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Preparation of transition-state analogues of sterol 24-methyl transferase as potential anti-parasitics

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Abstract—There is an urgent need for new drugs to treat leishmaniasis and Chagas disease. One important drug target in these organisms is sterol biosynthesis. In these organisms the main endogenous sterols are ergosta- and stigmata-like compounds in contrast to the situation in mammals, which have cholesterol as the sole sterol. In this paper we discuss the design, synthesis and evaluation of potential transition state analogues of the enzyme $\Delta^{24(25)}$-methyltransferase (24-SMT). This enzyme is essential for the biosynthesis of ergosterol, but not required for the biosynthesis of cholesterol. A series of compounds were successfully synthesised in which mimics of the S-adenosyl methionine co-factor were attached to the sterol nucleus. Compounds were evaluated against recombinant *Leishmania major* 24-SMT and the parasites *L. donovani* and *Trypanosoma cruzi* in vitro, causative organisms of leishmaniasis and Chagas disease, respectively. Some of the compounds showed inhibition of the recombinant *Leishmania major* 24-SMT and induced growth inhibition of the parasites. Some compounds also showed anti-parasitic activity against *L. donovani* and *T. cruzi*, but no inhibition of the enzyme. In addition, some of the compounds had anti-proliferative activity against the bloodstream forms of *Trypanosoma brucei rhodesiense,* which causes African trypanosomiasis.

1. Introduction

Chagas disease and leishmaniasis are major health problems in the developing world,[1] and increasingly leishmaniasis is found as a complication of AIDS in both the developing world and industrialised world.[2] There is an urgent need for the development of new drugs[3] to treat these diseases, as the existing drugs suffer from poor clinical efficacy (Chagas disease) and increasing problems due to resistance (leishmaniasis). One target of interest is sterol biosynthesis. The main sterol found in mammalian cells is cholesterol, whilst the pathogens which cause Chagas disease and leishmaniasis (*Trypanosoma cruzi* and species of *Leishmania*, respectively), synthesise ergosterol and related 24-alkylated sterols.[4–6] These sterols have differences in their biosynthetic pathway that are attractive for drug design. The presence of ergosterol has been shown to be essential for *T. cruzi*; replacement of ergosterol by cholesterol is not possible for this organism.[7] The ergosterol is thought to have two roles within the parasite; it has a structural role in the cell membrane and is thought to have a 'sparking' or 'hormonal' role similar to that seen in yeast. In *Leishmania* the case is less certain. It has been shown that various inhibitors of sterol biosynthesis show activity against leishmania. For example, inhibitors of 14-α-demethylase and sterol 24-methyltransferase (24-SMT) have been shown to have anti-parasitic activity in vitro and in vivo.[6,8] However, Goad and Chance have shown that it is possible over many generations to replace ergosterol by cholesterol in *L. donovani* promastigotes (promastigotes are the vector form) and still maintain cell viability, albeit under laboratory conditions. *L. donovani* promastigotes were cultured in the presence of azasterol, an inhibitor of 24-SMT, together with

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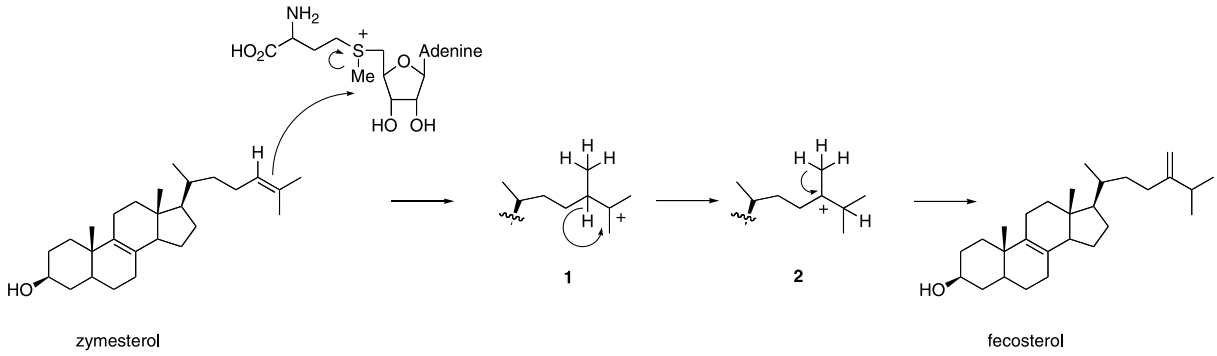


Figure 1.

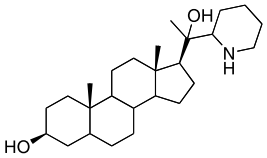


Figure 2. Structure of AZA.

a bis-triazole inhibitor of the the 14α-demethylase. Gradual depletion of 24-alkylsterols was observed and subsequent replacement by Δ^{24}-cholesta-type sterols.[9]

One enzyme which is required for ergosterol biosynthesis is 24-SMT, which has no direct counterpart in mammalian sterol biosynthesis pathways. The proposed mechanism of action of the enzyme is shown in Figure 1.[10–17] The sterol is alkylated on the 24-position by S-adenosylmethionine (SAM), by electrophilic attack of the methyl group on the 24–25 double bond. A number of inhibitors of 24-SMT are known.[19] They have been investigated as inhibitors of yeast and plant 24-SMT. Most of the known inhibitors are mimics of the high-energy intermediates **1** and **2** (Fig. 1) and contain carbocations in the side chain at the 24- or 25-positions. Typically compounds are azasterols, where the side chain is protonated at physiological pH. These presumably bind strongly to the enzyme, as the enzyme increases the rate of reaction by stabilising these high-energy intermediates.

One compound of particular interest is AZA (20-piperidin-2-yl-5α-pregnane-3β,20-diol) (Fig. 2). This compound has been investigated as a potential anti-parasitic compound.[9,20,21]

In this article we describe a strategy in which we have prepared mimics of the transition state (TS). In the TS, there is a molecule of sterol interacting with a molecule of SAM. The binding of these two substrates appears to be independent from the kinetic analysis of the *Candida albicans* enzyme.[11] Also transfer of the methyl group appears to occur directly from SAM to the substrate (concerted) and not via alkylation of the enzyme (ping pong). We decided to prepare compounds that would bind to both the sterol and the SAM binding sites. In order to achieve this, we linked sterol moieties to SAM moieties (Fig. 3). The moieties were coupled to the sterol by either an amide bond or an amine bond. Coupling the entire SAM moiety to the sterol is likely to give rise to compounds that do not have good drug-like properties. Therefore only fragments of the SAM moiety were coupled to the sterol.

These TS mimics should have a number of advantages as an inhibitor: firstly interaction with both the sterol and SAM binding sites should produce stronger binding to the target enzyme. Secondly, one of the disadvantages of current azasterols is that in addition to inhibition of 24-SMT, they also inhibit sterol 24-reductase, an enzyme found in cholesterol biosynthesis. Inhibition of this enzyme is associated with accumulation of desmosterol.[22] In the approach described here, inhibitors should be more specific for 24-SMT, because they bind in both the sterol and SAM binding site, the latter is not present in the reductase.

We proposed to link the amines to the sterol via an amide or an amine bond. The amide linker should provide a neutral linker for coupling the amine to the sterol.

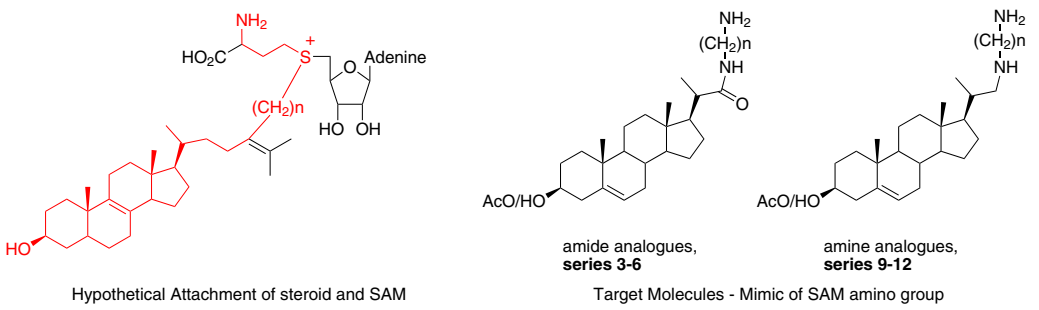


Figure 3.

The amine linker is likely to be protonated at physiological pH and should be a mimic of the high-energy intermediates of type **1** or **2** (Fig. 1), which may produce an additional interaction with the enzyme active site.

We have recently reported the results of the biological assays for series **9**–**12** (amine analogues).[23] In this paper we describe the preparation of these compounds, the synthesis and biological assays for series **3**–**6** (amide analogues), and a comparison of the two series.

2. Chemistry

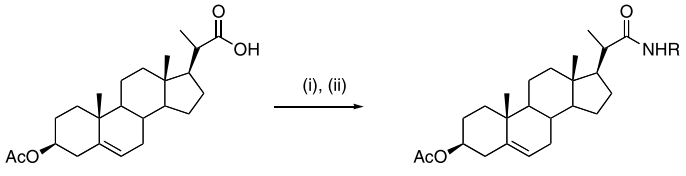
2.1. Preparation of amides

To establish the chemical methodology, some simple amines were coupled to the sterol by an amide bond. A variety of simple amides were chosen (butyl, benzyl, piperidine) to investigate the effect of size and aromaticity on the activity of the compounds. Once the methodology was optimised, coupling of diamines with the sterol nucleus was conducted.

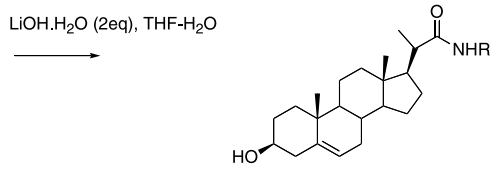
The starting material was the commericially available 3β-acetoxy-5-cholenic acid. Several methods for coupling the sterol to the amines were investigated. The best method seemed to use 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and HOBt. Amines were coupled as shown in Scheme 1 to give the corresponding amides **3a**–**c**.

However, when the same methodology was applied to diamines ($H_2N(CH_2)_nNH_2$), a number of products were isolated (Scheme 2). The major product was the dimer (**4f** or **4g**), due to reaction of the required products (**4b** and **4d**) with excess activated 3β-acetoxy-5-cholenic acid. The required products were isolated in low yield, together with another product, shown to be the guanidine derivatives **4h** and **4i**. This latter compound is thought to be due to attack of the product on the TBTU. This has been observed before when using an excess of uronium-based activation agents in peptide coupling reactions.[24]

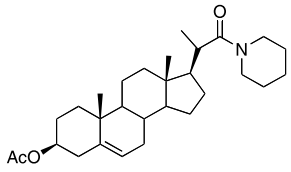
In order to circumvent this problem, the method was modified to give procedure B. The amount of TBTU



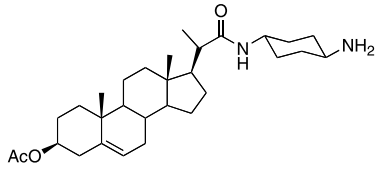
3a, R = Bu%, 70, procedure A
3b, R = N-piperidyl, 80%, procedure A
3c, R = CH_2Ph, 80%, procedure A
4a, R = $(CH_2)_4NH_2$, 55%, procedure B
4b, R = $(CH_2)_6NH_2$, 60%, procedure B
4c, R = $(CH_2)_8NH_2$, 40%, procedure B
4d, R = $(CH_2)_{10}NH_2$, 60%, procedure B
4e R = 1,4-*trans*-$(C_6H_{10})NH_2$, 70%, procedure B



5a, R = Bu, 97%
5b, R = N-piperidyl, 70%
5c, R = CH_2Ph, 65%
6a, R = $(CH_2)_4NH_2$, 95%
6b, R = $(CH_2)_6NH_2$, 90%
6c, R = $(CH_2)_8NH_2$, 75%
6d, R = $(CH_2)_{10}NH_2$, 90%
6e, R = 1,4-*trans*-$(C_6H_{10})NH_2$, 70%

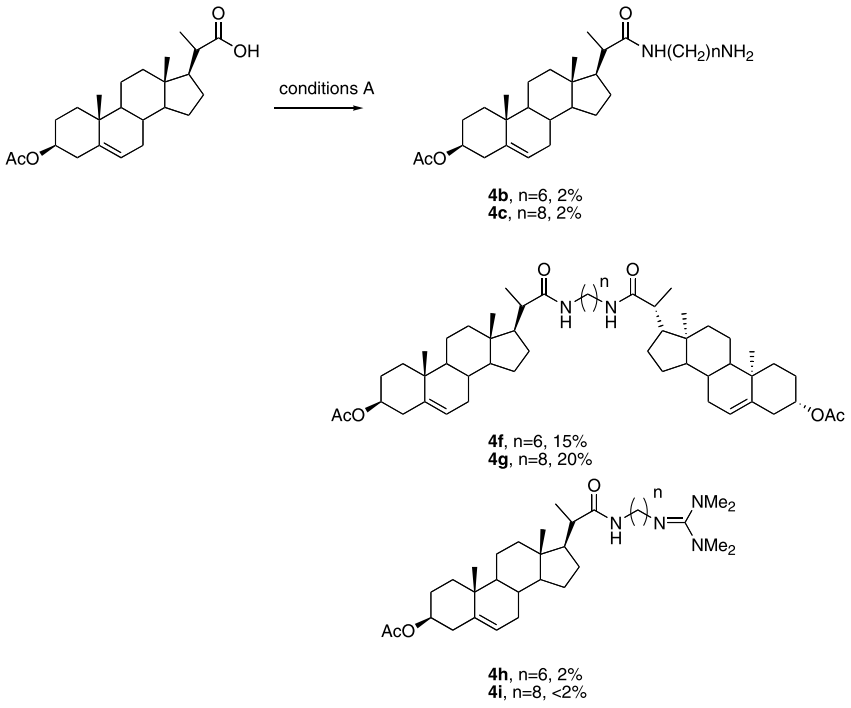


structure of 3b



structure of 4e

Scheme 1. Reagents and conditions: Procedure A: (i) DIPEA (3 equiv), TBTU (2 equiv), HOBT (2 equiv), DMF, rt 30 min; (ii) R′R″NH (2 equiv), rt. Procedure B: (i) DIPEA (3 equiv), TBTU (1.1 equiv), HOBT (1.1 equiv), DMF, rt 30 min; (ii) $H_2N(CH_2)_nNH_2$ (4 equiv), rt.



Scheme 2.

was reduced to 1 equiv which should avoid the coupling of terminal amino group with excess TBTU to give guanidines. Then the activated acid was added dropwise to a solution of the diamine, in order to keep the diamine in excess, preventing formation of the dimers. This strategy was successful and led to formation of the required compounds **4a**–**e** in good yields (Scheme 1).

The acetate groups were then deprotected by treatment with lithium hydroxide in THF–water.[25]

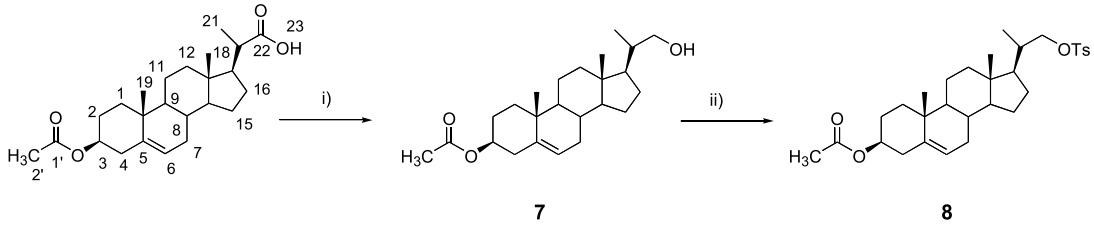
2.2. Preparation of amines

The starting point for the synthesis was the commercially available sterol 3β-acetoxy-5-cholenic acid. This was reduced to alcohol **7** using borane-dimethyl sulfide.[26,27] This agent turned to be selective for the carboxylic acid functionality, and did not seem to affect the acetate group or the double bond in the sterol. The temperature and the amount of reagent used in the reduction appeared to affect the final yield. After a number of different experiments, the optimum reaction conditions were found to be those shown in Scheme 3. The alcohol was then tosylated following a standard procedure[28] for the introduction of the amines.

