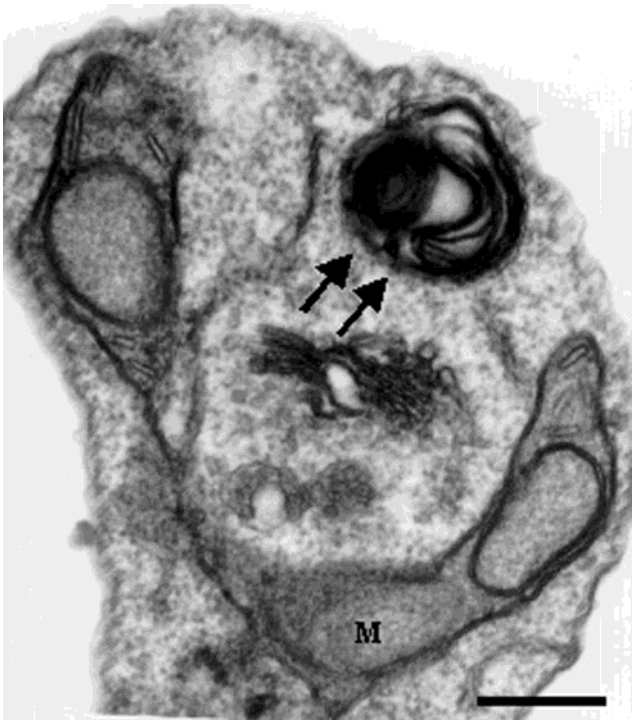


Figure 6. Electron micrograph of promastigote form treated with 5.0 μM compound **12** for 72 h. This image shows the presence of a large myelin-like figure in the cytoplasm (arrows). M, mitochondrion. Bar, 0.5 μm.

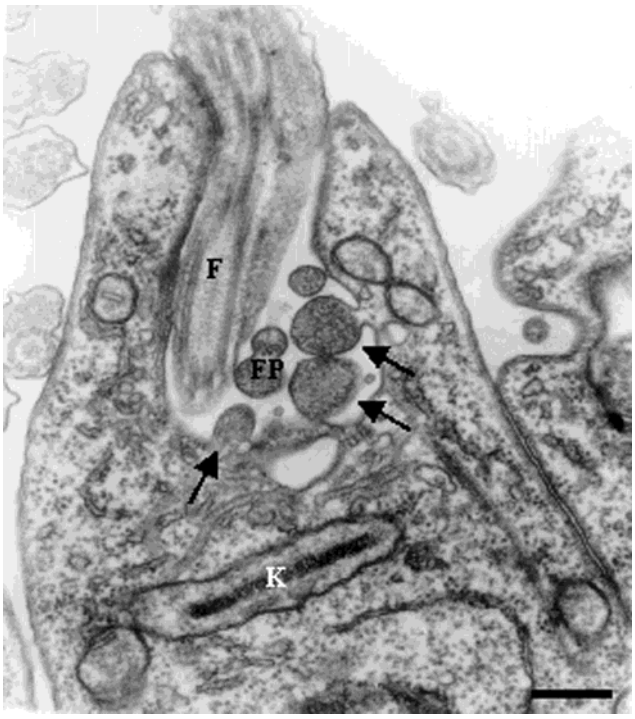


Figure 7. Promastigote form treated with 1.0 μM compound **12** for 72 h, showing the body protrusions toward the flagellar pocket (arrows). F, flagellum; FP, falgellar pocket; K, kinetoplast. Bar, 0.5 μm.

Leishmania should also have an effect in *T. cruzi*. Similar results were, in fact, obtained with epimastigotes of *T. cruzi* and promastigotes of *L. amazonesis*, with all the compounds tested. This may imply some feature of the promastigote/ epimastigote stage is susceptible to steroids in a nonspecific manner. The situation with the *T. cruzi* amastigotes was similar to that with the

L. donovani amastigotes, with compounds showing inhibition of 24-SMT also showing antiparasitic activity (**1**, **9**, **14**), although compound **5**, which does not inhibit 24-SMT is active against *T. cruzi*.

The sterol biosynthesis pathway, both in *T. cruzi* and *Leishmania* parasites, has been chemically validated at many different steps of the pathway, including 24-SMT. It has been shown in all cases that the complete depletion of the parasite's mature sterols (ergosterol and its 24-ethyl analogue in *T. cruzi*, episterol and 5-dehydroepisterol in *Leishmania*) leads not only to growth arrest but also to loss of cell viability and rapid lytic effects (see the description of the breakdown of internal organelles induced by the azasterols in the Ultrastructural Effects section). This takes place typically after exposure of the cells to the sterol biosynthesis inhibitors for two to three generations, when the pool of preformed sterols is exhausted (see refs 3−9). We have demonstrated that compounds which inhibit 24-SMT will strongly kill the parasites. However, some of the compounds in this study may also act through an alternative mode of action.

There appeared to be no general correlation between inhibition of parasitic growth (amastigote or procyclic stages) and toxicity to vertebrate cells, implying specific action of the compounds rather than general cytotoxicity. However, of the compounds showing the greatest activity against amastigotes (**1**, **5**, **9**, **13**, **14**), compounds **1**, **13**, and **14** also showed quite high toxicity against mammalian KB cells. This gave low selectivity values. For **1** and **13**, activity against *L. donovani* and *T. cruzi* amastigotes was similar to cellular toxicity (1.3 and 1.6-fold-selectivity, respectively, for **1**; 0.39 and 0.06, respectively, for **13**). However, for **14**, the selectivity increased for *L. donovani* and *T. cruzi* (6.1 and 1.6-fold, respectively), suggesting that **14** is a more promising lead than **1**.

Compounds **1**[6] and **12** were investigated for their effects on the structure of promastigotes and were shown to have effects on the structure of the membranes, in particular the mitochondrial membrane and around the flagellar pocket. The alterations in the mitochondrion were observed in a previous study when *L. amazonensis* promastigotes[6] and *T. cruzi*[3] were incubated with compound **1** (22,26-azasterol), as well as *T. cruzi* and *L. amazonensis* when treated with ICI 195,739,[9] ketoconazole, and terbinafine,[10] other inhibitors of ergosterol biosynthesis. Concluding, these observations suggest that the presence of ergosterol and 24-methenylated analogues is essential for the preservation of a normal structural organization of the mitochondrial membrane in trypanosomatids. Recent studies have shown a large concentration of endogenous and exogenous sterol in the mitochondrion of *T. cruzi* epimastigotes,[11] in contrast to what has been observed in mammalian cells. Our results indicate that the mitochondrion is an important target in the chemotherapy of leishmaniasis and Chagas's disease.