Synthesis of the azasterols was conducted by coupling the tosyl sterol **8** with amines varying in the nature and length of the side chain. Initially it was decided to prepare some simple amines to establish the methodology and to derive some structure–activity relationship data. Butylamine, piperidine and benzyl were chosen as they represent straight-chain, lipophilic and aromatic substituents. Once the method was optimized, coupling of diamines with the sterol nucleus was conducted.

Displacement of the tosyl group by the different amines was achieved by four different methods (Scheme 2). Firstly, the tosyl-ether was reacted with a large amount of excess amine (10–20 equiv) and diisopropylethylamine (DIPEA) in DMF.[29] Secondly, the displacement was carried out using a small amount of excess amine (3–4 equiv), an inorganic base (potassium carbonate) and the phase transfer catalyst tetrabutylammonium iodide in DMF.[30]

Due to the low yield of the reactions when attaching the diamines, it was decided to try an alternative method that used milder conditions, refluxing ethanol.[31] On account of the low solubility of the tosylate **8** in ethanol,



Scheme 3. Reagents and conditions: (i) $BH_3{\cdot}SMe_2$ (1 M CH_2Cl_2) (1 equiv), THF, −10 °C → 0 °C, 8 h, 57%; (ii) TsCl (2 equiv), pyridine:DCM (1:1), 0 °C → 4 °C, 24 h, 90%.

the third method was modified and DMF was added as co-solvent in the reaction in an attempt to increase the yield of the reaction. However, when DMF was added, not only the desired compound was obtained but also a second product was stemming from the reaction of the free terminal amino group in the side chain of the sterol with DMF (Scheme 5).

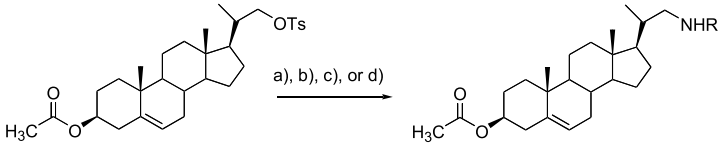
Analogues **11a**–**12e** with a free hydroxyl group in position 3β of the sterol were prepared by the hydrolysis of the corresponding acetates using lithium hydroxide (Scheme 4).[25]

3. Biology

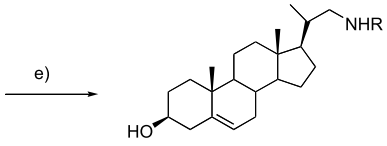
It has been proposed by Nes[10] that inhibitors of 24-SMT must have a free 3β-OH for binding to the active site. The compounds protected with an acetate at the 3β-OH were also assayed for activity, as the acetate group will probably be cleaved by esterases in vitro or in vivo to yield the active agent.

3.1. Enzyme inhibition

Compounds were evaluated against the recombinant *L. major* 24-SMT (Table 1). The enzyme was over expressed in *E. coli*, and the enzyme assays were conducted using *E. coli* cell-free extracts containing soluble protein as the enzyme source. Essentially none of the amide-linked compounds, either the 3β-OH protected with acetate (compounds **3** and **4**) or those with the acetate removed (compounds **5** and **6**) showed significant inhibition of the enzyme. For the amine-linked series, those protected with an acetate at the 3β-OH (series **9** and **10**) showed little inhibition as predicted by Nes. However, the compounds with the 3β-OH deprotected (**11** and **12**) showed good inhibition of the enzyme.

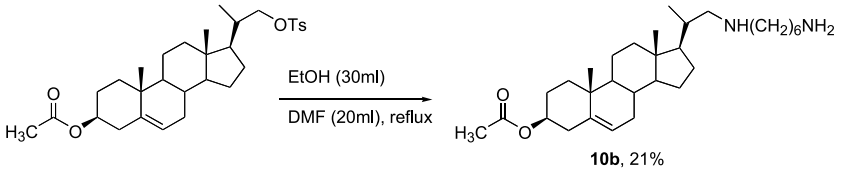


9a, R = butyl, 86% (method a)
9b, R = piperidyl, 51% (method a); 46% (method b)
9c, R = benzyl, 49% (method b)
10a, R = $(CH_2)_4NH_2$, 36% (method b)
10b, R = $(CH_2)_6NH_2$, 24% (method d)
10c, R = $(CH_2)_8NH_2$, 36% (method a); 37% (method b); 56% (method c)
10d, R = $(CH_2)_{10}NH_2$, 47% (method b); 10% (method b)
10e, R = 1,4-*trans*-$(C_6H_{10})NH_2$, 50% (method b)



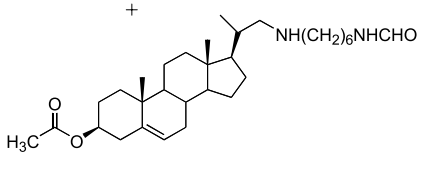
11a, R = butyl, 78%
11b, R = piperidyl, 84%
12c, R = $(CH_2)_8NH_2$, 70%
12d, R = $(CH_2)_{10}NH_2$, 93%
12e, R = 1,4-*trans*-$(C_6H_{10})NH_2$, 60%

Scheme 4. Reagents and conditions: (a) Amine (10–20 equiv), DIPEA (3 equiv), DMF, 60 °C, 48 h; (b) Amine (3–4 equiv), K_2CO_3 (1.5 equiv), $Bu_4N^+I^-$ (cat.), DMF, 100 °C, 4 h; (c) Diamine (4 equiv), EtOH (40 ml), reflux; (d) Diamine (4 equiv), EtOH (30 ml), DMF (20 ml), reflux; (e) LiOH.H_2O (2 equiv), THF: H_2O (3:1), 50 °C, 24 h.



10b, 21%

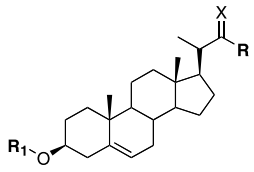
+



10f, 10%

Scheme 5.

Table 1. Inhibition of *L. major* 24-SMT and anti-parasitic activities against intracellular *T. cruzi* amastigotes, intracellular *L. donovani* amastigotes, *T. b. rhodesiense* trypomastigotes and mammalian cells



	R_1	X	R	WSP	IC_{50} (μM) *L. major* 24-SMT	ED_{50} (μM) *T. cruzi*	ED_{50} (μM) *L. donovani*	ED_{50} (μM) *T. brucei*	TD_{50} (μM) Toxicity
3a	Ac	O	NHbutyl	564	>100	>68.00	>68.00	33.00	>676
3b	Ac	O	Piperidyl	566	>100	>66.00	>66.00	>66.00	>658
3c	Ac	O	$NHCH_2Ph$	568	>100	>62.80	>62.80	>62.80	>628.03
4a	Ac	O	$NH(CH_2)_4NH_2$	697	>100	>65.00	>65.00	16.15	162.86
4b	Ac	O	$NH(CH_2)_6NH_2$	695	>100	>61.00	35.56	>61.00	131.08
4c	Ac	O	$NH(CH_2)_8NH_2$	694	51	38.65	58.28	15.50	110.73
4d	Ac	O	$NH(CH_2)_{10}NH_2$	696	>100	>55.00	10.63	2.52	53.42
4e	Ac	O	*Trans*-1,4-$NHC_6H_{10}NH_2$	698	>100	48.48	14.30	<2.29	48.3
4f	Ac	O	$NH(CH_2)_6$NH-Sterol	692	>100	>35.00	>35.00	19.60	<0.35
4g	Ac	O	$NH(CH_2)_8$NH-Sterol	691	N.D.	>34.00	>34.00	21.72	>339
4h	Ac	O	$NH(CH_2)_6NC(N(CH_3)_2)_2$	693	N.D.	8.49	22.81	4.97	75.63
5a	H	O	NHbutyl	565	>100	>75.00	>75.00	>75.00	>747
5b	H	O	Piperidyl	567	>100	>69.00	>72.00	>72.00	>725
5c	H	O	$NHCH_2Ph$	563	>100	>70.00	>70.00	>70.00	>688
6a	H	O	$NH(CH_2)_4NH_2$	702	93	>72.00	>72.00	15.20	117.00
6b	H	O	$NH(CH_2)_6NH_2$	700	>100	48.57	38.90	<2.50	108.60
6c	H	O	$NH(CH_2)_8NH_2$	699	>100	61.55	44.42	63.46	155.47
6d	H	O	$NH(CH_2)_{10}NH_2$	701	>100	>60.00	16.11	>20.00	51.70
6e	H	O	*Trans*-1,4-$NHC_6H_{10}NH_2$	703	>100	>67.80	13.08	2.51	19.0
9a	Ac	H_2	NHbutyl		>100	21.41	7.70	0.2	6.30
9b	Ac	H_2	Piperidyl		>100	16.00	2.50	3.93	83
9c	Ac	H_2	$NHCH_2Ph$		>100	>64.70	8.90	0.47	10.56
10a	Ac	H_2	$NH(CH_2)_4NH_2$		16	3.37	>67.46	8.7	14.8
10b	Ac	H_2	$NH(CH_2)_6NH_2$			57.11	>63.00	2.31	40
10c	Ac	H_2	$NH(CH_2)_8NH_2$		35	4.49	3.85	3.27	51.7
10d	Ac	H_2	$NH(CH_2)_{10}NH_2$			56.73	45.96	27.08	165
10e	Ac	H_2	*Trans*-1,4-$C_6H_{10}NH_2$		>100	8.9	12.30	3.95	11.3
10f	Ac	H_2	$NH(CH_2)_6NHCHO$			>60.00	34.57	1.64	<0.60
11a	H	H_2	NHbutyl		0.97	<0.95	3.45	1.24	36.2
11b	H	H_2	Piperidyl		6.4	10.28	>75	5.7	36
12c	H	H_2	$NH(CH_2)_8NH_2$		2	10.9	5.88	<2.4	109
12d	H	H_2	$NH(CH_2)_{10}NH_2$			33.49	>61.00	25.7	—
12e	H	H_2	*Trans*-1,4-$NHC_6H_{10}NH_2$		45	19.2	30.35	1.45	8.6
AZA					0.028	7.4	8.9	3.3	11.9

Data for series **9**–**12** have been reported previously.[23]

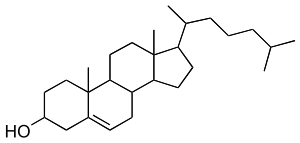
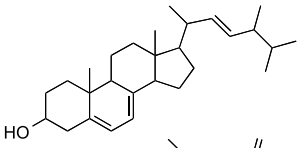
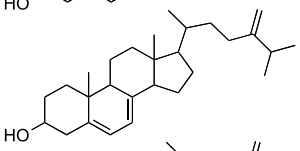
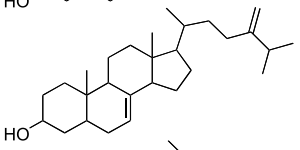
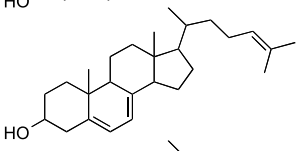
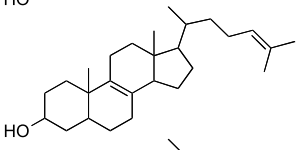
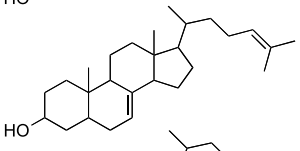
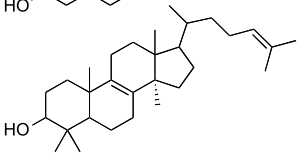
It is interesting to compare compounds **5a** and **5b** with **11a** and **11b**. This indicates that the presence of a positively charged functionality in the side chain is essential for inhibition of the enzyme, as in compounds **11a** and **11b** there is an amino group which is positively charged at physiological pH whilst, in compounds **5a** and **5b** the amine is replaced by an amide. Although the amide is polar it is not positively charged.

3.2. Sterol composition

A number of compounds were also investigated for their ability to disrupt sterol biosynthesis in intact *Leishmania mexicana* promastigotes (Table 2). Essentially the parasites are cultured in the presence of inhibitor and the effect on sterol composition is monitored. Inhibitors of 24-SMT will reduce the proportion of 24-alkylated sterols (e.g., ergosterol, 5-dehydroepisterol, episterol), whilst there is a concomitant increase in the levels of non-alkylated, cholesta-derived sterols.[21,23]

The amide-based compounds (**5a**–**b**) showed little effect on sterol composition at 1 μM concentration. Compound **5c** showed a small decrease in the levels of 24-alkylated sterols. These results are consistent with their lack of activity against enzyme. In contrast, the amino derived compounds **9a** and **11a** showed a significant effect on the levels of 24-alkylated azasterols (Table 3).[23] They caused a large reduction in the levels of the 24-alkylated sterols (ergosta-5,24(24^1)-dien-3β-ol, 5-dehydroepisterol and episterol) compared to control values at submicromolar concentrations with a concomitant increase in the levels of non-alkylated (cholesterol-like

Table 2. Effects of amide-based compounds on the free sterol composition of *Leishmania mexicana* promastigotes

		Compound			
		Control	**5a**	**5b**	**5c**
Concentration (μM)		0	1	1	1
Cholesterol (exogenous)		9.8	13.1	10.9	11.3
Ergosterol		6.7	7.6	3.3	6.1
Ergosta-5,7,24(24[1])-trien-3β-ol (5-dehydroepisterol)		73.0	66.0	72.2	48.8
Ergosta-7,24(24[1])-dien-3β-ol (episterol)		8.3	8.5	10.0	5.7
Cholesta-5,7,24-trien-3β-ol		<1	1.2	<1	19.0
Cholesta-8,24-dien-3β-ol (zymosterol)		<1	<1	<1	2.1
Cholesta-7,24-dien-3β-ol		<1	1.8	1.9	4.8
Lanosterol (exogenous)		2.1	1.8	1.7	2.2

sterols) which is consistent with inhibition of 24-SMT in the intact parasites.

3.3. In vitro anti-proliferative assays

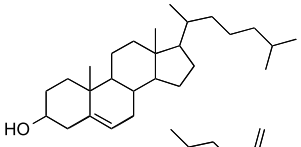
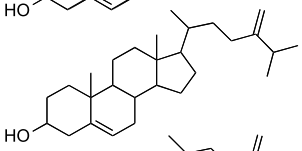
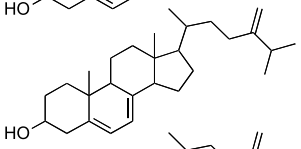
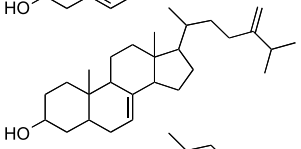
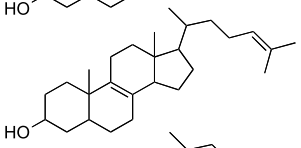
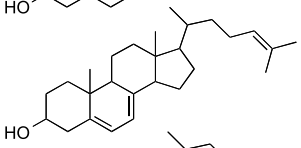
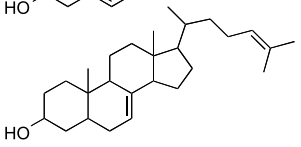
Compounds were assayed against the clinically relevant stage of the parasites *T. cruzi* and *L. donovani* (Table 1). In addition, compounds were investigated for their toxicity against mammalian cells (KB cells). *T. cruzi* and *Leishmania* undergo complex life cycles with each stage having different metabolism. It is important to ensure that compounds are active against the clinically relevant stage of the parasite. In the case of *T. cruzi* and *L. donovani* parasites, it is the intra-cellular amastigote form. Compounds were also evaluated against the related parasite *T. b. rhodesiense* as the trypomastigote form. In this organism, ergosterol biosynthesis is reported in the vector stage of the parasite,[32] but sequestration of sterols via LDL receptors is thought to occur in the bloodstream form trypomastigote.

3.4. Amide-linked series (3–6)

For the amide-linked series of compounds, those with a simple alkyl substituent (series **3** and **5**) showed no significant activity or toxicity with the acetate protecting group on the 3β-OH position (**3a**–**c**) or as the free 3β-OH (**5a**–**c**). Similarly the dimeric compounds **4f** and **4g** showed no activity as would be expected, except slight activity against *T. brucei*.

For the diamines, the compounds showed weak activity against *T. cruzi* with or without an acetate protecting group on the 3β-OH (compounds **4** and **6**). The only compound which showed some activity against *T. cruzi* was the guanidine analogue **4h**. However, compounds

Table 3. The effects of compounds, **9a** and **11a** on the free sterol composition of *L. amazonensis* promastigotes[a23]

		Compound						
		Control	**9a**			**11a**		
Concentration (μM)		0	0.01	0.1	1.0	0.01	0.1	1.0
Sterol detected								
Cholesterol (exogenous)		9.6	9.2	10.8	8.5	6.7	7.6	11.2
Ergosta-5,24(24[1])-dien-3β-ol		n.d.	n.d.	19.7	2.1	n.d.	n.d.	n.d.
Ergosta-5,7,24(24[1])-trien-3β -ol(5-dehydroepisterol)		68.0	62.8	7.0	3.1	43.4	2.6	n.d.
Ergosta-7,24(24[1])-dien-3β-ol(episterol)		17.0	22.7	15.8	n.d.	19.5	2.8	n.d.
Cholesta-8,24-dien-3β-ol(zymosterol)		n.d.	n.d.	29.9	83.8	n.d.	6.2.	84.4
Cholesta-5,7,24-trien-3β-ol		2.6	3.2	5.2	2.6	24.2	51.9	n.d.
Cholesta-7,24-dien-3β-ol		2.8	2.1	11.4	4.4	6.2	28.9	4.4

[a] Sterols were extracted from cells exposed to the indicated drug concentration for 96 h; they were separated from polar lipids by silicic acid column chromatography and analyzed by quantitative capillary gas-liquid chromatography and mass spectrometry. Composition is expressed as mass percentages. n.d., not detected. Data reported previously but reproduced here for comparative purposes.[23]

4d, **4e** and **6d**, **6e**, showed weak activity against *L. donovani*, which were the longer and the more lipophilic amino substituents that were investigated. In contrast, against *T. b. rhodesiense*, a number of compounds showed growth inhibition, in general the more lipophilic compounds showing the greatest inhibition. The compounds showing the greatest inhibition were **4d**, **4e** and **4h** from the acetate-protected derivatives; and the hexyl (**6b**) and *trans*-cyclohexylamino (**6e**) derivatives being the most active for the unprotected series. These derivatives show ED_{50} values less than 10 μM.

Given the lack of activity of these compounds against the *L. major* enzyme, the activity of compounds against the parasites is probably associated with a mechanism of action different from inhibition of the 24-SMT.

3.5. Amine-linked series (9–12)

The activity of the amine-linked series of compounds contrasts with that of the amide-linked series. For the amines, those with a simple alkyl substituent (series **9** and **11**) showed generally strong anti-parasitic activity. There were changes in the activity on removal of the acetate protecting group from the 3β-OH; in some cases the activity increased and in some cases decreased. Compound **9a**–**c** showed relatively low activity against *T. cruzi*, the activity increasing on removal of the acetate (**11a**–**b**). But against *L. donovani* and *T. b. rhodesiense*, compounds **9** and **11** showed good inhibition of growth, with compound **9a** being particularly potent against *T. b. rhodesiense* (ED_{50} 0.2 μM) and compound **9b** against *L. donovani* (ED_{50} 2.5 μM).

Addition of the alkylamino side chain (compounds **10** and **12**) gave rise to compounds showing moderate growth inhibition of the parasites. The presence of the acetate on the 3β-OH position appeared to have little effect on activity. The compounds were selective for the parasites over the mammalian cells.

4. Discussion

Simple amide-linked derivatives (**3a**–**c** and **5a**–**c**) showed no activity against either 24-SMT or the parasites. In the case of **5a**–**c** there was also no effect on sterol composition of the parasites at 1 μM concentration. This implies that the compounds are not active against 24-SMT. Presumably the partial positive charge on the nitrogen due to delocalisation of the amide bond is not sufficient to cause strong interaction with the enzyme active site.

On attaching functionality found in SAM (the amino group) no inhibition of the enzyme was observed in the series with the 3β-OH protected with acetate (**4a**–**e**) or with the 3β-OH unprotected (**6a**–**e**). This implies that the terminal amino groups are not undergoing significant interaction in the SAM binding pocket, perhaps due to the amide bond reducing the conformational flexibility of the compound.

Interestingly some of these compounds showed moderate to weak to moderate activity against parasites with the greatest activity against *T. brucei*. The lack of inhibition of the *L. major* enzyme suggests that the anti-parasitic activity is due to some other mode of action than inhibition of 24-SMT.

When the amide functionality (series **3**–**6**) was replaced with an amine in the side chain (series **9**–**12**) there was a clear inhibitory effect on the recombinant enzyme and a significant increase in the anti-parasitic activity. This anti-parasitic activity was independent of the presence of an acetate protecting group on the 3β-OH. Presumably this acetate group is readily removed by esterases present, to give activity against the enzyme. However, it is possible that there are other modes of action.

These results allow us to draw some conclusions on the structure–activity relationships required for inhibition of 24-SMT. Firstly, an amino group is required in the sterol side-chain rather than an amide (compare series **9**–**12** with **3**–**6**). Secondly, for strong inhibition of the enzyme, the 3β-OH group cannot be protected. However, if an acetate protecting group is placed at this position, hydrolysis occurs in cellular systems, leading to release of the unprotected compound that can then inhibit 24-SMT.[23]

The activity of some of the azasterols described here against *T. b. rhodesiense* trypomastigotes was unexpected. According to the literature,[32–34] whilst the vector form of the parasite biosynthesises ergosterol in a similar way to *Leishmania* and *T. cruzi*, the bloodstream form (bsf) of *T. b. rhodesiense* is unable to biosynthesise sterols de novo but takes up cholesterol via LDL receptors. The activity of these compounds against bloodstream form *T. b. rhodesiense* could imply that ergosterol biosynthesis is still important in the bloodstream form of the parasite, or that the compounds have an alternate mechanism of action.

In summary we have prepared some potential transition state analogues as potential inhibitors of 24-SMT. Compounds with an amino-linker in the side chain and a free 3β-OH group gave inhibition of the *L. major* 24-SMT and had marked anti-parasitic activity (series **11** and **12**). In contrast, compounds with an amide-linker in the side chain were inactive (series **5** and **6**) demonstrating the importance of the positive charge in the sterol side chain. In series **10**, there is an acetate in the 3β-OH position. However, the compound gave some inhibition of the 24-SMT. This may be due to additional interactions with the primary amine of the side chain with the SAM binding pocket of the enzyme, overcoming the lack of interaction at the 3β-OH. The amine-linked series of compounds protected with an acetate at the 3β-OH (series **9**) did not inhibit the enzyme directly. However, in cell culture these compounds inhibited the enzyme presumably due to hydrolysis of the acetates by esterases.[23] Furthermore, many of the compounds inhibited the growth of *T. b. rhodesiense*, probably by some mechanism other than inhibition of 24-SMT. These compounds represent good drug leads for further development.

5. Experimental

All reactions were carried out under nitrogen atmosphere and they were monitored by TLC using pre-coated silica gel 60 F_{254} plates (Merck). The solvents used in the reactions were purchased from Aldrich or Fluka in SureSeal bottles. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Brüker Avance DPX 300 MHz spectrometer, operating at 300 and 75 MHz, respectively. Chemical shifts are reported downfield in parts per million and coupling constants (*J* values) are in Hertz. IR spectra were recorded on a Perkin Elmer 1600 series FTIR spectrometer. Low resolution mass spectra (LRMS) were recorded on a Fison VG Platform II spectrometer using the Electrospray (ES) (methanol as solvent) or Atmospheric Pressure Chemical Ionization (APCI) (dichloromethane as solvent) technique. High resolution mass spectra (HRMS) were determined by the EPSRC Mass Spectroscopy Center (Swansea, UK) using ES technique. Elemental analysis were determined on a Perkin-Elmer 240C elemental analyser.

5.1. Procedure A

A solution of 3β-acetoxy-5-cholenic acid (1.00 g, 2.57 mmol), DIPEA (0.998 g, 7.72 mmol), TBTU (1.653 g, 5.14 mmol) and HOBt (0.695 g, 5.14 mmol) in dry DMF (35 ml) was stirred at room temperature for 30 min to form the activated benzotriazol-ester. Two equivalents of butylamine, piperidine and benzylamine were added to the above mixture and stirred overnight at room temperature. The resulting solution was diluted with chloroform, washed with water, dried over sodium sulfate and reduced in vacuo. Chromatography over silica gel [EtOAc/hexane (10% → 30%)] afforded compounds **3a**–**3c** as white solids.

5.1.1. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-butylamine (3a). 0.802 g, 70%; mp 183–185 °C; $R_f = 0.44$ (40% EtOAc/hexane); IR (KBr) 3323 (CO*N*H

str), 2940 (CH), 1732 (CH_3CO), 1645 (*CO*NH), 1543 (NHδ), 1458 (CH_2), 1371(CH_3), 1248 (C–O), 1039 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.73 (3H, s, 18-CH_3), 0.97 (3H, t, $J = 7$, 27-CH_3), 1.05 (3H, s, 19-CH_3), 1.22 (3H, d, $J = 7$, 21-CH_3), 1.27–2.04 (23H, 9× CH_2, 5× CH), 2.07 (3H, s, 2′-CH_3), 2.36 (2H, d, $J = 7$, 4-CH_2), 3.26 (2H, m, 24-CH_2), 4.64 (1H, m, 3-CH), 5.41 (1H, d, $J = 5$, 6-CH), 5.49 (1H, m, 23-NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.9 (18-CH_3), 12.5 (27-CH_3), 14.2 (21-CH_3), 18.1 (19-CH_3), 19.7 (2′-CH_3), 20.5 (CH_2), 21.4 (CH_2), 21.9 (CH_2), 24.7 (CH_2), 27.9 (CH_2), 28.1 (CH_2), 32.1 (CH_2), 32.2 (CH_2), 32.3 (CH), 37.0 (10-C), 37.4 (CH_2), 38.5 (CH_2), 39.4 (12-CH_2), 39.9 (24-CH_2), 42.7 (13-C), 45.4 (20-CH), 50.3 (9-CH), 53.2 (17-CH), 56.6 (14-CH), 74.3 (3-CH), 122.9 (6-CH), 140.0 (5-C), 170.9 (1′-C=O), 176.9 (22-C=O); MS ($APCI^+$) *m*/*z* (rel intensity) 444 ($M + H^+$, 100), 383.9 ($M-AcO^+$, 40); HRMS calcd for $C_{28}H_{46}NO_3$ ($[M + H]^+$): 444.3477; found: 444.3468; Anal. Calcd for $C_{28}H_{45}NO_3$: C, 75.80; H, 10.22; N, 3.16; Found: C, 75.48; H, 10.14; N, 3.17.

5.1.2. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-piperidylamine (3b). 0.948 g, 81%; $R_f = 0.42$ (40% EtOAc/hexane); IR (KBr) 2931 (CH), 1729 (CH_3CO), 1634 (*CO*NH), 1434 (CH_2), 1368 (CH_3), 1247 (C–O), 1120, 1037 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.80 (3H, s, 18-CH_3), 1.10 (3H, s, 19-CH_3), 1.20 (3H, d, $J = 7$, 21-CH_3), 1.26–2.06 (24H, 10× CH_2, 4× CH), 2.11 (3H, s, 2′-CH_3), 2.40 (2H, d, $J = 7$, 4-CH_2), 2.84 (1H, m, 20-CH), 3.60 (4H, m, 24-CH_2, 25-CH_2), 4.68 (1H, m, 3-CH), 5.45 (1H, d, $J = 5$, 6-CH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.7 (18-CH_3), 17.9 (21-CH_3), 19.7 (19-CH_3), 21.4 (2′-CH_3), 21.9 (11-CH_2), 24.8 (CH_2), 25.2 (CH_2), 26.8 (CH_2), 28.1 (CH_2), 28.2 (CH_2), 32.2 (CH_2), 32.3 (CH), 37.0 (10-C), 37.4 (CH_2), 38.5 (12-CH_2), 39.9 (24-CH_2, 25-CH_2), 42.6 (13-C), 50.3 (9-CH), 53.2 (17-CH), 56.5 (14-CH), 74.6 (3-CH), 123.0 (6-CH), 140.0 (5-C), 170.9 (1′-C=O), 175.4 (22-C=O); MS ($APCI^+$) *m*/*z* (rel intensity) 455 (M^+, 70), 396 ($M-AcO^+$, 100); HRMS calcd for $C_{29}H_{46}NO_3$ ($[M + H]^+$): 456.3477; found: 456.3483; Anal. Calcd for $C_{29}H_{45}NO_3$: C, 76.44; H, 9.95; N, 3.07; Found: C, 76.17; H, 9.92; N, 3.01.

5.1.3. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-benzylamine (3c). 0.988 g, 80%; mp 186–187 °C; $R_f = 0.5$ (40% EtOAc/hexane); IR (KBr) 3336 (CO*NH* str), 2936 (CH), 1731 (CH_3CO), 1646 (*CO*NH), 1534 (NHδ), 1451 (CH_2), 1370 (CH_3), 1247 (C–O), 1037, 739, 696 (ArCH) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.78 (3H, s, 18-CH_3), 1.11 (3H, s, 19-CH_3), 1.32 (3H, d, $J = 7$, 21-CH_3), 1.35–2.06 (19H, 7× CH_2, 5× CH), 2.12 (3H, s, 2′-CH_3), 2.42 (2H, d, $J = 7$, 4-CH_2), 4.52 (2H, dd, $J = 5$, $J = 5$, 24-CH_2), 4.70 (1H, m, 3-CH), 5.47 (1H, d, $J = 5$, 6-CH), 5.90 (1H, 23-NH), 7.41 (5H, m, 26-CH, 27-CH, 28-CH, 29-CH, 30-CH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.5 (18-CH_3), 18.1 (21-CH_3), 19.7 (19-CH_3), 21.4 (2′-CH_3), 21.9 (11-CH_2), 24.8 (CH_2), 28.0 (CH_2), 28.2 (CH_2), 32.2 (CH_2), 32.3 (CH), 37.0 (10-C), 37.4 (CH_2), 38.5 (12-CH_2), 39.9 (CH_2), 42.8 (13-C), 43.9 (24-CH_2), 45.3 (20-CH), 50.3 (9-CH), 53.2 (17-CH), 56.6 (14-CH), 74.3 (3-CH), 122.9 (6-CH), 127.9 (26-CH, 27-CH), 128.3 (30-CH), 129.1 (28-CH, 29-CH), 138.9 (25-C), 140.0 (5-C), 171.0 (1′-C=O), 176.9 (22-C=O); MS ($APCI^+$) *m*/*z* (rel intensity) 436 ($[M-Ac]^+$, 100), 418 ($[M-AcO]^+$, 75); HRMS calcd for $C_{31}H_{44}NO_3$ ($[M + H]^+$): 478.3321; found: 478.3315. Anal. Calcd for $C_{31}H_{43}NO_3$: C, 78.0; H, 9.1; N, 2.9; Found: C, 77.5; H, 9.0; N, 2.9.