The presence of myelin-like figures in the cytoplasm could be related to the absence of sterols essential to the maintenance of the membrane structure in the endoplasmic reticulum. This structure was also observed in the flagellar pocket of parasites treated with different drugs and is characteristic of an exocytic process. In previous studies with *L. amazonensis* promastigotes cultured in the presence of 12-*O*-tetradecanoyl phorbol-13-acetate, an activator of proteinase kinase C, an increase in the exocytic process was observed, with the appearance of membranous structures within the flagellar pocket[12] similar to those observed in the parasites treated with ergosterol biosynthesis inhibitors. We also observed the presence of protrusions of the cell body toward the flagellar pocket (Figure 7, arrows). These protrusions were formed by the plasma membrane lining the flagellar pocket region. It is important to remember that the membrane of flagellar pocket does not contain a layer of subpellicular microtubules, making this region more susceptible to morphological changes. The presence of concentric membranes in the flagellar pocket cannot exclude the possibility that they result from the secretion into this region of abnormal lipids, which accumulate as a consequence of the drug action and the assembly of the concentric membranes and myelin-like figures, probably due to their different physical properties.[13]

In conclusion, 24-SMT is a potential drug target in *Leishmania* and *T. cruzi*; potent inhibitors of the enzyme are likely to have antiproliferative effects. However, there may well be other modes of action of these azasterols, as compounds which are not strong inhibitors of 24-SMT, also have antiparasitic effects. We have demonstrated an array of tools to monitor the effect of these inhibitors. However, further work is needed to improve the activity and selectivity of compounds.

Experimental Section

Chemicals were obtained from Sigma-Aldrich, Fluka, and Lancaster. Where applicable, glassware was oven dried, and reactions were carried out under a nitrogen atmosphere. Dry solvents were purchased from Aldrich or Fluka. Reactions were monitored by TLC using silica gel 60 F254 plates (Merck). NMR spectra were recorded on a Bruker avance 300MHz spectrometer at 300 MHz for ^{1}H and 75 MHz for ^{13}C. Infrared spectra were recorded on a Perkin-Elmer 1600 series FTIR spectrometer. Low-resolution mass spectra were recorded on a Micromass Platform 2 spectrometer. Accurate mass spectra were determined by the EPSRC Mass Spectrometry Centre, Swansea, UK. Elemental analysis was carried out by Medac Limited.

3*β*-*tert*-Butyldimethylsilanyloxy-5α-pregnan-20-one (2). To a suspension of 5α-pregnan-3*β*-ol-20-one (5.0 g, 15.7 mmol) in dry DMF (120 mL) under nitrogen at 0 °C was added a solution of TBS chloride (2.8 g, 18.5 mmol) and imidazole (1.35, 19.8 mmol) in dry DMF (40 mL) over a period of 10 min. The mixture was allowed to warm to RT overnight under stirring. The resulting suspension was diluted with Et_2O (400 mL) and washed with $NaHCO_3$ sat. (200 mL × 3), HCl 1 M (200 mL × 2) and water (200 mL × 2). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to yield 6.63 g (98%) of pure 3*β*-*tert*-butyldimethylsilanyloxy-5α-pregnan-20-one as a white solid. R_f 0.61 (AcOEt:hexane 1:4); ^{1}H NMR δ 3.60 (m, 1 H, 3-H), 2.55 (m, 1 H, 17-CH), 2.2 (s, 3 H, 21-Me), 2.05 (m, 1 H), 1.8−0.6 (m, 22 H), 0.95 (s, 9 H, Si-tBu), 0.85 (s, 3 H, 18-Me), 0.65 (s, 3H, 19-Me), 0.1 (s, 6 H, $Si(Me)_2$); ^{13}C NMR δ 210.1 (C=O), 72.5 (3-CH), 64.3 (17-CH), 57.2 (14-CH), 54.7 (CH), 45.4 (CH), 44.7 (C-13), 39.5 (CH_2), 39.0 (CH_2), 37.6, 36.0 (C-10), 35.9, 32.5, 32.3, 31.9, 29.1, 26.4 (Si-tBu), 24.8, 23.2, 21.7, 18.7 ($SiCMe_3$), 13.9, 12.8, −4.1 ($Si(Me)_2$); *m*/*z* (APCI+): 433 (M^+, 5%), 415 ($M^+ - H_2O$, 30%), 283.1 (100%); mp 139−140 °C; ν_{max} cm^{-1}: 1709 (C=O).

(20*R*)-3*β*-*tert*-Butyldimethylsilanyloxy-5α-pregnan-20-(pyridin-2-yl)-20-ol (3). A solution of 2-bromopyridine (0.51

mL, 5.3 mmol) in 25 mL of dry Et_2O under nitrogen was cooled at −78 °C. A solution of butyllithium (3.4 mL of a 1.6 M solution in hexane, 5.4 mmol) was added dropwise under rapid stirring over 5 min. The dark-red solution was stirred at −78 °C for 15 min. A solution of **2** (1.45 g, 3.35 mmol) in dry Et_2O (20 mL) was added dropwise to the cold solution over a period of 30 min. Afterward the temperature was allowed to rise to room temperature, and stirring was continued for 3 h. The mixture was treated with 5 mL of HCl 1 M followed by addition of K_2CO_3 solution until basic pH. The organic layer was washed twice with water, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was washed with cold acetone to afford 1.23 g (71%) of **3** as a white solid. R_f 0.6 (CH_2Cl_2); ^{1}H NMR δ 8.55 (d, 1 H, 6′-H, J = 6), 7.7 (td broad, 1 H, 4′-H), 7.35 (d, 1 H, 3′-H, J = 8.4), 7.15 (dd broad, 1 H, 5′-H), 5.6 (s, 1 H, OH), 3.6 (m, 1 H, 3-H), 2.25 (m, 1 H), 1.7 (s, 3 H, 21-CH_3), 0.95 (s, 9 H, t-Bu-Si), 0.85 (s, 3 H, 18-CH_3), 0.85 (s, 3 H, 19-CH_3), 1.9−0.6 (m, 28 H), 0.1 (s, 6 H, Si(CH_3)$_2$); ^{13}C NMR δ 166.5 (22-C), 146.7 (24-CH), 137.5 (26-CH), 121.9 (25-CH), 119.8 (27-CH), 75.4 (20-C), 72.6 (3-CH), 60.9, 57.2, 55.0, 45.5, 44.0 (13-C), 41.1, 39.1, 37.6, 35.9 (10-C), 35.3, 32.5, 32.4, 29.2, 26.4 (SiCMe_3), 24.1, 22.9, 21.6, 18.7, 13.9, 12.8, −4.1 (SiMe_2); *m*/*z* (ES+) 512.1 (M + H$^+$ 100%); mp 219.5−221.5 °C; Anal. ($C_{32}H_{53}NO_2Si$) C, H, N.