5.2. Procedure B

A solution of 3β-acetoxy-5-cholenic acid (0.5 g, 1.28 mmol), DIPEA (0.67 ml, 3.86 mmol), TBTU (0.454 g, 1.4 mmol) and HOBt (0.191 g, 1.4 mmol) in dry DMF (30 ml) was stirred at room temperature for 30 min. The mixture was added dropwise over 60 min to a solution of diamine (4 equiv) in dry DMF (20 ml) and stirred overnight at room temperature. The resulting solution was diluted with chloroform, washed with water, dried over sodium sulfate and reduced in vacuo to yield a white solid. Chromatography over silica gel [$CHCl_3$:MeOH/NH_4OH (94:5:1 → 85:10:5)] furnished compounds **4a**–**4e** as white solids.

5.2.1. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-(1,4)-diaminobutyl (4a). 0.321 g, 55% yield; mp 171–173 °C; $R_f = 0.16$ ($CHCl_3$:MeOH/NH_4OH, 85:10:5); IR (KBr) 3299 (CO*NH* str), 2935 (CH), 1731 (CH_3CO), 1643 (*CO*NH), 1552 (NHδ), 1444 (CH_2), 1371 (CH_3), 1247 (C–O str), 1039, 632 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.72 (3H, s, 18-CH_3), 1.05 (3H, s, 19-CH_3), 1.21 (3H, d, $J = 7$, 21-CH_3), 1.27–2.00 (23H: 9× CH_2, 5× CH), 2.07 (3H, s, 2′-CH_3), 2.35 (2H, d, $J = 7$, 4-CH_2), 2.76 (2H, t, $J = 7$, 27-CH_2), 3.27 (2H, m, 24-CH_2), 4.64 (1H, m, 3-CH), 5.40 (1H, d, $J = 5$, 6-CH), 5.87 (1H, t, 23-NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.5 (18-CH_3), 18.1 (21-CH_3), 19.7 (19-CH_3), 21.4 (11-CH_2), 21.9 (2′-CH_3), 24.7 (CH_2), 27.5 (CH_2), 28.0 (CH_2), 28.1 (CH_2), 31.2 (CH_2), 32.2 (CH_2), 32.3 (CH), 37.0 (10-C), 37.4 (CH_2), 38.5 (CH_2), 39.5 (CH_2), 39.9 (CH_2), 42.1 (CH_2), 42.7 (13-C), 45.3 (20-CH), 50.3 (9-CH), 53.2 (17-CH), 56.6 (14-CH), 74.3 (3-CH), 122.9 (6-CH), 140.0 (5-C), 171.0 (1′-C=O), 177.0 (22-C=O); MS (ES^+) *m*/*z* (rel intensity) 459 (M^+, 100); HRMS calcd for $C_{28}H_{47}N_2O_3$ ($[M + H]^+$): 459.3587; found: 459.3593. Anal. Calcd for $C_{28}H_{46}N_2O_3 \cdot 1.02H_2O$: C, 70.5; H, 10.2; N, 5.9; Found: C, 70.5; H, 9.9; N, 5.7.

5.2.2. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-1,6-diaminohexyl (4b). 0.374 g, 60% yield; mp 148–149 °C; $R_f = 0.26$ ($CHCl_3$:MeOH/NH_4OH, 85:10:5); IR (KBr) 3302 (CO*NH* str), 2936 (CH), 1731 (CH_3CO), 1644 (*CO*NH), 1547 (NHδ), 1462 (CH_2), 1371 (CH_3), 1247 (C–O), 1039 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.71 (3H, s, 18-CH_3), 1.04 (3H, s, 19-CH_3), 1.20 (3H, d, $J = 7$, 21-CH_3), 1.28–2.02 (27H: 11× CH_2, 5× CH), 2.05 (3H, s, 2′-CH_3), 2.34 (2H, d, $J = 7$, 4-CH_2), 2.70 (2H, t, $J = 7$, 29-CH_2), 3.24 (2H, m, 24-CH_2), 4.62 (1H, m, 3-CH), 5.39 (1H, d, $J = 5$, 6-CH), 5.53 (1H, m, 23-NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.5 (18-CH_3), 18.1 (21-CH_3), 19.7 (19-CH_3), 21.3 (11-CH_2), 21.9 (2′-CH_3), 24.7 (CH_2), 26.9 (CH_2), 27.1 (CH_2), 27.6 (CH_2), 28.0 (CH_2), 28.1 (CH_2), 29.7 (CH_2), 30.1 (CH_2), 32.2 (CH_2), 32.3

(CH_2), 34.1 (CH), 37.0 (10-C), 37.3 (CH_2), 38.5 (CH_2), 39.5 (CH_2), 39.9 (CH_2), 42.7 (13-C), 45.4 (20-CH), 50.3 (9-CH), 51.8 (29-CH_2), 53.2 (17-CH), 56.6 (14-CH), 74.3 (3-CH), 122.9 (6-CH), 140.0 (5-C), 170.9 (1′-C=O), 176.9 (22-C=O); MS (ES^+) *m/z* (rel intensity) 487 (M^+, 100); HRMS calcd for $C_{30}H_{51}N_2O_3$ ($[M + H]^+$): 487.3900; found: 487.3900. Anal. Calcd for $C_{30}H_{50}N_2O_3 \cdot 0.72\ H_2O$: C, 72.1; H, 10.4; N, 5.6; Found: C, 72.1; H, 10.2; N, 5.3.

5.2.3. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-(1,8)-diaminooctyl (4c). 0.250 g, 40% yield; mp 147–149 °C; $R_f = 0.38$ ($CHCl_3$:MeOH/NH_4OH, 85:10:5); IR (KBr) 3302 (CO*NH* str), 2929 (CH), 1728 (CH_3CO), 1643 (*CO*NH), 1551 (NHδ), 1246 (C–O), 1038 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.78 (3H, s, 18-CH_3), 1.11 (3H, s, 19-CH_3), 1.18 (3H, d, $J = 7$, 21-CH_3), 1.40 (12H, s, 25-CH_2, 26-CH_2, 27-CH_2, 28-CH_2, 29-CH_2, 30-CH_2), 1.48–2.10 (19H, 7× CH_2, 5× CH), 2.13 (3H, s, 2′-CH_3), 2.41 (2H, d, $J = 7$, 4-CH_2), 2.77 (2H, t, $J = 7$, 31-CH_2), 3.28 (2H, m, 24-CH_2), 4.69 (1H, m, 3-CH), 5.47 (1H, d, $J = 5$, 6-CH), 5.53 (1H, m, 23-NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.5 (18-CH_3), 18.1 (21-CH_3), 19.7 (19-CH_3), 21.4 (11-CH_2), 21.9 (2′-CH_3), 24.7 (CH_2), 27.2 (CH_2), 27.3 (CH_2), 28.0 (CH_2), 28.1 (CH_2), 29.7 (CH_2), 29.8 (CH_2), 30.1 (CH_2), 31.3 (CH), 32.2 (CH_2), 32.3 (CH), 34.2 (CH_2), 37.0 (10-C), 37.4 (CH_2), 38.5 (CH_2), 39.6 (CH_2), 39.9 (CH_2), 42.7 (13-C), 45.4 (20-CH), 50.3 (9-CH), 52.0 (31-CH_2), 53.2 (17-CH), 56.6 (14-CH), 74.3 (3-CH), 122.9 (6-CH), 140.0 (5-C), 170.9 (1′-C=O), 176.9 (22-C=O); MS (ES^+) *m/z* (rel intensity) 515 (M^+, 100); HRMS calcd for $C_{32}H_{55}N_2O_3$ ($[M + H]^+$): 515.4213; found: 515.4199. Anal. Calcd for $C_{32}H_{54}N_2O_3 \cdot 1.01H_2O$: C, 72.1; H, 10.6; N, 5.3; Found: C, 72.1; H, 10.3; N, 4.8.

5.2.4. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-(1,10)-diaminodecyl (4d). 0.430 g, 62% yield; mp 153–155 °C; $R_f = 0.44$ ($CHCl_3$:MeOH/NH_4OH, 85:10:5); IR (KBr) 3289 (CO*NH* str), 2927 (CH), 1728 (CH_3CO), 1642 (*CO*NH), 1557 (NHδ), 1462 (CH_2), 1248 (C–O str), 1036 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.67 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 1.16 (3H, d, $J = 7$, 21-CH_3), 1.26 (16H, s, 25-CH_2, 26-CH_2, 27-CH_2, 28-CH_2, 29-CH_2, 30-CH_2, 31-CH_2, 32-CH_2), 1.37–1.99 (19H: 7× CH_2, 5× CH), 2.01 (3H, s, 2′-CH_3), 2.30 (2H, d, $J = 7$, 4-CH_2), 2.65 (2H, t, $J = 7$, 33-CH_2), 3.20 (2H, m, 24-CH_2), 4.58 (1H, m, 3-CH), 5.35 (1H, d, $J = 4$, 6-CH), 5.44 (1H, m, 23-NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.5 (18-CH_3), 18.1 (21-CH_3), 19.7 (19-CH_3), 21.4 (11-CH_2), 21.9 (2′-CH_3), 24.7 (CH_2), 27.3 (CH_2), 28.0 (CH_2), 28.1 (CH_2), 29.7 (CH_2), 29.8 (CH_2), 29.9 (CH_2), 30.1 (CH_2), 32.2 (CH_2), 32.3 (CH_2), 34.2 (CH_2), 37.0 (10-C), 37.4 (CH_2), 38.5 (CH_2), 39.6 (CH_2), 39.9 (CH_2), 42.7 (13-C), 45.4 (20-CH), 50.3 (9-CH), 53.2 (17-CH), 56.6 (14-CH), 74.3 (3-CH), 122.9 (6-CH), 140.0 (5-C), 171.0 (1′-C=O), 176.9 (22-C=O); MS (ES^+) *m/z* (rel intensity) 543 (M^+, 100); HRMS calcd for $C_{34}H_{59}N_2O_3$ ($[M + H]^+$): 543.4526; found: 543.4522 ($M + H^+$). Anal. Calcd for $C_{34}H_{58}N_2O_3 \cdot 0.42\ H_2O$: C, 74.2; H, 10.8; N, 5.1; Found: C, 74.2; H, 10.6; N, 4.8.

5.2.5. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-(*trans*-1,4)-diaminocyclohexyl (4e). 0.450 g, 72% yield; mp 230–231 °C; $R_f = 0.26$ ($CHCl_3$:MeOH/NH_4OH, 85:10:5); IR (KBr) 3319 (CO*NH* str), 2939 (CH), 1729 (CH_3CO), 1643 (*CO*NH), 1533 (NHδ), 1452 (CH_2), 1373 (CH_3), 1248 (C–O str), 1037, 607 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.68 (3H, s, 18-CH_3), 1.02 (3H, s, 19-CH_3), 1.16 (3H, d, $J = 7$, 21-CH_3), 1.21–1.99 (27H: 11× CH_2, 5× CH), 2.04 (3H, s, 2′-CH_3), 2.32 (2H, d, $J = 7$, 4-CH_2), 2.62 (1H, m, 29-CH), 3.68 (1H, m, 24-CH), 4.60 (1H, m, 3-CH), 5.37 (1H, d, $J = 4$, 6-CH), 5.66 (1H, d, $J = 8$, 23-NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.4 (18-CH_3), 17.8 (21-CH_3), 19.6 (19-CH_3), 21.3 (11- CH_2), 21.8 (2′-CH_3), 24.7 (CH_2), 27.7 (CH_2), 28.1 (CH_2), 31.9 (CH_2), 32.1 (CH_2), 32.1 (CH_2), 32.2 (CH), 35.2 (CH_2), 36.9 (10-C), 37.3 (CH_2), 38.4 (CH_2), 39.8 (CH_2), 42.7 (13-C), 45.1 (CH), 47.8 (CH), 47.9 (29-CH), 50.0 (24-CH), 50.2 (9-CH), 53.2 (17-CH), 56.5 (14-CH), 74.5 (3-CH), 122.9 (6-CH), 140.0 (5-C), 171.3 (1′-C=O), 176.8 (22-C=O); MS (ES^+) *m/z* (rel intensity) 485 (M^+, 100); HRMS calcd for $C_{30}H_{49}N_2O_3$ ($[M + H]^+$): 485.3743; found: 485.3738. Anal. Calcd for $C_{30}H_{48}N_2O_3 \cdot 0.88\ H_2O$: C, 72.0; H, 10.0; N, 5.6; Found: C, 71.9; H, 9.8; N, 5.3.

5.2.6. Bis-3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-(1,6)-diaminohexyl (4f). Compound **4f** (0.163 g, white solid) was obtained as by-product during the synthesis of the compound **4b** by procedure A: mp 264–266 °C; $R_f = 0.42$ (5% MeOH/$CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 0.81 (6H, s, 18-CH_3), 1.13 (6H, s, 19-CH_3), 1.30 (6H, d, $J = 7$, 21-CH_3), 1.36–2.12 (46H: 18× CH_2, 10× CH), 2.15 (6H, s, 2′-CH_3), 2.43 (4H, d, $J = 7$, 4-CH_2), 3.34 (4H, m, 24-CH_2), 4.68 (2H, m, 3-CH), 5.49 (2H, d, $J = 5$, 6-CH), 5.77 (2H, m, 23-NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.5 (18-CH_3), 18.2 (21-CH_3), 19.7 (19-CH_3), 21.4 (11-CH_2), 21.9 (2′-CH_3), 24.8 (CH_2), 26.0 (CH_2), 28.0 (CH_2), 28.1 (CH_2), 29.9 (CH_2), 32.2 (CH_2), 32.3 (CH_2), 37.0 (10-C), 37.4 (CH_2), 38.5 (CH_2), 38.8 (CH_2), 39.0 (CH_2), 39.9 (CH_2), 42.7 (13-C), 45.3 (20-CH), 50.3 (9-CH), 53.2 (17-CH), 56.7 (14-CH), 74.3 (3-CH), 122.9 (6-CH), 140.0 (5-C), 170.9 (1′-C=O), 177.1 (22-C=O); MS (ES^+) *m/z* (rel intensity) 879 ($M+Na^+$, 100); HRMS calcd for $C_{54}H_{85}N_2O_6$ ($[M + H]^+$): 857.6407; found: 857.6415.

5.2.7. Bis-3β-acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-(1,8)-diaminooctyl (4g). Compound **4g** (0.241 g, white solid) was obtained as by-product during the synthesis of the compound **4c** by procedure A: mp 238 °C; $R_f = 0.54$ (5% MeOH/$CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 0.64 (6H, s, 18-CH_3), 0.97 (6H, s, 19-CH_3), 1.13 (6H, d, $J = 7$, 21-CH_3), 1.25 (12H, s, 25-CH_2, 26-CH_2, 27-CH_2), 1.34–1.92 (38H, 14× CH_2, 10× CH), 1.99 (6H, s, 2′-CH_3), 2.27 (4H, d, $J = 7$, 4-CH_2), 3.16 (4H, m, 24-CH_2), 4.56 (2H, m, 3-CH), 5.33 (4H, m, 6-CH, 23-NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.5 (18-CH_3), 18.1 (21-CH_3), 19.7 (19-CH_3), 21.4 (11-CH_2), 21.9 (2′-CH_3), 24.8 (CH_2), 27.1 (CH_2), 28.0 (CH_2), 28.1 (CH_2), 29.4 (CH_2), 30.0 (CH_2), 32.2 (CH_2), 32.3 (CH_2), 37.0 (10-C), 37.4 (CH_2), 38.5 (CH_2), 39.0 (CH_2), 39.6 (CH_2), 39.9 (CH_2), 42.7 (13-C), 45.4

(20-CH), 50.3 (9-CH), 53.2 (17-CH), 56.6 (14-CH), 74.3 (3-CH), 122.9 (6-CH), 140.0 (5-C), 170.9 (1′-C═O), 176.9 (22-C═O); MS (ES^+) *m/z* (rel intensity) 907 ($M+Na^+$, 100); HRMS calcd for $C_{56}H_{89}N_2O_6$ ($[M + H]^+$): 885.6720; found: 885.6724.