(20*R*)-3β-*tert*-Butyldimethylsilanyloxy-5α-pregnan-20-(pyridin-3-yl)-20-ol (4). To a solution of 3-bromopyridine (0.4 mL, 4.2 mmol) in dry Et_2O (15 mL) under nitrogen at −78 °C was added a solution of 2 M butyllithium (2.4 mL, 4.8 mmol) in pentane over a period of 5 min under rapid stirring. The yellow suspension was stirred at −78 °C for 25 min. A solution of **2** (0.81 g, 1.9 mmol) in dry Et_2O (8 mL) was added dropwise to the cold solution over a period of 30 min. After 1 h the temperature was allowed to rise to room temperature, and stirring was continued for 1 h. The mixture was treated with water (5 mL), and the organic layer was washed with water (2 × 50 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was washed with cold acetone and afforded 0.59 g (61%) of **4** as a white solid. R_f 0.31 (AcOEt:hexane 1:1); ^{1}H NMR δ 8.65 (d, 1 H, 23-H, J = 1.9), 8.4 (dd, 1 H, 25-H, J_3 = 4.8, J_4 = 1.5), 7.9 (s, 1 H, 27-H), 7.4 (m, 1 H, 26-H), 3.6 (m, 1 H, 3-H), 1.95 (m, 1 H, 17-CH), 1.7 (s, 3 H, 21-CH_3), 0.95 (s, 9 H, t-Bu-Si), 0.85 (s, 3 H, 18-CH_3), 0.85 (s, 3 H, 19-CH_3), 1.9−0.6 (m, 25 H), 0.1 (s, 6 H, Si(CH_3)$_2$); ^{13}C NMR δ 146.7 (23-CH),146.3 (25-CH), 146.1 (C-22), 133.9 (27-CH), 123.5 (26-CH), 75.8 (20-C), 72.6 (3-CH), 61.2, 57.0, 54.8, 45.4, 43.8 (13-C), 40.9, 39.1, 37.6, 35.9 (10-C), 35.2, 32.3, 30.1, 29.1, 26.4 (SiCMe_3), 23.8, 23.1, 21.5, 18.7, 14.3, 12.8, −4.1 (Si(CH_3)$_2$); *m*/*z* (ES+): 512.1 (100%, M + H$^+$); mp 251−253 °C; *m*/*z* (ES+) found 512.3916 (M + H)$^+$, expected $C_{32}H_{54}NO_2Si$ 512.3924; Anal. ($C_{32}H_{53}NO_2Si$) C, H, N.

(20*R*)-3β-*tert*-Butyldimethylsilanyloxy-5α-pregnan-20-(pyridin-4-yl)-20-ol (5). To a suspension of 4-bromopyridine hydrochloride (2.63 g, 13.5 mmol) in dry Et_2O (60 mL) under nitrogen cooled at −78 °C was added a solution of butyllithium (20 mL, 1.6 M in hexane, 32 mmol) over a period of 5 min under rapid stirring. The yellow suspension was stirred at −78 °C for 25 min. A solution of **2** (2.5 g, 5.8 mmol) in dry Et_2O (40 mL) was added dropwise to the cold solution over a period of 30 min. After 1 h the temperature was allowed to rise to room temperature, and stirring was continued for another 1 h. The mixture was then treated with water, and the organic layer was washed twice with water, dried over Na_2SO_4, and concentrated under reduced pressure. The orange residue was washed with acetone. The insoluble residue was subjected to silica gel chromatography (AcOEt:hexane 1:1) to yield 0.34 g (11%) of **4** as white solid. R_f 0.3 (AcOEt:hexane 1:1); ^{1}H NMR δ 8.6 (m, 2 H, 24 and 26-CH), 7.45 (d, 2 H, 23 and 27 CH, J = 4.6), 3.6 (m, 1 H, 3-H), 2.15 (m, 1 h, 17-CH), 1.7 (s, 3 H, 21-CH_3), 1.0 (s, 3-H, 18 CH_3), 0.95 (s, 9 H, Si-t-Bu), 0.85 (s, 3 H, 19-CH_3), 1.8−0.6 (m, 28 H), 0.1 (s, 6 H, $SiMe_2$); ^{13}C NMR δ 148.6 (24 and 26 CH), 120.8 (23 and 27 CH), 76.4 (20-C), 72.5 (3-CH), 60.4, 57.0, 54.8, 45.4, 44.0 (13-C), 41.0, 39.0, 37.6, 35.9 (10-C), 35.2, 32.3, 29.8, 29.1, 26.4 (SiCMe_3), 23.9, 23.0, 22.1, 21.5, 18.7, 14.2, 12.8, −4.1 (SiMe_2); *m*/*z* (ES+): 512.0 (100% M + H$^+$); mp 261−262 °C; Anal. ($C_{32}H_{53}NO_2Si$) C, H, N.

(20*R*)-3β-*tert*-Butyldimethylsilanyloxy-5α-pregnan-20-(3-chloropyridin-4-yl)-20-ol (6). To a solution of dry diisopropylamine (1.8 mL, 12.5 mmol) in 30 mL of dry THF under nitrogen at −78 °C was added a solution of butyllithium (7.8 mL, 1.6 M in hexane, 12.5 mmol) over a period of 1 min under rapid stirring. The yellowish solution was stirred at −78 °C for 20 min. A solution of 3-chloropyridine (1.2 mL, 12.5 mmol) in dry THF (10 mL) was added dropwise to the cold solution over 15 min, maintaining temperature below −70 °C. Stirring of the dark orange solution was continued for 25 min; a precipitate formed though, due to 3-chloro-4-lithiopyridine partially precipitating out of the solution. A solution of **2** (5 g, 11.5 mmol) in 20 mL of dry THF was added over 5 min, maintaining temperature below −60 °C. The resulting pale-red solution was stirred overnight at room temperature, quenched with a saturated solution of NH_4Cl (5 mL), and diluted with $CHCl_3$ (100 mL). The organic layer was washed with water (50 mL) and a saturated solution of K_2CO_3 (50 mL) and dried over Na_2SO_4. The solvent was removed under reduced pressure, and the crude solid was washed with acetone and $CHCl_3$ to give 1.29 g (31%) of **6** as a white solid. R_f 0.28 (AcOEt:$CHCl_3$ 1:4); ^{1}H NMR δ 8.55 (s, 1 H, 24-CH), 8.45 (d, 1 H, 26-CH, J = 4.8), 7.73 (d, 1 H, 27-CH, J = 4.8), 3.55 (m, 1 H, 3-CH), 2.45 (t, 1 H, 17-CH, J = 7.2), 2.15 (m, 1 H), 1.92 (s, 3 H, 21-Me), 1.8−0.8 (m, 21 H), 1.0 (s, 3 H, 18-Me), 0.9 (s, 9 H, Si-t-Bu), 0.8 (s, 3 H, 19-Me), 0.1 (s, 6 H, $SiMe_2$); ^{13}C NMR δ 154.9 (23-CH), 151.3, 148.4, 128.6 (22-CH), 122.4, 77.7 (3-CH), 72.6 (20-C), 56.9, 54.7, 54.4, 45.4, 44.0 (13-C), 40.5, 39.1, 37.6, 35.9 (10-C), 35.2, 32.4, 29.1, 27.0, 26.4 (t-Bu), 23.9, 22.9, 21.5, 18.7 (SiC), 14.6, 12.8, −4.1 (SiMe_2); *m*/*z* (ES+): 546.1 (100%, M$^+$, ^{35}Cl), 547.0 (40%, M + H$^+$, ^{35}Cl), 548.0 (40%, M$^+$, ^{37}Cl), 549.1 (15%, M + H$^+$, ^{35}Cl); mp 255−257 °C; Anal. ($C_{32}H_{52}ClNO_2Si$) C, H, N, Cl.

(20*R*)-3β-*tert*-Butyldimethylsilanyloxy-5α-pregnan-20-(3-fluoropyridin-4-yl)-20-ol (7). To a solution of dry diisopropylamine (1.4 mL, 10 mmol) in dry THF (30 mL) under nitrogen at −78 °C was added a solution of 1.6 M butyllithium (6.3 mL, 10 mmol) in hexane over a period of 1 min under rapid stirring. The yellowish solution was stirred at −78 °C for 20 min. Over the next 15 min, a solution of 3-fluoropyridine (0.87 mL, 10 mmol) in dry THF (10 mL) was added dropwise to the cold solution maintaining the temperature below −70 °C. Stirring of the pale yellow solution was continued for a further 25 min; a precipitated formed, thought to be due to 3-fluoro-4-lithiopyridine. A solution of **2** (4 g, 9.2 mmol) in dry THF (20 mL) was added over a period of 5 min, maintaining the temperature below −60 °C. The resulting solution was stirred for 4 h, allowing it to warm to room temperature, then quenched with water. The mixture was extracted with $CHCl_3$, and the organic layer washed with water and dried over Na_2SO_4. The solvent was removed under reduced pressure, followed by silica gel chromatography (AcOEt:$CHCl_3$ 1:4) to afford 3.2 g (65%) of **7** as a white solid. R_f 0.28 (AcOEt:$CHCl_3$ 1:4); ^{1}H NMR δ 8.4 (m, 2 H, 24 and 26 CH), 7.55 (m, 1 H, 27-CH), 3.55 (m, 1 H, 3-CH), 2.2−2.0 (m, 1 h, 17-CH) 1.8−0.9 (m, 22 H), 1.8 (s, 3 H, 21-Me), 0.95 (s, 3 H, 18-Me), 0.9 (s, 9 H, Si-tBu), 0.8 (s, 3 H, 19-Me), 0.65 (m, 1 H); ^{13}C NMR δ 157.6 (d, 23-CF, J_1 = 390), 146.2 (d, 26-CH, J_4 = 5), 145.1 (d, 22-C, J_2 = 10), 138.8 (d, 24-CH, J_2 = 27), 121.9 (27-CH), 75.5 (d, 20-C, J_3 = 5), 72.7 (3-CH), 57.0, 54.8 (17-CH), 45.4, 44.0 (13-C), 40.7, 39.1, 37.6, 35.9 (10-C), 35.2, 32.3, 29.1, 28.2, 26.4 (tBu), 23.9, 23.2, 21.5, 18.7 (SiCMe_3), 14.4, 12.8, −4.1 (SiMe_2); ^{19}F NMR δ −127.7 (s); mp 240−242 °C

(20*R*)-5α-Pregnan-20-(pyridn-2-yl)-3β,20-diol (8).[14] To a solution of 2-bromopyridine (4.9 mL, 50 mmol) in dry Et_2O (130 mL) under nitrogen at −78 °C was added a solution of butyllithium (25 mL, 2 M in pentane, 50 mmol) over a period of 5 min dropwise, and the resulting dark-red solution was stirred at −78 °C for 30 min. A solution of 3β-yl-pregnan-20-one acetate (4.0 g, 11.1 mmol) in dry Et_2O (120 mL) was added over 10 min. The temperature was allowed to rise to room temperature over 1 h, water was added (5 mL), and the organic

layer was washed with water (2 × 100 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was washed with cold hexane (2 × 50 mL) followed by silica gel chromatography (AcOEt:hexane 2:8) to afford 2.44 g (55%) of **8** as white solid. R_f 0.37 (AcOEt:hexane, 1:4); ^{1}H NMR δ 8.55 (d, 1 H, 6′-H, J = 6), 7.7 (td broad, 1 H, 4′-H), 7.35 (d, 1 H, 3′-H, J = 8.4), 7.15 (dd broad, 1 H, 5′-H), 5.6 (s, 1 H, OH), 3.6 (m, 1 H, 3-H), 2.25 (m, 1 H), 1.7 (s, 3 H, 21-CH_3), 0.85 (s, 3 H, 18-CH_3), 0.85 (s, 3 H, 19-CH_3), 1.9−0.6 (m, 23 H); ^{13}C NMR δ 166.5 (22-CH), 146.8 (24-CH), 137.7 (26-CH), 122.0 (27-CH), 119.9 (25-CH), 75.5 (3-CH), 71.7 (20-C), 60.8 (17-CH), 57.2, 54.9, 45.3, 44.0, 41.1 (13-C), 38.6, 37.5, 35.9 (10-C), 35.3, 32.3, 31.9, 29.1, 24.1, 22.9, 21.6, 13.9, 12.8; *m*/*z* (ES+): 398.6 (M + H$^+$, 30%), 380.1 (M$^+$ − H_2O, 100%); mp 249−250 °C.

(20*R*)-5α-Pregnan-20-(pyridn-3-yl)-3β,20-diol (9). A suspension of **4** (0.59 g, 1.15 mmol) in a mixture AcOH:THF:water (9:3:3, 15 mL) was stirred at 75 °C overnight. The solution was then left cool to room temperature, and most of the solvent was removed under reduced pressure. The resultant solid was suspended in CH_2Cl_2, and the organic phase was washed with NaOH 0.5 M (3 × 10 mL), then dried over Na_2SO_4. Removal of the solvent under reduced pressure, followed by gel chromatography (AcOEt:hexane, 1:1) gave 0.16 g (35%) of **9** as white solid. R_f 0.12 (AcOEt:hexane, 1:1); ^{1}H NMR δ 8.65 (d, 1 H, 23-H, J = 2), 8.4 (m, 1 H, 25-H), 7.9 (1 H, 27-H), 7.4 (m, 1 H, 26-H), 3.6 (m, 1 H, 3-H), 1.95 (m, 1 H, 17-CH), 1.7 (s, 3 H, 21-CH_3), 0.85 (s, 3 H, 18-CH_3), 0.85 (s, 3 H, 19-CH_3), 1.9−0.6 (m, 25 H); ^{13}C NMR δ 146.7 (23-CH), 146.3 (25-CH), 146.1 (C-22), 133.9 (27-CH), 123.5 (26-CH), 75.8 (20-C), 72.1 (3-CH), 61.2, 57.0, 54.8, 45.4, 42.8 (13-C), 40.9, 39.1, 37.6, 35.7 (10-C), 35.5, 32.3, 30.1, 29.1, 23.5, 23.1, 21.5, 18.7, 14.3, 12.8; *m*/*z* (ES+): 398.0 (100% M$^+$); mp 249−252 °C; Anal. ($C_{26}H_{39}NO_2$·0.25H_2O) C, H, N.