5.2.8. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-oxo-22-(1,6)-diamino-6-*N*,*N*-dimethyl hexyl (4h). Compound **4h** (0.012 g, white solid) was obtained as by-product during the synthesis of the compound **4b** by procedure A: $R_f = 0.15$ (5% MeOH/$CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 0.65 (3H, s, 18-CH_3), 0.96 (3H, s, 19-CH_3), 1.11 (3H, d, $J = 7$, 21-CH_3), 1.20–1.91 (27H: 11× CH_2, 5× CH), 1.98 (3H, s, 2′-CH_3), 2.26 (2H, d, $J = 7$, 4-CH_2), 2.94 (12H, s, 33-CH_3), 3.14 (4H, m, 24-CH_2, 29-CH_2), 4.64 (1H, m, 3-CH), 5.31 (1H, d, $J = 5$, 6-CH), 6.54 (1H, m, 23-NH); MS (ES^+) *m/z* (rel intensity) 586 ($M + H^+$, 100); HRMS calcd for $C_{35}H_{61}N_4O_3$ ($[M + H]^+$): 585.4743; found: 585.4741.

5.3. Procedure C

5.3.1. 3β-Hydroxy-23,24-bisnor-chol-5-en-22-oxo-22-butylamine (5a). A mixture of **3a** (0.600 g, 1.35 mmol) and lithium hydroxide monohydrate (0.113 g, 2.70 mmol) in THF:H_2O (3:1, 32 ml) was stirred at 50 °C for 24 h. The resulting solution was diluted with chloroform, washed several times with water, dried over sodium sulfate and reduced in vacuo. Chromatography over silica gel [EtOAc/Hexane (10% → 50%)] afforded **5a** (0.562 g, 97%) as a white solid: mp 202–203 °C; $R_f = 0.16$ (40% EtOAc/Hexane); IR (KBr) 3287 (OH, CO*NH* str), 2930 (CH), 1641 (*CO*NH), 1551 (NHδ), 1458 (CH_2), 1369 (CH_3), 1068 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.83 (3H, s, 18-CH_3), 1.07 (3H, t, $J = 7$, 27-CH_3), 1.14 (3H, s, 19-CH_3), 1.32 (3H, d, $J = 7$, 21-CH_3), 1.37–2.37 (23H, 9× CH_2, 5× CH), 2.42 (2H, d, $J = 5$, 4-CH_2), 3.37 (2H, m, 24-CH_2), 3.66 (1H, m, 3-CH), 5.48 (1H, d, $J = 5$, 6-CH), 5.64 (1H, m, 23-NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.5 (18-CH_3), 14.2 (27-CH_3), 18.1 (21-CH_3), 19.8 (19-CH_3), 20.5 (11-CH_2), 21.4 (CH_2), 24.8 (CH_2), 28.0 (CH_2), 32.0 (CH_2), 32.1 (CH_2), 32.2 (CH_2), 32.3 (CH), 36.9 (10-C), 37.7 (CH_2), 39.4 (CH_2), 39.9 (12-CH_2), 42.7 (24-CH_2), 42.7 (13-C), 45.4 (20-CH), 50.4 (9-CH), 53.2 (17-CH), 56.7 (14-CH), 72.1 (3-CH), 121.9 (6-CH), 141.2 (5-C), 177.1 (22-C═O); MS (ES^+) *m/z* (rel intensity) 402 ($[M + H]^+$, 100), 384 ($[M-H_2O]^+$, 70); HRMS calcd for $C_{26}H_{44}NO_2$ ($[M + H]^+$): 402.3372; found: 402.3368; Anal. Calcd for $C_{26}H_{43}NO_2$: C, 77.75; H, 10.79; N, 3.40; Found: C, 77.63; H, 10.78; N, 3.38.

5.3.2. 3β-Hydroxy-23,24-bisnor-chol-5-en-22-oxo-22-piperidylamine (5b). A mixture of **3b** (0.711g, 1.56 mmol) and lithium hydroxide monohydrate (0.130 g, 3.12 mmol) in THF/H_2O (3:1, 40 ml) was stirred at 50 °C for 24 h. The resulting solution was diluted with chloroform, washed several times with water, dried over sodium sulfate and reduced in vacuo to yield a white solid. Chromatography over silica gel [EtOAc/Hexane (10% → 50%)] afforded **5b** (0.450, 70%) as a white solid: mp 218–219 °C; $R_f = 0.18$ (40% EtOAc/Hexane); IR (KBr) 3454 (OH), 2931 (CH), 1622 (*CO*N), 1444 (CH_2), 1368 (CH_3), 1246 (*C–O*H), 1133, 1066, 1013, 955 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.76 (3H, s, 18-CH_3), 1.05 (3H, s, 19-CH_3), 1.18 (3H, d, $J = 7$, 21-CH_3), 1.26–2.33 (24H, 10× CH_2, 4× CH), 2.84 (1H, m, 20-CH), 2.32 (2H, d, $J = 5$, 4-CH_2), 3.58 (5H, m, 3-CH, 24-CH_2, 25-CH_2), 5.38 (1H, d, $J = 5$, 6-CH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.7 (18-CH_3), 17.9 (21-CH_3), 19.8 (19-CH_3), 21.5 (11-CH_2), 24.7 (CH_2), 25.1 (CH_2), 26.8 (CH_2), 28.2 (CH_2), 32.0 (CH_2), 32.2 (CH_2), 32.3 (CH), 36.9 (10-C), 37.7 (CH_2), 37.9 (12-CH_2), 40.0 (24-CH_2, 25-CH_2), 42.7 (CH_2), 50.5 (9-CH), 53.2 (17-CH), 56.6 (14-CH), 72.1 (3-CH), 121.9 (6-CH), 141.3 (5-C), 175.6 (22-C═O); MS ($APCI^+$) *m/z* (rel intensity) 414 (M^+, 100), 396 ($[M-H_2O]^+$, 75); HRMS calcd for $C_{27}H_{44}NO_2$ ($[M + H]^+$): 414.3372; found: 414.3373. Anal. Calcd for $C_{27}H_{43}NO_2 \cdot 0.42\ H_2O$: C, 77.0; H, 10.5; N, 3.3; Found: C, 77.0; H, 10.4; N, 3.2.

5.3.3. 3β-Hydroxy-23,24-bisnor-chol-5-en-22-oxo-22-benzylamine (5c). A mixture of **3c** (0.787 g, 1.65 mmol) and lithium hydroxide monohydrate (0.138 g, 3.29 mmol) in THF/H_2O (3:1, 40 ml) was stirred at 50 °C for 24 h. The resulting solution was diluted with chloroform, washed several times with water, dried over sodium sulfate and reduced in vacuo to yield a white solid. Chromatography over silica gel [EtOAc/hexane (10% → 50%)] afforded **5c** (0.476, 65%) as a white solid: $R_f = 0.20$ (40% EtOAc/hexane); IR (KBr) 3413 (OH, CO*NH* str), 2924 (CH), 1651 (*CO*NH), 1521 (NHδ), 1456 (CH_2), 1346 (CH_3), 1209 (*C–O*H), 1069 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.73 (3H, s, 18-CH_3), 1.05 (3H, s, 19-CH_3), 1.27 (3H, d, $J = 7$, 21-CH_3), 1.30–2.32 (19H, 7× CH_2, 5× CH), 2.35 (2H, d, $J = 5$, 4-CH_2), 3.57 (1H, m, 3-CH), 4.47 (2H, dd, $J = 5$, $J = 5$, 24-CH_2), 5.40 (1H, d, $J = 5$, 6-CH), 5.89 (1H, m, 23-NH), 7.34 (5H, m, 26-CH, 27-CH, 28-CH, 29-CH, 30-CH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.5 (18-CH_3), 18.1 (21-CH_3), 19.8 (19-CH_3), 21.4 (11-CH_2), 24.8 (CH_2), 28.0 (CH_2), 32.0 (CH_2), 32.2 (CH_2), 32.3 (CH), 36.9 (10-C), 37.6 (CH_2), 40.0 (CH_2), 42.7 (CH_2), 42.8 (13-C), 43.9 (24-CH_2), 45.4 (20-CH), 50.4 (9-CH), 53.2 (17-CH), 56.7 (14-CH), 72.1 (3-CH), 122.0 (6-CH), 127.9 (26-CH, 27-CH), 128.3 (30-CH), 129.1 (28-CH, 29-CH), 138.8 (25-C), 141.2 (5-C), 177.0 (22-C═O); MS (ES^+) *m/z* (rel intensity) 436 (M^+, 100), 418 ($[M-H_2O]^+$, 75); HRMS calcd for $C_{29}H_{42}NO_2$ ($[M + H]^+$): 436.3215; found: 436.3209.

5.3.4. 3β-Hydroxy-23,24-bisnor-chol-5-en-22-oxo-22-(1,4)-diaminobutyl (6a). Compound **4a** (0.205 g, 0.44 mmol) was hydrolysed as described in the synthesis of compound **18**, affording the hydroxyl derivative **6a** (0.180 g, 95%) as a white solid. TLC showed only one spot, identified by MS and NMR as the compound **6a**: mp 132–133 °C; $R_f = 0.17$ ($CHCl_3$:MeOH/NH_4OH, 85:10:5); IR (KBr) 3370 (OH, CO*NH*, NH_2), 2918 (CH), 1645 (*CO*NH), 1549 (NHδ), 1452 (CH_2), 1372 (CH_3), 1058 cm^{-1}; ^1H NMR (300 MHz, CD_3OD): δ 0.71 (3H, s, 18-CH_3), 0.99 (3H, s, 19-CH_3), 1.10 (3H, d, $J = 7$, 21-CH_3), 1.26–2.08 (23H: 9× CH_2, 5× CH), 2.19 (2H, m, 4-CH_2), 2.78 (2H, m, 27-CH_2), 3.13 (2H, m, 24-CH_2), 3.36 (1H, m, 3-CH), 5.29 (1H, d, $J = 5$,

6-CH); ^{13}C NMR (75 MHz, CD_3OD): δ 12.9 (18-CH_3), 18.3 (21-CH_3), 20.3 (19-CH_3), 22.5 (11-CH_2), 25.7 (CH_2), 28.6 (CH_2), 28.8 (CH_2), 29.1 (CH_2), 31.1 (CH_2), 32.7 (CH_2), 33.4 (CH_2), 33.7 (CH), 38.1 (10-C), 38.9 (CH_2), 41.4 (CH_2), 43.4 (CH_2), 43.8 (13-C), 45.4 (20-CH), 51.9 (CH_2), 52.1 (9-CH), 54.4 (17-CH), 58.2 (14-CH), 72.8 (3-CH), 122.8 (6-CH), 142.6 (5-C), 179.0 (22-C=O); MS (ES^+) *m/z* (rel intensity) 417 (M^+, 100); HRMS calcd for $C_{26}H_{45}N_2O_3$ ($[M + H]^+$): 417.3481; found: 417.3476.

5.3.5. 3β-Hydroxy-23,24-bisnor-chol-5-en-22-oxo-22-(1,6)-diaminohexyl (6b). Compound **4b** (0.165 g, 0.33 mmol) was hydrolysed as described in the synthesis of compound **18**, affording the hydroxyl derivative **6b** (0.136 g, 90%) as a white solid. TLC showed only one spot, identified by MS and NMR as the compound **6b**: mp 129–131 °C; $R_f = 0.12$ ($CHCl_3$:MeOH/NH_4OH, 85:10:5); IR (KBr) 3289 (OH, CO*NH*, NH_2), 2935 (CH), 1645 (*CO*NH), 1548 (NHδ), 1455 (CH_2), 1371 (CH_3), 1058 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.64 (3H, s, 18-CH_3), 0.95 (3H, s, 19-CH_3), 1.12 (3H, d, $J = 7$, 21-CH_3), 1.16–2.18 (27H: 11× CH_2, 5× CH), 2.23 (2H, d, $J = 3$, 4-CH_2), 2.63 (2H, m, 29-CH_2), 3.15 (2H, m, 24-CH_2), 3.47 (1H, m, 3-CH), 5.29 (1H, d, $J = 5$, 6-CH), 5.46 (1H, m, 23-NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.5 (18-CH_3), 18.1 (21-CH_3), 21.4 (19-CH_3), 24.7 (11-CH_2), 26.9 (CH_2), 27.1 (CH_2), 27.2 (CH_2), 27.6 (CH_2), 28.0 (CH_2), 30.1 (CH_2), 31.4 (CH_2), 32.0 (CH_2), 32.2 (CH_2), 32.3 (CH_2), 33.9 (CH), 36.9 (10-C), 37.7 (CH_2), 39.5 (CH_2), 39.9 (CH_2), 42.4 (CH_2), 42.7 (13-C), 45.4 (20-CH), 50.4 (9-CH), 51.8 (29-CH_2), 53.2 (17-CH), 56.7 (14-CH), 72.0 (3-CH), 121.9 (6-CH), 141.3 (5-C), 177.0 (22-C=O); MS (ES^+) *m/z* (rel intensity) 445 (M^+, 100); HRMS calcd for $C_{28}H_{49}N_2O_2$ ($[M + H]^+$): 445.3794; found: 445.3801.

5.3.6. 3β-Hydroxy-23,24-bisnor-chol-5-en-22-oxo-22-(1,8)-diaminooctyl (6c). A mixture of **4c** (0.100 g, 0.19 mmol) and lithium hydroxide monohydrate (0.016 g, 0.38 mmol) in THF/H_2O (3:1, 16 ml) was stirred at 50 °C for 24 h. The resulting solution was diluted with chloroform, washed several times with water, dried over sodium sulfate and reduced in vacuo to yield a white solid. TLC showed only one spot, identified by MS and NMR as the compound **6c** (0.070, 75%): mp 133–135 °C; $R_f = 0.21$ ($CHCl_3$:MeOH/NH_4OH, 85:10:5); IR (KBr) 3300 (OH, CO*NH*, NH_2), 2929 (CH), 1642 (*CO*NH), 1548 (NHδ), 1057 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.72 (3H, s, 18-CH_3), 1.03 (3H, s, 19-CH_3), 1.20 (3H, d, $J = 7$, 21-CH_3), 1.32 (12H, s, 25-CH_2, 26-CH_2, 27-CH_2, 28-CH_2, 29-CH_2, 30-CH_2), 1.41–2.24 (19H: 7× CH_2, 5× CH), 2.41 (2H, m, 4-CH_2), 2.70 (2H, t, $J = 7$, 31-CH_2), 3.24 (2H, m, 24-CH_2), 3.54 (1H, m, 3-CH), 5.36 (1H, d, $J = 5$, 6-CH), 5.68 (1H, m, 23-NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.5 (18-CH_3), 18.0 (21-CH_3), 19.8 (19-CH_3), 21.4 (11-CH_2), 24.7 (CH_2), 27.1 (CH_2), 27.2 (CH_2), 27.9 (CH_2), 29.5 (CH_2), 29.7 (CH_2), 30.0 (CH_2), 30.1 (CH_2), 31.9 (CH), 32.2 (CH_2), 32.3 (CH_2), 36.9 (10-C), 37.6 (CH_2), 39.5 (CH_2), 39.6 (CH_2), 39.9 (CH_2), 42.6 (CH_2), 42.7 (13-C), 45.3 (20-CH), 50.0 (9-CH), 50.4 (CH), 53.2 (17-CH), 56.7 (14-CH), 71.9 (3-CH), 121.9 (6-CH), 141.2 (5-C), 177.3 (22-C=O); MS (ES^+) *m/z* (rel intensity) 473 (M^+, 100); HRMS calcd for $C_{30}H_{53}N_2O_2$ ($[M + H]^+$): 473.4107; found: 473.4107.

5.3.7. 3β-Hydroxy-23,24-bisnor-chol-5-en-22-oxo-22-(1,10)-diaminodecyl (6d). Compound **4d** (0.200 g, 0.36 mmol) was hydrolysed as described in the synthesis of compound **18**, affording the hydroxyl derivative **6d** (0.162 g, 90%) as a white solid. TLC showed only one spot, identified by MS and NMR as the compound **6d**: mp 158–159 °C; $R_f = 0.35$ ($CHCl_3$/MeOH/NH_4OH, 85:10:5); IR (KBr) 3290 (OH, CO*NH*, NH_2), 2928 (CH), 1642 (*CO*NH), 1555 (NHδ), 1465 (CH_2), 1373 (CH_3), 1060 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.62 (3H, s, 18-CH_3), 0.92 (3H, s, 19-CH_3), 1.09 (3H, d, $J = 7$, 21-CH_3), 1.20 (16H, s, 25-CH_2, 26-CH_2, 27-CH_2, 28-CH_2, 29-CH_2, 30-CH_2, 31-CH_2, 32-CH_2), 1.36–2.11 (19H: 7× CH_2, 5× CH), 2.18 (2H, m, 4-CH_2), 2.59 (2H, m, 33-CH_2), 3.13 (2H, m, 24-CH_2), 3.41 (1H, m, 3-CH), 5.25 (1 H, d, $J = 3$, 6-CH), 5.75 (1H, m, 23-NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.4 (18-CH_3), 17.8 (21-CH_3), 19.7 (19-CH_3), 21.3 (11-CH_2), 24.7 (CH_2), 27.1 (CH_2), 27.2 (CH_2), 27.8 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 29.8 (CH_2), 31.6 (CH_2), 32.1 (CH), 32.2 (CH_2), 33.1 (CH_2), 36.8 (10-C), 37.6 (CH_2), 39.5 (CH_2), 39.9 (CH_2), 41.9 (CH_2), 42.3 (CH_2), 42.6 (13-C), 45.0 (20-CH), 50.4 (9-CH), 53.1 (17-CH), 56.6 (14-CH), 71.7 (3-CH), 121.8 (6-CH), 141.2 (5-C), 177.7 (22-C=O); MS (ES^+) *m/z* (rel intensity) 501 (M^+, 100); HRMS calcd for $C_{32}H_{57}N_2O_2$ ($[M + H]^+$): 501.4420; found: 501.4418.

5.3.8. 3β-Hydroxy-23,24-bisnor-chol-5-en-22-oxo-22-(*trans*-1,4)-diaminocyclohexyl (6e). Compound **4e** (0.250 g, 0.51 mmol) was hydrolysed as described in the synthesis of compound **18**, affording the hydroxyl derivative **6b** (0.162 g, 70%) as a white solid. TLC showed only one spot, identified by MS and NMR as the compound **6e**: mp 225 °C; $R_f = 0.19$ ($CHCl_3$/MeOH/NH_4OH, 85:10:5); IR (KBr) 3325 (OH, CO*NH*, NH_2), 2935 (CH), 1655 (*CO*NH), 1508 (NHδ), 1457 (CH_2), 1372 (CH_3), 1202, 1071, 1005 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.61 (3H, s, 18-CH_3), 0.92 (3H, s, 19-CH_3), 1.08 (3H, d, $J = 7$, 21-CH_3), 1.13–2.14 (27H: 11× CH_2, 5× CH), 2.19 (2H, d, $J = 5$, 4-CH_2), 2.56 (1H, m, 29-CH), 3.42 (1H, m, 24-CH), 3.59 (1H, m, 3-CH), 5.26 (1H, d, $J = 3$, 6-CH), 5.63 (1H, m, 23-NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.4 (18-CH_3), 17.9 (21-CH_3), 19.7 (19-CH_3), 21.4 (11-CH_2), 24.7 (CH_2), 27.7 (CH_2), 31.7 (CH_2), 31.8 (CH_2), 32.0 (CH_2), 32.1 (CH_2), 32.3 (CH), 35.1 (CH_2), 36.8 (10-C), 37.6 (CH_2), 39.9 (CH_2), 42.4 (CH_2), 42.7 (13-C), 45.1 (CH), 47.8 (CH), 47.9 (24-CH), 50.0 (29-CH), 50.4 (9-CH), 53.1 (17-CH), 56.6 (14-CH), 71.7 (3-CH), 121.8 (6-CH), 141.2 (5-C), 176.8 (22-C=O); MS (ES^+) *m/z* (rel intensity) 443 (M^+, 100); HRMS calcd for $C_{28}H_{47}N_2O_2$ ($[M + H]^+$): 443.3638; found: 443.3639.

5.3.9. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-ol (7). A solution of borane-methyl sulfide complex (1 M DCM) (38.61 ml, 38.61 mmol) was added drop-wise over 1 h to a solution of 3β-acetoxy-5-cholenic acid (10.0 g, 25.74 mmol) in THF (100 ml) and kept below 0 °C by

using a bath mixture of ice and sodium chloride. The reaction mixture was stirred for 8 h. Then it was hydrolysed with water and potassium carbonate, filtered, diluted with diethylether, washed with sodium chloride (satd), dried over sodium sulfate and concentrated. Chromatography over silica gel [MeOH/$CHCl_3$ ($0 \rightarrow 1\%$)] afforded compound **7** as a white solid (5.45 g, 57%): mp 148–149 °C; $R_f = 0.40$ (2% MeOH/$CHCl_3$); IR (KBr) 3487, 3258 (CH_2OH), 2940 (CH), 1711 (CH_3CO), 1463 (CH_2), 1378 (CH_3), 1261 (CH_3CO str), 1033, 741 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.65 (3H, s, 18-CH_3), 0.97 (3H, s, 19-CH_3), 1.01 (3H, d, $J = 7$, 21-CH_3), 1.98 (3H, s, 2′-CH_3), 1.05–1.97 (19H: 7× CH_2, 5× CH), 2.26 (2H, d, $J = 7$, 4-CH_2), 3.31 (1H, dd, $J = 3$, $J = 3$, 22-CH), 3.59 (1H, dd, $J = 3$,$J = 3$, 22′-CH), 4.55 (1H, m, 3-CH), 5.32 (1H, d, $J = 5$, 6-CH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.3 (18-CH_3), 17.2 (21-CH_3), 19.7 (19-CH_3), 21.4 (11-CH_2), 21.9 (2′-CH_3), 24.8 (CH_2), 28.2 (CH_2), 32.3 (CH_2), 37.0 (10-C), 37.4 (CH_2), 38.5 (CH_2), 39.2 (20-CH), 40.0 (12-CH_2), 42.8 (13-C), 50.4 (9-CH), 52.8 (17-CH), 56.8 (14-CH), 68.4 (22-CH_2), 74.4 (3-CH), 123.0 (6-CH), 140.1 (5-C), 171.0 (1′-C=O); MS (APCI $^+$) *m/z* (rel intensity) 315 ($[M-AcO]^+$, 100), 329 ($[M-Ac]^+$, 75); HRMS (ES^+) Calcd for $C_{24}H_{42}NO_3$ ($[M+NH_4]^+$): 392.3165; found: 392.3169; Anal. Calcd for $C_{24}H_{38}O_3$: C, 77.0; H, 10.2; Found: C, 76.8; H, 10.3.

5.3.10. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-p-toluensulfonyloxy (8). Compound**7** (0.682 g, 1.82 mmol) was dissolved in pyridine/DCM (10:10 ml) and cooled to 0 °C. *p*-Toluensulfonylchloride (0.694 g, 3.64 mmol) was added as a solid at 0 °C. The solution was stirred at 4 °C for 24 h. A white precipitate was formed. The reaction mixture was poured into water, extracted with chloroform, the organic extract washed with water, copper (II) sulfate hydrate (satd), dried over magnesium sulfate and the solvent removed to afford 0.844 g (88%) of **8** as a white solid. TLC analysis revealed a single spot different to the starting material. No further purification of the product was performed. mp 124–126 °C; $R_f = 0.3$ (10% EtOAc/hexane); IR (KBr) 2938 (CH), 1727 (CH_3CO), 1462 (CH_2), 1371 (CH_3), 1242 (C–O), 1177 (S=O), 1022, 929, 847 (ArCH) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.70 (3H, s, 18-CH_3), 0.95 (3H, d, $J = 7$, 21-CH_3), 1.00 (3H, s, 19-CH_3), 1.10–2.09 (1× CH_3, 7× CH_2, 5× CH), 2.10 (3H, s, 2′-CH_3), 2.38 (2H, d, $J = 7$, 4-CH_2), 2.51 (31-CH_3), 3.80 (1H, m, 22-CH), 4.15 (1H, m, 22′-CH), 4.65 (1H, m, 3-CH), 5.40 (1H, d, $J = 5$, 6-CH), 7.40 (2H, d, $J = 8$, 28-CH, 29-CH), 7.82 (2H, d, $J = 5$, 26-CH, 27-CH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.2 (18-CH_3), 17.3 (21-CH_3), 19.7 (19-CH_3), 21.3 (11-CH_2), 21.9 (2′-CH_3), 22.1 (31-CH_3), 24.7 (CH_2), 27.9 (CH_2), 28.1 (CH_2), 32.21 (CH_2), 32.25 (8-CH), 36.6 (CH_2), 37.0 (10-C), 37.4 (CH_2), 38.5 (CH_2), 39.8 (12-CH_2), 42.8 (13-C), 50.3 (9-CH), 52.2 (17-CH), 56.7 (14-CH), 74.3 (3-CH), 76.0 (22-CH_2), 122.9 (6-CH), 128.3 (28-CH, 29-CH), 130.2 (26-CH, 27-CH), 133.5 (30-CH), 140.1 (5-C), 145.0 (25-C), 171.0 (1′-C=O); MS (APCI $^+$) *m/z* (rel intensity) 297 ($[M-OAc-Ts-H_2O]^+$, 100), 298 ($[M-AcO-H_2O+H]^+$, 55); MS (APCI $^-$) *m/z* (rel intensity) 171 ($-OTs^-$, 100).

5.3.11. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-butylamine (9a). A solution of **8** (0.383 g, 0.72 mmol), butylamine (0.72 ml, 7.24 mmol) and DIPEA (0.38 ml, 2.17 mmol) in DMF (10 ml) was stirred at 60 °C for 48 h. The reaction mixture was poured into water, extracted with chloroform, dried over magnesium sulfate and the solvent removed to afford 0.332 g of crude. Chromatography over silica gel [MeOH/$CHCl_3$ (10%)] afforded 0.239 g (86%) of **9a** as a white solid: mp 132 °C; $R_f = 0.26$ (10% MeOH/$CHCl_3$); IR (KBr) 2933 (CH), 1731 (CH_3CO), 1462 (CH_2), 1372 (CH_3), 1248 (C–O), 1129, 1030 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.78 (3H, s, 18-CH_3), 0.95 (3H, d, $J = 7$, 21-CH_3), 1.00 (3H, t, $J = 7$, 27-CH_3), 1.10 (3H, s, 19-CH_3), 1.11–2.10 (23H: 9-CH_2, 5-CH), 2.12 (3H, s, 2′-CH_3), 2.40 (2H, d, $J = 7$, 4-CH_2), 2.61–2.78 (2H, m, 22-CH_2), 3.39 (2H, m, 24-CH_2), 4.68 (1H, m, 3-CH), 5.46 (1H, d, $J = 4$, 6-CH), 8.25 (1H, 23-NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.3 (18-CH_3), 14.4 (27-CH_3), 18.1 (21-CH_3), 19.7 (19-CH_3), 20.9 (11-CH_2), 21.9 (2′-CH_3), 24.7 (CH_2), 28.2 (CH_2), 28.4 (CH_2), 32.0 (CH_2), 32.3 (CH_2), 36.7 (CH), 37.00 (10-C), 37.4 (CH_2), 38.3 (CH_2), 38.5 (4-CH_2), 40.1 (12-CH_2), 42.9 (13-C), 45.4 (20-CH), 50.3 (24-CH_2), 50.4 (9-CH), 54.7 (17-CH), 55.8 (22-CH_2), 56.9 (14-CH), 74.4 (3-CH), 123.0 (6-CH), 140.0 (5-C), 171.0 (1′-C=O); MS (APCI $^+$) *m/z* (rel intensity) 429 (M^+, 100); HRMS (ES^+) Calcd for $C_{28}H_{48}NO_2$ ($[M + H]^+$): 430.3685; found: 430.3685; Anal. Calcd for $C_{28}H_{47}NO_3 \cdot 0.14\ H_2O$: C, 77.8; H, 11.0; N, 3.2; Found: C, 77.8; H, 11.1; N, 3.1.

5.3.12. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-piperidyl amine (9b). To a solution of **8** (0.218 g, 0.41 mmol) in DMF (3 ml) was added piperidine (0.12 ml, 1.24 mmol), potassium carbonate (0.085 g, 6.18 mmol) and tetrabutylammonium iodide, as the catalyst (0.07 g) and stirred at 100 °C for 4 h. The reaction mixture was poured into water, extracted with ether, washed with NaCl (satd) and dried over magnesium sulfate. Chromatography over silica gel [MeOH/$CHCl_3$ (1%)] afforded 0.083 g (46%) of **9b** as a white solid: mp 155–157 °C; $R_f = 0.58$ (20% MeOH/$CHCl_3$); IR (KBr) 2933 (CH), 1736 (CH_3CO), 1443 (CH_2), 1368 (CH_3), 1247 (CH_3CO str), 1034 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.74 (3H, s, 18-CH_3), 1.07 (3H, s, 19-CH_3), 1.10 (3H, d, $J = 7$, 21-CH_3), 1.13–2.07 (25H: 10-CH_2, 5× CH), 2.08 (3H, s, 2′-CH_3), 2.10–2.37 (6H, m, 24-CH_2, 25-CH_2, 4-CH_2), 2.54 (2H, m, 22-CH_2), 4.66 (1H, m, 3-CH), 5.42 (1H, d, $J = 4$, 6-CH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.4 (18-CH_3), 18.7 (21-CH_3), 19.7 (19-CH_3), 21.4 (11-CH_2), 21.9 (2′-CH_3), 24.9 (CH_2), 26.0 (CH_2), 28.2 (CH_2), 28.7 (CH_2), 32.2 (CH_2), 32.3 (8-CH), 34.5 (CH), 37.0 (10-C), 37.4 (CH_2), 38.5 (CH_2), 40.1 (CH_2), 43.1 (13-C), 50.4 (9-CH), 55.6 (17-CH, 24-CH_2, 25-CH_2), 56.9 (14-CH), 65.8 (22-CH_2), 74.4 (3-CH), 123.0 (6-CH), 140.1 (5-C), 171.0 (1′-C=O); MS (APCI $^+$) *m/z* (rel intensity) 442 (M^+, 100); HRMS (ES^+) calcd for $C_{29}H_{48}NO_2$ ($[M + H]^+$): 442.3685; found: 442.3686.