(20*R*)-5α-Pregnan-20-(pyridn-4-yl)-3β,20-diol (10). Dowex 50WX4−200 ion-exchange resin (strong acidic cation, Aldrich) (2 g) was washed with 1 M HCl (10 mL × 3), water (10 mL × 3) and MeOH (10 mL × 1), added to a solution of **5** (0.08 g, 0.15 mmol) in MeOH:$CHCl_3$ (10:10), and stirred at room temperature overnight. The resin was filtered and washed with MeOH:$CHCl_3$ (1:1, 3 × 10 mL), 2 M H_2SO_4 (2 × 10 mL), and MeOH (10 mL). The washings were combined and made basic by careful addition of NaOH pellets, followed by extraction with $CHCl_3$. The organic layer was dried over Na_2SO_4, and removal of solvent under reduced pressure afforded 0.04 g (65%) of **10** as a white solid. R_f 0.06 (AcOEt:hexane 1:4); ^{1}H NMR δ 8.55 (d, 2 H, 24 and 26-CH, J = 3), 7.4 (d, 2 H, 23 and 28-CH, J = 3), 3.65 (m, 1 H, 3-CH), 2.3 (m, 1 H), 1.65 (s, 3 H, 21-CH_3), 0.95 (s, 3 H, 19-CH_3), 0.85 (s, 3 H, 18- CH_3), 1.9−0.85 (m, 20 H), 0.6 (m, 1 H); ^{13}C NMR δ 148.2 (24 and 26 CH), 121.8 (23 and 27 CH), 76.1 (20-C), 72.6 (3-CH), 61.2, 57.0, 55.1, 45.0, 42.9 (13-C), 41.1, 39.1, 37.6, 35.9 (10-C), 35.3, 32.5, 32.4, 29.2, 24.1, 22.9, 21.6, 18.7, 13.9, 12.8; *m*/*z* (CI+): 398.0 (M + H$^+$, 100%); mp 281−282 °C; *m*/*z* (ES+) Found 398.3059 (M + H)$^+$, expected $C_{26}H_{40}NO_2$ 398.3059.

(20*R*)-5α-Pregnan-20-(3-chloropyridn-4-yl)-3β,20-diol (11). To a suspension of **6** (0.49 g, 0.9 mmol) in MeOH (15 mL) was added 0.2 mL of 10 M HCl, and the solution was stirred for 15 min. The solvent was removed under reduced pressure, and the residue was suspended in a K_2CO_3 solution (10 g in 100 mL water), extracted with $CHCl_3$, and dried over Na_2SO_4. The solvent was removed under reduced pressure to yield a crude yellow solid that was further purified by gel chromatography to give 0.3 g (78%) of **11** as a white solid. R_f 0.27 (AcOEt:$CHCl_3$ 1:1); ^{1}H NMR δ 8.6 (s, 1 H, 24-CH), 8.5 (d, 1 H, 26-CH, J = 5), 7.7 (d, 1 H, 27-CH, J = 5), 3.65 (m, 1 H, 3-CH), 2.5 (t, 1 H, 17-CH, J = 7.2), 2.15 (m, 1 H), 1.9 (s, 3 H, 21-Me), 1.8−0.8 (m, 20 H), 1.0 (s, 3 H, 18-Me), 0.9 (s, 3 H, 19-Me), 0.7 (m, 1 H); ^{13}C NMR δ 154.9 (23-CH), 151.3, 148.4, 128.6 (22-CH), 122.4, 76.8 (3-CH), 71.7 (20-C), 56.9, 54.6, 54.4, 45.2, 43.9 (13-C), 40.4, 38.6, 37.4, 35.9 (10-C), 35.2, 31.9, 29.1, 27.0, 23.9, 22.9, 21.5, 14.6, 12.8; *m*/*z* (ES+) 433.3 (100%, M + H$^+$); mp 276−278 °C; *m*/*z*: Found: 432.2678 (M + H)$^+$, expected $C_{26}H_{39}NO_2Cl$ 432.2669.

(20*R*,22*R*)-3β-*tert*-Butyldimethylsilanyloxy-5α-pregnan-20-(piperidin-2-yl)-20-ol (12). To a solution of **3** (1.1 g, 2.15 mmol) in 100 mL of glacial acetic acid was added PtO_2 (0.105 g), and then the suspension was shaken under hydrogen (55 psi) at room temperature for 60 h. The reaction mixture was diluted with 100 mL of *i*-propanol:ethanol (10:1), and the catalyst was removed by filtration. The solvent was removed under reduced pressure, and the crude solid was suspended in diethyl ether, washed with sodium hydroxide, and dried over sodium sulfate to yield a solid that was crystallized from acetonitrile to afford 0.98 g (88%) of **12** as white solid. R_f 0.66 (AcOEt:NEt_3 4:1); ^{1}H NMR δ 3.55 (m, 1 H, 3-CH), 3.25 (d, 1 H, J = 10), 2.6 (t, 1 H, J = 10), 2.4 (d, 1 H, J = 10), 2.1 (d, 1 H, J = 10), 1.95−1.0 (m, 28 H), 1.35 (s, 3 H, 21-Me), 0.9 (s, 9 H, Si-t-Bu), 0.85 (s, 3 H, 18-Me), 0.8 (s, 3 H, 19-Me), 0.1 (s, 6 H, $SiMe_2$); ^{13}C NMR δ 75.4 (20-C), 72.6 (3-CH), 68.3 (17-CH), 56.7, 55.5, 55.1, 54.8, 48.4, 45.4, 43.8 (13-C), 40.9, 39.1, 37.6, 35.9 (10-C), 35.3, 32.4, 29.2, 27.4, 26.4 (Si-t-Bu), 25.5, 25.1, 24.6, 23.2, 21.6, 18.7, 14.1, 12.8, −4.1 ($SiMe_2$); *m*/*z* (ES+): 518.4 (100% M$^+$); mp 208−210 °C; Anal. ($C_{32}H_{59}NO_2Si$) C, H, N.

(20R,22ξ)-3β-*tert*-Butyldimethylsilanyloxy-5α-pregnan-20-(piperidin-3-yl)-3β,20-diol (13). To a solution of **4** (1.52 g, 3 mmol) in 80 mL of glacial acetic acid was added PtO_2 (0.125 g), and the suspension was placed under hydrogen (50 psi) and shaken at room temperature for 5 days. The catalyst was filtered, the solvent removed under reduced pressure, and the crude solid was suspended in diethyl ether, washed with sodium hydroxide solution, and dried over sodium sulfate. The solid was crystallized from acetone−acetonitrile to afford 1.37 g (89%) of **13** as white solid. R_f 0.53 (MeOH:NEt_3 4:1); ^{1}H NMR δ 3.6 (m, 1 H, 3-CH), 3.0 (m, 1 H), 2.5−1.8 (m, 4 H, 23-CH_2 and 25-CH_2), 1.75−0.9 (m, 25 H), 1.15 (s, 3 H, 21-CH_3), 0.85 (s, 9 H, t-Bu), 0.82 (s, 3 H, 18-CH_3), 0.75 (s, 3 H, 19-CH_3) 0.65 (m, 1 H, 5-CH), 0.1 (s, 6 H, $SiMe_2$); ^{13}C NMR δ 76.3 (20-C), 72.6 (3-CH), 68.3 (17-CH), 57.1, 56.3, 55.9, 54.8, 49.3, 47.4, 45.4, 43.6 (13-C), 41.1, 39.1, 37.6, 35.9 (10-C), 35.2, 32.4, 29.2, 26.4 (Si-tBu), 24.8, 22.9, 22.5, 21.5, 18.7, 14.3, 12.8, −4.1 ($SiMe_2$); *m*/*z* (ES+): 518.1 (M + H$^+$, 100%); mp 218−220 °C; Anal. ($C_{32}H_{59}NO_2Si$) C, H, N.