5.3.13. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-benzyl amine (9c). To a solution of **8** (0.428 g, 0.81 mmol) in DMF (20 ml) was added benzylamine (0.26 ml, 2.43 mmol), potassium carbonate (0.170 g, 1.21 mmol)

and tetrabutylammonium iodide, as the catalyst, (0.14 g) and stirred at 100 °C for 4 h. The reaction mixture was poured into water, extracted with chloroform, washed with NaCl (satd) and dried over magnesium sulfate. Chromatography over silica gel [MeOH/$CHCl_3$ (10%)] afforded 0.183 g (49%) of compound **9c** as a white solid: mp 132–134 °C; $R_f = 0.51$ (10% MeOH/$CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.74 (3H, s, 18-CH_3), 0.90 (3H, s, 19-CH_3), 1.05 (H, d, $J = 7$, 21-CH_3), 1.09–2.07 (19H: 7× CH_2, 5× CH), 2.08 (3H, s, 2′-CH_3), 2.35 (2H, d, $J = 7$, 4-CH_2), 2.40 (1H, d, $J = 3$, 22-CH), 2.74 (1H, d, $J = 3$, 22′-CH), 3.78 (1H, d, $J = 13$, 24-CH), 3.89 (1H, d, $J = 13$, 24′-CH), 4.59 (1H, m, 3-CH), 5.42 (1H, d, $J = 4$, 6-CH), 7.29–7.39 (5H, CH aromatic), 8.31 (1H, 23-NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.3 (18-CH_3), 18.2 (21-CH_3), 19.7 (19-CH_3), 21.4 (11-CH_2), 21.9 (2′-CH_3), 24.7 (CH_2), 28.2 (CH_2), 28.3 (CH_2), 32.3 (CH_2), 36.8 (CH), 37.0 (10-C), 37.4 (CH_2), 38.5 (CH_2), 40.0 (12-CH_2), 42.9 (13-C), 50.4 (9-CH), 54.5 (24-CH_2), 54.5 (17-CH), 55.2 (22-CH_2), 56.9 (14-CH), 74.4 (3-CH), 123.0 (6-CH), 127.4 (30-CH), 128.1–129.2 (26-CH, 27-CH), 140.1 (25-C), 140.6 (5-C), 171.0 (1′-C=O); MS (APCI $^+$) *m/z* (rel intensity) 464 (M^+, 100); HRMS (ES^+) calcd for $C_{31}H_{46}NO_2$ ($[M + H]^+$): 464.3529; found: 464.3522.

5.3.14. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-1,4-diaminobutyl (10a). To a solution of **8** (0.290 g, 0.55 mmol) in DMF (40 ml) was added 1,4-diaminobutane (0.22 ml, 2.19 mmol), potassium carbonate (0.113 g, 0.82 mmol) and tetrabutylammonium iodide as the catalyst (0.10 g), and stirred at 110 °C for 4 h. The reaction mixture was poured into water, extracted with chloroform, washed with sodium chloride (satd) and dried over sodium sulfate. Chromatography over silica gel using DCM/MeOH/NH_4OH (95:5:0 → 85:10:5) as the eluent afforded a yellow solid identified as the diamine **10a** (0.087 g, 36% yield): mp 121–124 °C; $R_f = 0.27$ (DCM/MeOH/NH_4OH, 85:10:5); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.63 (3H, s, 18-CH_3), 0.93 (6H, m, 19-CH_3, 21-CH_3), 0.97–1.95 (23H: 9× CH_2, 5× CH), 1.96 (3H, s, 2′-CH_3), 2.34 (2H, d, $J = 7$, 4-CH_2), 2.61 (4H, m, 22-CH_2, 24-CH_2), 3.13 (2H, m, 27-CH_2), 4.53 (1H, m, 3-CH), 5.30 (1H, d, $J = 4$, 6-CH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.3 (18-CH_3), 17.8 (21-CH_3), 19.6 (19-CH_3), 21.3 (11-CH_2), 21.7 (2′-CH_3), 24.6 (CH_2), 27.1 (CH_2), 27.6 (CH_2), 28.0 (CH_2), 28.3 (CH_2), 28.5 (CH_2), 32.1 (CH_2), 36.2 (8-CH), 36.9 (10-C), 37.3 (CH_2), 38.4 (CH_2), 39.9 (27-CH_2), 42.8 (13-C), 50.3 (9-CH), 51.2 (24-CH_2), 54.6 (17-CH), 54.3 (22-CH_2), 56.8 (14-CH), 74.5 (3-CH), 122.9 (6-CH), 140.0 (5-C), 171.4 (1′-C=O); MS (ES^+) *m/z* (rel intensity) 485 (100); 445 (M^+, 40); HRMS (ES^+) calcd for $C_{28}H_{49}N_2O_2$ ($[M + H]^+$): 445.3794; found: 445.3785.

5.3.15. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-1,6-diaminohexyl (10b). Compound **8** (0.400 g, 0.75 mmol) and 1,6-diaminohexane (0.352 g, 3.02 mmol) were dissolved in ethanol (30 ml) and DMF (15 ml). The solution was stirred overnight under reflux. The reaction mixture was poured into water, extracted with chloroform and dried over sodium sulfate. MS analysis of the crude showed formation of compound **10b** and its formamide derivative **10f** (see below). Chromatography over silica gel using DCM/MeOH/NH_4OH (80:20:0 → 92:6:2) as the eluent afforded compound **10b** (0.074 g, 20% yield) as a white solid: $R_f = 0.27$ (DCM/MeOH/NH_4OH, 85:10:5); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.73 (3H, s, 18-CH_3), 1.06 (6H, m, 19-CH_3, 21-CH_3), 1.10–2.07 (27H: 11× CH_2, 5× CH), 2.07 (3H, s, 2′-CH_3), 2.36 (2H, d, $J = 8$, 4-CH_2), 2.69 (6H, m, 22-CH_2, 24-CH_2, 29-CH_2), 4.63 (1H, m, 3-CH), 5.41 (1H, d, $J = 4$, 6-CH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.3 (18-CH_3), 18.2 (21-CH_3), 19.7 (19-CH_3), 21.4 (11-CH_2), 21.9 (2′-CH_3), 24.8 (CH_2), 27.17 (CH_2), 27.58 (CH_2), 28.15 (CH_2), 28.39 (CH_2), 30.2 (CH_2), 31.4 (CH_2), 32.2 (CH), 36.71 (CH), 36.97 (10-C), 37.4 (CH_2), 38.5 (CH_2), 40.0 (29-CH_2), 42.9 (13-C), 50.36 (9-CH), 50.53 (24-CH_2), 54.7 (17-CH), 55.8 (22-CH_2), 56.9 (14-CH), 74.4 (3-CH), 122.9 (6-CH), 140.1 (5-C), 170.9 (1′-C=O); MS (ES^+) *m/z* (rel intensity) 473 (M^+, 100); HRMS (ES^+) calcd for $C_{30}H_{53}N_2O_2$ ($[M + H]^+$): 473.4107; found: 473.4103.

5.3.16. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-1,8-diaminooctyl (10c). To a solution of **8** (0.370 g, 0.69 mmol) in DMF (30 ml) was added 1,8-diaminooctane (0.403 g, 2.79 mmol), potassium carbonate (0.145 g, 1.048 mmol) and tetrabutylammonium iodide as the catalyst (0.07 g) and stirred at 110 °C for 4 h. The reaction mixture was poured into water, extracted with ether, washed with sodium chloride (satd) and dried over sodium sulfate. Chromatography over silica gel first using EtOAc and then MeOH/EtOAc (5% → 10%, 5%Et_3N) as the eluent afforded a yellow solid, which was identified as the diamine **10c** (0.130 g, 37% yield): mp 132–133 °C; $R_f = 0.30$ (DCM/MeOH/NH_4OH, 85:10:5); IR (KBr) 3286 (NH str), 2921 (CH), 1730 (CH_3CO), 1470 (CH_2), 1372 (CH_3), 1259 (C–O), 1040, 802 cm^{-1}; ^{1}H NMR (300 MHz, CD_3OD): δ 0.77 (3H, s, 18-CH_3), 1.04 (3H, d, $J = 7$, 21-CH_3), 1.06 (3H, s, 19-CH_3), 1.09–2.00 (31H: 13× CH_2, 5× CH), 2.02 (3H, s, 2′-CH_3), 2.33 (2H, d, $J = 7$, 4-CH_2), 2.59 (4H, m, 24-CH_2, 22-CH_2), 2.69 (2H, m, 31-CH_2), 4.55 (1H, m, 3-CH), 5.45 (1H, d, $J = 5$, 6-CH); ^{13}C NMR (75 MHz, CD_3OD): δ 12.8 (18-CH_3), 18.4 (21-CH_3), 20.1 (19-CH_3), 21.6 (2′-CH_3), 22.5 (11-CH_2), 25.8 (CH_2), 28.3 (CH_2), 28.8 (CH_2), 29.2 (CH_2), 30.4 (CH_2), 30.8 (CH_2), 31.0 (CH_2), 33.6 (8-CH), 37.7 (CH), 38.1 (10-C), 38.6 (CH_2), 39.5 (CH_2), 42.6 (CH_2), 44.1 (CH_2), 49.4 (CH_2), 49.7 (31-CH_2), 50.0 (13-C), 50.2 (24-CH_2), 52.0 (9-CH), 56.1 (17-CH, 22-CH_2), 58.3 (14-CH), 75.8 (3-CH), 123.9 (6-CH), 141.5 (5-C), 172.8 (1′-C=O); MS (ES^+) *m/z* (rel intensity) 501 (M^+, 100); HRMS (ES^+) calcd for $C_{32}H_{57}N_2O_2$ ($[M + H]^+$): 501.4420; found: 501.4420; Anal. Calcd for $C_{32}H_{56}N_2O_2 \cdot 0.27\ H_2O$: C, 76.0; H, 11.3; N, 5.5; Found: C, 76.0; H, 11.2; N, 5.4.

5.3.17. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-1,10-diaminodecyl (10d). 1,10-Diaminodecane (0.391 g, 2.27 mmol), potassium carbonate (0.117 g, 0.85 mmol) and tetrabutylammonium iodide, as the catalyst (0.117 g), were added to a solution of **8** (0.300 g, 0.56 mmol) in DMF (40 ml) and stirred at 110 °C for 8 h. The reaction mixture was poured into water,

extracted with chloroform, washed with sodium chloride (satd) and dried over sodium sulfate. Chromatography over silica gel using firstly EtOAc and then MeOH/EtOAc (5% → 10%, 5%Et_3N) as the eluents afforded a yellow solid identified as the diamine **10d** (0.141 g, 47% yield): mp 131 °C; R_f = 0.18 (MeOH/Et_3N/EtOAc, 30:5:65); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.74 (3H, s, 18-CH_3), 1.06 (3H, s, 19-CH_3), 1.10 (3H, d, J = 7, 21-CH_3), 1.32 (16H, 25-CH_2, 26-CH_2, 27-CH_2, 28-CH_2, 29-CH_2, 30-CH_2, 31-CH_2, 32-CH_2), 1.34–2.06 (19H: 7× CH_2, 5× CH), 2.07 (3H, s, 2′-CH_3), 2.36 (2H, d, J = 7, 4-CH_2), 2.69 (2H, m, 22-CH_2), 2.74 (2H, m, 24-CH_2), 3.28 (5H, m, 33-CH_2, 23-NH, 34-NH_2), 4.70 (1H, m, J = 5, 3-CH), 5.41 (1H, d, J = 4, 6-CH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.3 (18-CH_3), 18.2 (21-CH_3), 19.7 (19-CH_3), 21.4 (11-CH_2), 21.9 (2′-CH_3), 24.7 (CH_2), 27.2 (CH_2), 27.7 (CH_2), 28.2 (CH_2), 28.4 (CH_2), 29.8 (CH_2), 32.2 (CH_2), 36.4 (8-CH), 37.0 (10-C), 37.4 (CH_2), 38.5 (CH_2), 40.0 (CH_2), 42.1 (33-CH_2), 42.9 (13-C), 50.3 (9-CH), 50.4 (24-CH_2), 54.6 (17-CH), 55.5 (22-CH_2), 56.9 (14-CH), 74.4 (3-CH), 123.0 (6-CH), 140.1 (5-C), 171.0 (1′-C═O); MS (ES^+) m/z (rel intensity) 529 (M^+, 82), 265 ($[M/2]^+$, 100); HRMS (ES^+) calcd for $C_{34}H_{61}N_2O_3$ ($[M + H]^+$): 529.4733; found: 529.4728.

5.3.18. 3β-Acetoxy-23,24-bisnor-chol-5-en-22-1,4-*trans*-diaminocyclohexyl (10e). To a solution of **8** (0.400 g, 0.75 mmol) in DMF (30 ml) was added *trans*-1,4-diaminocyclohexane (0.345 g, 3.03 mmol), potassium carbonate (0.157 g, 3.03 mmol) and tetrabutylammonium iodide as the catalyst (0.05 g) and stirred at 110 °C for 3 days. The reaction mixture was poured into water, extracted with ether, washed with sodium chloride (satd) and dried over sodium sulfate. Chromatography over silica gel using DCM/MeOH/NH_4OH (95:5:0 → 85:10:5) as the eluent afforded a white solid identified as the diamine **10e** (0.179 g, 50% yield): mp 142–144 °C; R_f = 0.24 (DCM/MeOH/NH_4OH, 85:10:5); IR (KBr) 2935 (CH), 1730 (CH_3CO), 1455 (CH_2), 1370 (CH_3), 1250 (C–O), 1036 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.63 (3H, s, 18-CH_3), 0.92 (3H, d, J = 7, 21-CH_3), 0.94 (3H, s, 19-CH_3), 1.00–1.91 (19H: 7× CH_2, 5× CH), 1.96 (3H, s, 2′-CH_3), 2.22 (4H, m, 4-CH_2, 22-CH_2), 2.64 (2H, m, 24-CH, 29-CH), 4.51 (1H, m, 3-CH), 5.30 (1H, m, 6-CH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.3 (18-CH_3), 17.9 (21-CH_3), 19.6 (19-CH_3), 21.3 (11-CH_2), 21.8 (2′CH_3), 24.7 (CH_2), 28.1 (CH_2), 28.4 (CH_2), 31.3 (CH_2), 31.7 (CH_2), 32.2 (CH_2), 34.5 (CH_2), 36.5 (8-CH), 36.9 (10-C), 37.3 (CH_2), 38.4 (CH_2), 40.01 (CH_2), 42.86 (13-C), 50.3 (9-CH), 52.7 (22-CH_2), 54.7 (24-CH, 29-CH), 56.6 (17-CH), 56.9 (14-CH), 74.5 (3-CH), 122.9 (6-CH), 140.0 (5-C), 171.4 (1′-C═O); MS (ES^+) m/z (rel intensity) 471 (M^+, 100); HRMS (ES^+) calcd for $C_{30}H_{51}N_2O_2$ ($[M + H]^+$): 471.3950; found: 471.3952.

5.3.19. 3β-Acetoxy-23,24-bisnor-5-en-22-1,6-diamino-6-al-hexyl (10f). Diamine **10f** was isolated during the purification of compound **10b** as a white solid (0.037 g, 10% yield): R_f = 0.43 (DCM/MeOH/NH_4OH, 85:10:5); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.73 (3H, s, 18-CH_3), 1.06 (6H, m, 19-CH_3, 21-CH_3), 1.09–2.03 (27H: 11× CH_2, 5× CH), 2.07 (3H, s, 2′-CH_3), 2.35 (2H, d, J = 7, 4-CH_2), 2.66 (4H, m, 22-CH_2, 24-CH_2), 3.32 (2H, m, 29-CH_2), 4.63 (1H, m, 3-CH), 5.41 (1H, d, J = 4, 6-CH), 6.18 (1H, m, 30-NH), 8.19 (1H, s, 31-CHO); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.3 (18-CH_3), 18.2 (21-CH_3), 19.7 (19-CH_3), 21.4 (11-CH_2), 21.9 (2′-CH_3), 24.7 (CH_2), 27.2 (CH_2), 27.5 (CH_2), 28.1 (CH_2), 28.4 (CH_2), 29.4 (CH_2), 29.7 (CH_2), 32.2 (CH), 36.4 (CH), 36.9 (10-C), 37.4 (CH_2), 38.4 (CH_2), 38.5 (CH_2), 40.0 (29-CH_2), 42.9 (13-C), 50.1 (9-CH), 50.4 (24-CH_2), 54.6 (17-CH), 55.5 (22-CH_2), 56.9 (14-CH), 74.3 (3-CH), 123.0 (6-CH), 140.1 (5-C), 171.0 (1′-C═O); MS (ES^+) m/z (rel intensity) 501 (M^+, 100); HRMS (ES^+) calcd for $C_{31}H_{53}N_2O_3$ ($[M + H]^+$): 501.4065; found: 501.4055.

5.3.20. 3β-Hydroxy-23,24-bisnor-chol-5-en-22-butylamine (11a). A mixture of **9a** (0.05 g, 0.11 mmol) and lithium hydroxide monohydrate (0.010 g, 0.23 mmol) in THF:H_2O (3:1, 16 ml) was stirred at 50 °C for 24 h. The resulting solution was diluted with chloroform, washed several times with water, dried over sodium sulfate and reduced in vacuo to yield a white solid. Chromatography over silica gel with 5% MeOH/$CHCl_3$ as the eluent afforded **11a** (0.035, 78%) as a white solid: mp 137 °C; R_f = 0.14 (0% MeOH/$CHCl_3$); IR (KBr) 3384 (OH), 3279 (NH str), 2955 (CH), 1458 (CH_2), 1376 (CH_3), 1260 (*C–O*H), 1064, 801 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.63 (3H, s, 18-CH_3), 0.87 (3H, d, J = 7, 21-CH_3), 0.93 (3H, s, 19-CH_3), 0.97 (3H, t, J = 7, 27-CH_3), 1.11–2.10 (23H: 9× CH_2, 5× CH), 2.25 (4H, m, 4-CH_2, 22-CH_2), 2.62 (2H, m, 24-CH_2), 3.43 (1H, m, 3-CH), 5.28 (1H, m, 6-CH); ^{13}C NMR (75 MHz, $CDCl_3$): δ12.3 (18-CH_3), 14.3 (27-CH_3), 19.8 (21-CH_3), 20.8 (19-CH_3), 21.4 (11-CH_2), 24.7 (CH_2), 28.4 (CH_2), 31.0 (CH_2), 31.9 (CH_2), 32.3 (CH_2), 36.9 (10-C), 37.6 (CH_2), 40.1 (CH_2), 42.9 (13-C), 49.5 (24-CH_2), 50.4 (9-CH), 54.5 (17-CH), 55.0 (22-CH_2), 56.9 (14-CH), 71.9 (3-CH), 121.9 (6-CH), 141.2 (5-C); MS (ES^+) m/z (rel intensity) 388 (M^+, 100); HRMS (ES^+) calcd for $C_{26}H_{46}NO$ ($[M + H]^+$): 388.3579; found: 388.3579.

5.3.21. 3β-Hydroxy-23,24-bisnor-chol-5-en-22-piperidyl amine (11b). A mixture of **9b** (0.140 g, 0.32 mmol) and lithium hydroxide monohydrate (0.040 g, 0.95 mmol) in THF/H_2O (3:1, 16 ml) was stirred at 50 °C for 24 h. The resulting solution was diluted with chloroform, washed several times with water, dried over sodium sulfate and reduced in vacuo to yield a white solid. Chromatography over silica gel with 5% MeOH/$CHCl_3$ as the eluent afforded **11b** (0.106, 84%) as a white solid: mp 184–186 °C; R_f = 0.40 (20% MeOH/$CHCl_3$); IR (KBr) 3500 (OH), 2933 (CH), 1444 (CH_2), 1371 (CH_3), 1053 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.75 (3H, s, 18-CH_3), 1.05 (6H, m, 19-CH_3, 21-CH_3), 1.10–2.11 (25H: 10× CH_2, 5× CH), 2.19–2.33 (6H, m, 24-CH_2, 25-CH_2, 4-CH_2), 2.47 (2H, m, 22-CH_2), 3.54 (1H, m, 3-CH), 5.40 (1H, d, J = 5, 6-CH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.4 (18-CH_3), 18.6 (21-CH_3), 19.8 (19-CH_3), 21.5 (11-CH_2), 24.9 (CH_2), 25.0 (CH_2), 26.4 (CH_2), 28.6 (CH_2), 32.1 (CH_2), 32.3 (CH_2), 32.3 (8-CH), 34.6 (CH), 36.9 (10-C), 37.7 (CH_2), 40.1 (CH_2),

42.7 (CH_2), 43.1 (13-C), 50.5 (9-CH), 55.7 (17-CH, 24-CH_2, 25-CH_2), 56.9 (14-CH), 66.0 (22-CH_2), 72.2 (3-CH), 122.1 (6-CH), 141.2 (5-C); MS (ES^+) *m/z* (rel intensity) 400 (M^+, 100); HRMS (ES^+) calcd for $C_{27}H_{46}NO$ ($[M + H]^+$): 400.3579; found: 400.3577; Anal. Calcd for $C_{27}H_{45}NO.0.32\ H_2O$: C, 80.0; H, 11.3; N, 3.5; Found: C, 80.0; H, 11.2; N, 3.4.

5.3.22. 3β-Hydroxy-23,24-bisnor-chol-5-en-22-1,8- diaminooctyl (12c). A mixture of **10c** (0.073 g, 0.15 mmol) and lithium hydroxide monohydrate (0.012 g, 0.29 mmol) in THF:H_2O (3:1, 16 ml) was stirred at 50 °C for 24 h. The resulting solution was diluted with chloroform, washed several times with water, dried over sodium sulfate and reduced in vacuo to yield a yellow solid (0.061 g). Chromatography over silica gel using DCM/MeOH/NH_4OH (95:5:0 → 89:7:4) as the eluent afforded a white solid identified as the diamine **12c** (0.50 g, 70%): mp 136–137 °C; R_f 0.11 (DCM/MeOH/NH_4OH, 85:10:5); IR (KBr) 2930 (CH), 1452 (CH_2), 1376 (CH_3), 1060 cm^{-1}; ^1H NMR (300 MHz, CD_3OD): δ 0.75 (3H, s, 18-CH_3), 1.02 (6H, m, 19-CH_3, 21-CH_3), 1.35 (12H, s, 25-CH_2, 26-CH_2, 27-CH_2, 28-CH_2, 29-CH_2, 30-CH_2), 1.42–2.21 (19H: 7× CH_2, 5× CH), 2.25 (2H, m, 4-CH_2), 2.55 (2H, m, 22-CH_2), 2.64 (2H, m, 24-CH_2), 3.27 (2H, m, 31-CH_2), 3.38 (1H, m, 3-CH), 5.34 (1H, d, $J = 5$, 6-CH); MS (ES^+) *m/z* (rel intensity) 459 (M^+, 100); HRMS (ES^+) calcd for $C_{30}H_{55}N_2O$ ($[M + H]^+$): 459.431; found: 459.431; Anal. Calcd for $C_{30}H_{54}N_2O{\cdot}1.5\ H_2O$: C, 74.2; H, 11.8; N, 5.8; Found: C, 73.9; H, 11.2; N, 5.3.

5.3.23. 3β-Hydroxy-23,24-bisnor-chol-5-en-22-1,10- diaminodecyl (12d). Diamine **10d** (0.057 g, 0.11 mmol) was hydrolysed as described in the synthesis of diamine **12c** using lithium hydroxide monohydrate (0.014 g, 0.32 mmol). The analogous procedure yielded diamine **12d** as a white solid (0.049 g, 93%): $R_f = 0.30$ (DCM/MeOH/NH_4OH, 85:10:5); ^1H NMR (300 MHz, $CDCl_3$): δ 0.66 (3H, s, 18-CH_3), 0.96 (6H, m, 19-CH_3, 21-CH_3), 1.24 (16H, s, 25-CH_2, 26-CH_2, 27-CH_2, 28-CH_2, 29-CH_2, 30-CH_2, 31-CH_2, 32-CH_2), 1.40–2.14 (19H: 7× CH_2, 5× CH), 2.24 (4H, m, 4-CH_2, 22-CH_2), 2.61 (4H, m, 24-CH_2, 33-CH_2), 3.46 (1H, m, 3-CH), 5.31 (1H, d, $J = 4$, 6-CH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.3 (18-CH_3), 18.2 (21-CH_3), 19.8 (19-CH_3), 21.5 (11-CH_2), 24.8 (CH_2), 27.3 (CH_2), 27.8 (CH_2), 28.1 (CH_2), 28.4 (CH_2), 29.9 (CH_2), 30.0 (CH_2), 30.4 (CH_2), 30.7 (CH), 32.1 (CH_2), 32.3 (CH_2), 34.1 (CH_2), 36.8 (8-CH), 36.9 (10-C), 37.7 (CH_2), 40.1 (CH_2), 42.5 (4-CH_2), 42.7 (33-CH_2), 42.9 (13-C), 50.5 (9-CH), 50.7 (24-CH_2), 54.7 (17-CH), 55.9 (22-CH_2), 57.0 (14-CH), 71.9 (3-CH), 121.9 (6-CH), 141.3 (5-C); MS (ES^+) *m/z* (rel intensity) 487 (M^+, 100), 244 ($[M/2]^+$, 30); HRMS (ES^+) calcd for $C_{39}H_{59}N_2O$ ($[M + H]^+$): 487.4627; found: 487.4631.

5.3.24. 3β-Hydroxy-23,24-bisnor-chol-5-en-22-1,4- *trans*-diaminocyclohexyl (12e). Diamine **10e** (0.110 g, 0.23 mmol) was hydrolysed as described in the synthesis of diamine **12c** using lithium hydroxide monohydrate (0.029 g, 0.70 mmol). The analogous procedure yielded diamine **12e** as a white solid (0.060 g, 60%): mp 173–175 °C; $R_f = 0.14$ (DCM/MeOH/NH_4OH, 85:10:5); IR (KBr) 3260 (OH, NH str), 2930 (CH), 1452 (CH_2), 1375 (CH_3), 1066 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 0.79 (3H, s, 18-CH_3), 1.06 (6H, m, 19-CH_3, 21-CH_3), 1.14–2.12 (27H: 11× CH_2, 5× CH), 2.30 (4H, m, 4-CH_2, 22-CH_2), 2.77 (2H, m, 24-CH, 29-CH), 3.54 (1H, m, 3-CH), 5.42 (1H, d, $J = 4$, 6-CH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.9 (18-CH_3), 18.5 (21-CH_3), 20.4 (19-CH_3), 22.2 (11-CH_2), 25.5 (CH_2), 29.2 (CH_2), 32.1 (CH_2), 32.3 (CH_2), 32.5 (CH_2), 33.0 (CH_2), 35.1 (CH_2), 35.2 (CH_2), 37.3 (8-CH), 37.7 (10-C), 38.4 (CH_2), 40.0 (CH_2), 42.9 (CH_2), 43.7 (13-C), 51.1 (9-CH), 51.3 (22-CH_2), 55.6 (24-CH, 29-CH), 57.3 (17-CH), 57.8 (14-CH), 72.4 (3-CH), 122.6 (6-CH), 142.1 (5-C); MS (ES^+) *m/z* (rel intensity) 429 (M^+, 100%); HRMS (ES^+) calcd for $C_{28}H_{49}N_2O$ ($[M + H]^+$): 429.3845, found: 429.3840; Anal. Calcd for $C_{28}H_{48}N_2O{\cdot}0.83\ H_2O$: C, 75.8; H, 11.3; N, 6.3; Found: C, 75.8; H, 11.1; N, 6.2.

5.4. 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 *L. major SMT* gene in the pET28a vector (Novagen). *L. major* recombinant SMT is produced as a His-tagged fusion protein and is over-expressed when induced with IPTG 1 mM during 4 h. Cells were disrupted by sonication in a buffer containing Tris–HCl 50 mM pH 7.4, $MgCl_2$ 2 mM, CHAPS 4 mM, Tween 80 0.5% (v/v) and protease inhibitors (3×30 s, duty cycle 50%). The sonicate was centrifuged at 12,000 rpm for 30 min at 4 °C to obtain the soluble fraction, which contained the active form of the enzyme.

A standard SMT activity assay contained 1 mg of protein in the previously mentioned buffer, Tris–Hcl 50 mM, pH 7.4, $MgCl_2$ 2 mM, CHAPS 4 mM, Tween 80 0.5% (v/v), desmosterol 100 μM and ^{14}C-*S*-adenosyl-L-methionine 200 μM, 600,000 dpm per reaction. The inhibitor was re-suspended 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, ^3H-cholesterol (3 mg, 30,000 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. An amount of 1 ml of the organic layer was added to 10 ml of hydrofluor and the radioactivity measured in a scintillation counter.

IC_{50} values were obtained from plots of percentage of inhibition versus concentration of inhibitor.

5.4.1. Studies of lipid composition. *L. mexicana amazonensis* promastigotes were cultivated in LIT medium

supplemented with lactabulmin and 10% fetal calfserum (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.[34–37] 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% phenyl-methyl-siloxane, 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 choloroform and the column was kept at 50 °C for 1 min, 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.

5.5. In vitro assays

5.5.1. *L. donovani*. Peritoneal exudate macrophages were harvested from CD1 mice, 24 h after starch induction. After washing 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 ration of 5 parasites:1 macrophage. Infected cells were left for a further 24 h and then exposed to drug[39] for a total of five days, with the overlay being replaced on day 3.[40] The top concentration for the test compounds was 30 μg/ml and all concentrations were carried out in quadruplicate. On day 5 the overlay is removed, the slides fixed (100% methanol) and stained (10% Giemsa, 10 min) before being evaluated microscopically. ED_{50} (ED_{90}) values were calculated using Ms*xl*fit. The ED_{50} value for the positive control drug, Pentostam©, is usually 3–8 μg SbV/ml.

5.5.2. *T. cruzi*. Murine (CD1) peritoneal macrophages were harvested 24 h after starch induction. A total of 100 μl 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. 24 h later the infected cells were exposed to drug[20] for three days. A total of 50 μl of 500 μ MCPRG:1% nonidet P-40 was added to each well. The plates were read after 2–5 h, λ 570.[41] ED_{50} (ED_{90}) values were calculated using Ms *xl*fit. L6 fibroblasts are also used as host cells.

5.5.3. *T. brucei*. *Trypanosoma brucei rhodesiense* STIB900 form (bsf) trypomastigotes were maintained in HMI-18 medium[42] with 15% HIFCS [Harlan-SeraLab, UK] at 37 °C, 5%, CO_2/air mixture. Trypomastigotes were washed and resuspended in fresh medium at a concentration of 2×10^5/ml. A total of 100 μl was added to the drug dilutions. The top concentration for the test compounds was 30 μg/ml. The ED_{50} for pentamidine is usually between 1.0 and 0.1 ng/ml. Plates were incubated for 72 h at 37 °C, 5% CO_2. At 72 h the plates were assessed microscopically before Alamar Blue was added.[43] Plates were read after 5–6 h on a Gemini Fluorescent plate reader (Softmax Pro. 3.1.1, Molecular Devices, UK) at EX/EM 530/585 nm with a filter cutoff at 550 nm. ED_{50} values were calculated with Ms *xl*fit (IDBS, UK)

5.5.4. Cytotoxicity against mammalian cells. Plates were seeded with 100 μl KB cells @ 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 @ 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 assessed were microscopically for cell growth. The overlay was removed and wells washed three times with PBS (pH 7.0) × 3. Then 100 μl PBS + 10 μl 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.

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