(20R,22ξ)-5α-Pregnan-20-(piperidin-2-yl)-3β,20-diol (1). To a solution of **8** (4.0 g, 10.1 mmol) in glacial acetic acid (200 mL) was added PtO_2 (0.5 g), and then the suspension was placed under hydrogen (50 psi) and shaken at room temperature for 72 h. The catalyst was then filtered, and the solvent was removed under reduced pressure to give a cream white solid, which was suspended in diethyl ether, washed with sodium hydroxide solution, and dried over sodium sulfate. Crystallization from acetonitrile afforded 3.05 g (75%) of **20** as off-white solid. R_f 0.38 (MeOH:AcOEt:NEt_3, 4:4:2); ^{1}H NMR δ 3.6 (m, 1 H, 3-CH), 3.2 (d, 1 H, 24-CH_{eq}H, J_2 = 11.9), 2.6 (m, 1 H, 24-CHH_{ax}), 2.35 (d, 1 H, 22-CH_{ax}, J = 10.7), 2.1 (m, 1 H, 17-CH), 2.0−0.8 (m, 29 H), 1.35 (s, 3 H, 21-CH_3), 0.85 (s, 3 H, 18-CH_3), 0.8 (s, 3 H, 19-CH_3) 0.65 (m, 1 H, 5α-CH); ^{13}C NMR δ 75.3 (20-C), 71.7 (3-CH), 68.4 (22-CH), 56.7, 55.2, 54.8, 48.5 (24-CH_2), 45.3, 43.9 (13-C), 40.9, 38.6, 37.4, 35.9, 35.2 (10-C), 32.4, 31.9, 29.1, 28.0, 27.7, 25.8, 25.1, 24.6, 23.1, 21.6, 14, 12.7; *m*/*z* (ES+): 403.8 (75% M$^+$), 404.5 (55% M + H$^+$), 385.7 (100% M$^+$ − H_2O); mp 200−201 °C.

(20R,22ξ)-5α-Pregnan-20-(piperidin-3-yl)-3β,20-diol (14). To a suspension of **13** (0.3 g, 0.58 mmol) in methanol was added 0.5 mL of 10 M HCl (5 mmol) under vigorous stirring. After 20 min no trace of starting material was detected by mass spectroscopy. The solvent was removed under reduced pressure to give a white solid that was suspended in chloroform and extracted with a saturated solution of K_2CO_3 (2 × 20 mL). The organic layer was dried over Na_2SO_4, and removal of the solvent under reduced pressure gave 0.28 g (120%) of crude **14** that was washed with Et_2O to afford 78 mg (34%) of **14** as off-white solid. R_f 0.25 (MeOH:AcOEt:NEt_3, 4:4:2); ^{1}H NMR δ 3.55 (m, 1 H, 3-CH), 3.0 (s broad, 1 H), 2.5 (t, 1 H, J = 11), 2.25 (s, 3H), 2.1−1.9 (m, 2 H), 1.9−0.8 (m, 25 H), 1.2 (s, 3 H, 21-Me), 0.9 (s, 3 H, 18-Me), 0.85 (s, 3 H, 19-Me), 0.65 (m, 1 H, 5α-CH); ^{13}C NMR δ 76.2 (20-C), 71.5 (3-CH), 66.3 (25-CH_2),

57.1 (23-CH_2), 56.2, 54.7, 47.3, 47.0, 45.2, 43.5 (13-C), 41.1, 38.5, 37.4, 35.8 (10-C), 35.2, 32.3, 31.8, 29.1, 27.3, 24.7, 24.1, 22.7, 22.4, 21.0, 15.6, 14.2, 12.7; *m/z* (ES+): 404.1 (100% M + H^+), 386.2 (95% $M^+ - H_2O$), 807.1 (10%, 2 × M + H^+); mp 204−206 °C; *m/z*: Found: 404.3545 $(M + H)^+$, expected $C_{26}H_{46}NO_2$ 404.3529.

(20*R*,22*R*)-3β-*tert*-Butyldimethylsilanyloxy-5α-pregnan-20-[piperidin-1′-propionamide-2′-yl)-20-ol (15). To a solution of HOBt (0.375 g, 2.5 mmol) and propanoic acid (0.19 mL, 2.5 mmol) in THF (12 mL) under nitrogen at room temperature was added diisopropyl carbodiimide (0.47 mL, 3 mmol), and the solution was stirred for 15 min. This solution (3.8 mL, 0.8 mmol) was added to a solution of **12** (0.4 g, 0.77 mmol) in THF (2.5 mL), the reaction mixture was stirred at room temperature for 48 h, and then it was diluted with water (50 mL) and extracted with AcOEt (3 × 50 mL). The organic phase was washed with water (3 × 50 mL), dried over Na_2SO_4, and concentrated under reduced pressure. Gel chromatography (AcOEt:hexane 1:5) gave 0.15 g (34%) of **15** as a white solid. R_f 0.52 (AcOEt:hexane 1:1); ^{1}H NMR δ 4.45 (m, 1 H, 22-CH), 3.6−3.3 (m, 3 H, 3-CH and 24-CH_2), 2.85 (s, 1 H, OH), 2.3 (m, 2 H, O=CCH_2Me), 2.1−0.7 (m, 34 H), 1.15 (s, 21-Me), 1.1 (m, O=C−CH_2*Me*), 0.85 (s, 9 H, Si-tBu), 0.75 (d, 6 H, 13-Me and 10-Me), 0.62 (m, 1 H, 5-CH), 0.0 (s, 6 H, $SiMe_2$); ^{13}C NMR δ 174.9 (C=O), 80.5 (20-C), 72.6 (3-CH), 57.1 (22-CH), 56.9, 55.0, 54.8, 45.5, 43.4 (13-C), 40.3 (24-CH_2), 39.1, 37.6, 35.9 (10-C), 35.4, 32.5, 32.3, 29.2, 27.4, 26.4 (SiCMe_3), 24.9, 24.3, 24.0, 23.5, 21.6, 20.6, 18.7 (CMe_3), 14.1, 12.8, 10.1(O=C−CH_2*Me*), −4.1 ($SiMe_2$); *m/z* (ES+): 596.1 (100%, M + Na^+), 574.1 (20%, M^+); mp 168−170 °C; ν_{max} cm^{-1} 3392.3 (ν OH), 2918.5, 2855.2 (ν CH_2, CH_3), 1613.9 (ν C=O); Anal. ($C_{35}H_{63}NO_3Si$) C, H, N.

(20*R*,22*R*)-3β-ol-5α-Pregnan-20-[piperidin-1′-propionamide-2′-yl)-20-ol (16). To a suspension of **15** (0.11 g, 0.19 mmol) in MeOH (20 mL) was added HCl (10 M, 1 mL), and the mixture was stirred for 30 min. The solvent was removed under reduced pressure to yield 0.085 g (96%) of **57** as a white solid. R_f 0.51 (AcOEt); ^{1}H NMR (DMSO) δ 4.1 (d, 1 H, J = 13), 3.95 (d, 1 H, J = 13), 3.35 (m, 2 H, 3-CH and 22-CH), 2.95 (m, 1 H), 2.75 (m, 1 H), 2.5 (s, 0.9 H, OH), 2.25−0.7 (m, 33 H), 1.5 (s, 3 H, 21-Me), 1.15 (t, 3 H, ($COCH_2$*Me*, J = 7.2), 0.75 (s, 3 H, 18-Me), 0.7 (s, 3 H, 19-Me), 0.65 (m, 1 H, 5-CH); ^{13}C NMR (DMSO) δ 175.2 (NC=O), 97.9 (20-C), 71.2 (3-CH), 69.6 (22-CH), 55.3, 53.9, 45.3 (24-CH_2), 44.6, 43.6 (13-C), 40.1, 38.4, 37.0, 35.4 (10-C), 34.8, 31.6, 28.6, 26.4, 24.5, 21.7, 19.7, 12.8 (18-Me), 12.4 (19-Me), 8.4 ($COCH_2$*Me*); *m/z* (ES+) 442.1 (M − $H_2O)^+$, 100%), 483.2 (M + Na^+, 5%); mp 184−186 °C.

(20*R*,22ξ)-3β-*tert*-Butyldimethylsilanyloxy-5α-pregnan-20-[piperidin-1′-propionamide-3′-yl)-20-ol (17). To a solution of HOBt (0.375 g, 2.5 mmol) and propanoic acid (0.19 mL, 2.5 mmol) in THF (12 mL) under nitrogen at room temperature was added 0.47 mL (3 mmol) of diisopropyl carbodiimide, and the solution was stirred for 15 min. This solution (2.5 mL, 0.5 mmol) was added to a solution of **13** (0.27 g, 0.5 mmol) in THF (2.5 mL), and the reaction mixture was stirred at room temperature for 48 h and then diluted with water (40 mL) and extracted with AcOEt (3 × 50 mL). The organic phase was washed with water (3 × 50 mL), dried over Na_2SO_4, and concentrated under reduced pressure. Gel chromatography (AcOEt:hexane 1:4) gave 0.2 g (67%) of **17** as a white solid. R_f 0.31 (AcOEt:hexane 1:1); ^{1}H NMR δ 3.6 (m, 1 H, 3-CH), 2.8 (s, 0.7 H, OH), 2.4 (q, 2 H, O=CCH_2Me, J = 7), 2.1 (m, 2 H), 1.9−0.8 (m, 35 H), 1.25 (s, 21-Me), 1.2 (t, O=C−CH_2*Me*, J = 7), 0.95 (s, 9 H, Si-tBu), 0.9 (s, 3 H, 13-Me), 0.85 (s, 3 H, 10-Me), 0.65 (m, 1 H, 5-CH), 0.1 (s, 6 H, $SiMe_2$); ^{13}C NMR δ 172.7 (C=O), 76.1 (20-C), 72.6 (3-CH), 56.9, 56.3 (23-CH_2), 54.7, 46.2, 45.4 (25-CH_2), 43.6 (13-C), 41.0, 39.1, 37.6, 35.9 (10-C), 35.2, 32.4, 30.1, 29.1, 27.0, 26.4 (SiCMe_3), 24.7, 24.1, 22.8, 21.5, 18.7 (CMe_3), 14.3, 12.8, 10.0 (O=C−CH_2*Me*), −4.1 ($SiMe_2$); *m/z* (ES+): 612.2 (10%, M + K^+), 596.1 (100%, M + Na^+), 574.2 (5%, M^+); mp 231−233 °C; ν_{max} cm^{-1} 3405.1 (OH), 2927.1, 2852.4 (CH_2, CH_3), 1625.9 (C=O); Anal. ($C_{35}H_{63}NO_3Si$) C, H, N.

(20R,22*R*)-3β-ol-5α-Pregnan-20-[piperidin-1′-propionamide-3′-yl)-20-ol (18). To a suspension of **42** (0.16 g, 0.28 mmol) in MeOH (20 mL) was added HCl (10 M, 1 mL), and the mixture was stirred for 30 min. The solvent was removed under reduced pressure to yield 0.12 g (97%) of **57** as a white solid. R_f 0.32 (AcOEt); ^{1}H NMR (DMSO) δ 4.4 (M, 1 H), 3.8 (d, 1 H, J = 12), 3.35 (m, 1 H, 3-CH), 2.8 (m, 1 H), 2.5 (s, 1 H, OH), 2.3 (q, 2 H, O=CCH_2Me, J = 6.5), 2.0 (m, 2 H), 1.9−0.7 (m, 31 H), 1.1 (s, 21-Me), 1.0 (t, $COCH_2$*Me*, J = 6.5), 0.8 (s, 3 H, 18-Me), 0.7 (s, 3 H, 19-Me), 0.6 (m, 1 H, 5-CH); ^{13}C NMR (DMSO) δ 171.2 (N−C=O), 74.2 (20-C), 69.7 (3-CH), 56.8, 56.1, 54.2, 46.1 (23-CH_2), 44.7 (25-CH_2), 42.9 (13-C), 40.7, 40.1, 39.8, 38.5, 37.0, 35.4 (10-C), 34.7, 32.0, 31.7, 28.8, 26.0, 22.1, 21.1, 14.1 (18-Me), 12.5 (19-Me), 9.9 ($COCH_2$*Me*); *m/z* (ES+): 442.3 ($(M-H_2O)^+$, 100%), 460.2 (M + H^+, 50%); mp 235 − 237 °C (melted as brown liquid); *m/z*: Found: 482.3618 $(M + Na)^+$, expected $C_{29}H_{49}NO_3Na$ 482.3610.

Enzyme Assays. In assays of inhibition of 24-SMT, protein extracts from *E. coli* BL21 (DE3) pLysS/pET28a-HisLmSMT cells were used. Plasmid pET28a-HisLmSMT was obtained by cloning the entire coding sequence of the *Leishmania major SMT* gene in the pET28a vector (Novagen). *L. major* recombinant SMT is produced as a His-tagged fusion protein and is overexpressed when induced with IPTG 1mM during 4 h. Cells were disrupted by sonication in a buffer containing Tris-HCl 50mM pH 7.4, $MgCl_2$ 2mM, CHAPS 4mM, Tween 80 0.5% (v/v) and protease inhibitors (three times, 30 s, duty cycle 50%). The sonicate was centrifuged at 12000 rpm for 30 min at 4 °C to obtain the soluble fraction, which contained the active form of the enzyme. SMT was estimated by densitometric analysis to be approximately 1−2% of the soluble recombinant protein.

A standard SMT activity assay contained 1 mg of protein (which is equivalent to about 10−20 μg of pure enzyme) in the previously mentioned buffer, Tris-HCl 50mM pH 7.4, $MgCl_2$ 2mM, CHAPS 4mM, Tween 80 0.5% (v/v), desmosterol 100 μM, and ^{14}C-*S*-adenosyl-L-methionine 200 μM, 600000 dpm per reaction. The inhibitor was resuspended first in a minimal volume of its corresponding solvent and later added to the reaction mixture as an aqueous solution. The reaction was started with the enzyme. Incubations were performed at 30°C for 45 min and terminated with 0.5 mL of KOH 10% dissolved in 80% (v/v) methanol. To quantify the efficiency of the extraction, ^{3}H-cholesterol (3 mg, 30000 dpm per reaction) was added as an internal standard. The methylated sterol product was extracted three times with 1 mL of hexane and the resulting organic layer washed once with Tris-HCl buffer to remove the ^{14}C-*S*-adenosyl-L-methionine that was not incorporated. One milliliter of the organic layer was added to 10 mL of hydrofluor and the radioactivity measured in a scintillation counter.

Negative controls were reactions containing soluble extracts of *E. coli* BL21(DE3)pLysS cells transformed with pET28 (not overexpressing *L. major* SMT). No activity was observed using this as an enzyme source.

IC_{50} values were obtained from plots of percentage of inhibition versus concentration of inhibitor and were calculated by the equation:

$$\text{percentage inhibition} = \frac{100 \times \text{concentration of inhibitor}}{IC_{50} + \text{concentration of inhibitor}}$$

Studies of Lipid Composition. *L. mexicana amazonensis* promastigotes were cultivated in LIT medium supplemented with lactabulmin and 10% fetal calf-serum (Gibco) (3) at 26 °C, without agitation. The cultures were initiated with a cell density of 2 × 10^6 cells per mL, and the drug was added at a cell density of 0.5−1 × 10^7 cells per mL. Cell densities were measured with an electronic particle counter (model ZBI; Coulter Electronics Inc., Hielah, Fla.) and by direct counting with a haemocytometer. Cell viability was followed by Trypan blue exclusion using light microscopy.

For the analysis of the effects of drugs on the lipid composition of promastigotes, total lipids from control and drug-treated cells were extracted and fractionated into neutral and polar lipid fractions by silicic acid column chromatography and gas−liquid chromatography.[15−18] The neutral lipid fractions were

first analyzed by thin-layer chromatography (on Merck 5721 silica gel plates with heptane−isopropyl ether−glacial acetic acid [60:40:4] as developing solvent) and conventional gas−liquid chromatography (isothermic separation in a 4-m glass column packed with 3%OV-1 on Chromosorb 100/200 mesh, with nitrogen as carrier gas at 24 mL/min and flame ionization detection in a Varian 3700 gas chromatograph). For quantitative analysis and structural assignments, the neutral lipids were separated in a capillary high-resolution column (25 m × 0.20 mm i.d. Ultra-2 column, 5% phenylmethylsiloxane, 0.33 μm film thickness) in a Hewlett-Packard 6890 Plus gas chromatograph equipped with a HP5973A mass sensitive detector. The lipids were injected in chloroform, and the column was kept a 50 °C for 1 min, and then the temperature was increased to 270 °C at a rate of 25 °C min^{-1} and finally to 300 °C at a rate of 1 °C min^{-1}. The carrier gas (He) flow was kept constant at 0.5 mL min^{-1}. Injector temperature was 250 °C, and the detector was kept at 280 °C.

In Vitro Assays. Promastigote and Epimastigote Assays. The MHOM/BR/75/Josefa strain of *L. amazonensis* isolated from a patient with diffuse cutaneous leishmaniasis was used in the present study. It was maintained by passage in Balb/C mice. Promastigotes were cultured in Warren's medium (brain heart infusion plus hemin and folic acid) supplemented with 10% fetal bovine serum, at 25 °C. Epimastigotes of Y strain of *T. cruzi* were maintained in liver infusion tryptose medium[19] supplemented with 10% fetal bovine serum, at 28 °C. Parasites in exponential growth phase were cultivated in 96-multiwell plates and incubated with compounds at a concentration of 1.0 μM for 96 h. After 96 h the parasites were harvested, and the growth was determined microscopically by counting parasite numbers in a Neubauer hemocytometer. Inhibition was determined using the function:[20]

$$I = \frac{I_{max}C}{IC_{50} + C}$$

where I is the percent (%) of inhibition, $I_{max} = 100\%$ inhibition, C is the concentration of the inhibitor, and IC_{50} is the concentration for 50% growth inhibition.

***L. amazonensis* Amastigotes.** Peritoneal macrophages from Swiss mice were harvested by washing with DMEM (Sigma Chemical Co., St. Louis). Macrophages were plated in 24-well tissue culture chamber slides and allowed to adhere for 24 h at 37 °C in 5% CO_2. Adherent macrophages were infected with stationary phase promastigotes at a ratio of 1:10 at 35 °C in a 5% CO_2 for 2 h. After this time, the nonphagocytosed parasites were removed by washing, and infected cultures were incubated for 24 h with medium without drugs. The drugs were added after 24 h at a concentration of 1.0 μM, and the cultures were incubated with the compounds for 96 h. The cultures were fixed in Bouin solution and stained with Giemsa's stain. The percentage of infected cells was determined. IC_{50} was determined using the function mentioned above.[20]

L. donovani. Peritoneal exudate macrophages were harvested from CD1 mice, 24 h after starch induction. After being washed, the macrophages were dispensed into Lab-tek 16-well tissue culture slides and maintained in RPMI1640 + 10% heat-inactivated foetal calf serum (HIFCS) at 37 °C, 5% CO_2/air mixture for 24 h. *L. donovani* (MHOM/ET/67/L82) amastigotes were harvested from an infected Golden hamster spleen and were used to infect the macrophages at a ratio of five parasites: one macrophage. Infected cells were left for a further 24 h and then exposed to drug[21] for a total of 5 days, with the overlay being replaced on day 3.[22] The top concentration for the test compounds was 30μg/mL, and all concentrations were carried out in quadruplicate. On day 5 the overlay was removed, and the slides were fixed (100% methanol) and stained (10% Giemsa, 10 min) before being evaluated microscopically. ED_{50} values were calculated using Ms*xl*fit. The ED_{50} value for the positive control drug, Pentostam, is usually 3−8 μg Sb^V/mL.

T. cruzi. Murine (CD1) peritoneal macrophages were harvested 24 h after starch induction. One hundred microliters was dispensed into 96-well plates at a concentration of 4 × 10^5/mL. After 24 h the cells were infected with *T. cruzi* Tulahuan LAC-Z trypomastigotes, harvested from L6 feeder layer cultures. Twenty four hours later the infected cells were exposed to drug[20] for 3 days. Fifty microliters of 500 μM CPRG: 1% nonidet P-40 was added to each well. The plates were read after 2−5 h, λ570.[23] ED_{50} (ED_{90}) values were calculated using Ms*xl*fit. L6 fibroblasts are also used as host cells.

Cytotoxicity against Vertebrate Cells. Plates were seeded with 100 μL of KB cells at 4 × 10^4/mL, RPMI 1640 + 10% HIFCS and incubated at 37 °C, 5% CO_2, for 24 h. The overlay was removed and replaced by test drugs[20] in fresh medium at 300, 30, 3, and 0.3 μg/mL. The positive control drug was podophyllotoxin (Sigma, UK). Dilutions were carried out in triplicate. Plates were incubated for a further 72 h, at 37 °C, 5% CO_2. The wells were assessed microscopically for cell growth. The overlay was removed, and wells were washed with PBS (pH7.0) × 3. Then 100 μL of PBS + 10 μL of AlamarBlue were added per well and plates incubated for 2−4 h (37 °C, 5% CO_2) before reading at EX/EM 530/585 nm (cutoff 550 nm) in a Gemini plate reader. ED_{50} (ED_{90}) values were calculated compared to blanks and untreated controls.

Electron Microscopy. Control and treated parasites were fixed for 1 h in 2.5% glutaraldehyde (Sigma Chemical Co.) in 0.1 M cacodylate buffer (pH 7.2). After fixation, the cells were postfixed for 30 min in a solution containing 1% OsO_4 and 0.8% potassium ferrocyanide in 0.1 M cacodylate buffer, washed in the same buffer, dehydrated in acetone, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and were observed in a Zeiss 600 electron microscope.

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